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

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**References:** In the text, a reference identified by means of an author's name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author's name should be mentioned, followed by 'et al'. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like 'a' and 'b' after the date to distinguish the works.

Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998;

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 Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc.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

Examples:

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

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

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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**ARTICLES**

**Genetic Diversity Study Of Common Bean (*Phaseolus Vulgaris* L.)  
Germplasm From Ethiopia Using Inter Simple Sequence Repeat (ISSR)  
Markers**

Kefyalew Dagneu, Teklehaimanot Haileselassie and Tileye Feyissa

**Molecular Characterization Of *Trichoderma* Sp. Isolated From Rhizospheric  
Soils Of Uttar Pradesh (India) Based On Microsatellite Profiles**

Mohammad Shahid, Mukesh Srivastava, Sonika Pandey, Anuradha Singh and  
Vipul Kumar

**Evaluation Of Different Methods To Overcome In Vitro Seed Dormancy  
From Yellow Passion Fruit**

Rego, M. M., Rego, E. R., Nattrodt, L. P. U., Barroso, P. A., Finger, F. L. and  
Otoni, W. C.

**Canonical Correlations In Elephant Grass For Energy Purposes**

Drieli Aparecida Rossi, Bruna Rafaela da Silva Menezes, Rogério Figueiredo  
Daher, Geraldo de Amaral Gravina, Roberta Samara Nunes de Lima, Francisco  
José da Silva Lédo, Romildo Domingos Gottardo, Eliemar Campostrini and  
Cláudio Luiz Melo de Souza

**Evaluation Of Somatic Embryogenesis And Plant Regeneration In Tissue  
Culture Of Ten Sorghum (*Sorghum Bicolor* L.) Genotypes**

Shireen Kamal Assem, Mohamed Mahmoud Zamzam, Basita Abbas Hussein  
and Ebtissam Hussein Aly Hussein

**Micropropagation Of *Plectranthus Edulis* (Vatke) Agnew From Meristem  
Cultur**

Mesfin Tsegaw and Tileye Feyissa

**Technique For En-Masse Cryo-Fixation And Processing Of Second-Stage  
Juveniles Of *Meloidogyne Incognita* For Scanning Electron Microscopy**

Shikha Sharma, Rajdeep K. Sidhu and V. R. Parshad

## Table of Contents: Volume 13 Number 36, 3 September, 2014

***In Vitro* Evaluation Of Roots, Seeds And Leaves Of *Sesamum Indicum* L.  
For Their Potential Antibacterial And Antioxidant Properties**

Sandhya Sharma, Priti Gupta, Ashwani Kumar, Jaswant Ray, Bipin Kumar  
Aggarwal, Pankaj Goyal and Anubhuti Sharma

**Statistical Optimization Of Rapid Production Of Cellulases From  
*Aspergillus Niger* MA1 And Its Application In Bioethanol Production  
From Rice Hulls**

Mohammad M. El-Metwally, Amal M. El-Sharkawy, Adel A. El-Morsy and  
Salah M. El-Dohlob

**Bioethanol Production From *Curcubita Pepo* And *Opilia Amentacea* Fruits  
Using Four Strains Of *Saccharomyces Cerevisiae***

Kosi Mawuéna NOVIDZRO, Kokou Agbékonyi AGBODAN, Oudjaniyobi  
SIMALOU and Kossi Honoré KOUMAGLO

**Effect Of Various Concentrations Of Aloe Vera Coating On Postharvest  
Quality And Shelf Life Of Mango (*Mangifera Indica* L.) Fruits Var. 'Ngowe'**

Ochiki, Sophia, Wolukau, Joseph Ngwela and Gesimba, Morwani Robert

**Seed Protein Electrophoresis Of Some Members Of The Family Fabaceae**

Alege, G. O, Abu Ngozi E. and Sunday, C. E



## Full Length Research Paper

# Genetic diversity study of common bean (*Phaseolus vulgaris* L.) germplasm from Ethiopia using inter simple sequence repeat (ISSR) markers

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*Phaseolus vulgaris* L. (family Leguminosae), is a leguminous crop widely distributed in all parts of the world. In Ethiopia, common bean is cultivated as a source of protein for local consumption and for export. Mostly, it grows in the warm and lowland areas of the country. The aim of this research was to investigate the genetic diversity of *P. vulgaris* accessions from Ethiopia. Twelve accessions of *P. vulgaris* were used to estimate the genetic diversity among and within accessions using inter simple sequence repeat (ISSR) markers. Seven selected ISSR primers yielded 69 reproducible bands from 96 individuals studied. All the loci were found to be polymorphic. The total genetic diversity (H) and Shannon's diversity information index (I) for the entire accessions showed 0.35 and 0.53, respectively. Individuals from Shinile site showed the highest level of gene diversity (H = 0.29, I = 0.43); while the lowest variability was shown by accessions from Metekel (L) (H = 0.10, I = 0.15). Variation within accessions was higher (62.55%) as compared to that of inter accessions (37.45%) based on AMOVA. In PCoA, majority individuals of Metekel (L) tended to form separate group. The result of the study confirmed the presence of genetic diversity that can be exploited to improve the productivity. This calls for a concerted efforts in the collection, conservation and sustainable use of *P. vulgaris*.

**Key words:** Genetic diversity, ISSR, *Phaseolus vulgaris*

## INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is a diploid (2n=22) annual leguminous plant that belongs to the genus *Phaseolus*, and it is characterized by pinnately compound trifoliate leaves. There are about 50 species under the genus *Phaseolus* (Hedberg and Edwards, 1989). *P. vulgaris* was derived from wild ancestors distributed from Northern Mexico to Northwestern Argentina (Ibarra-Perez et al., 1997; Debouck, 1999). The ancestors of *P. vulgaris* appear to have formed two distinct gene pools one in

Mesoamerica (Mexico and Central America) and the other in the Southern Andes, a mountain range in South America (Zeven, 1997; Gepts and Bliss, 1988; Blair et al., 2007; Burle et al., 2010). An ancestral wild form is still found at the boundary between temperate and subtropical dry climatic regions (Debouck, 1999). They were brought to Europe and Africa during the 16th century by returning Spanish and Portuguese explorers (Gepts and Debouck, 1991; Ibarra-Perez et al., 1997). Since that time,

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**Table 1.** Passport data of *P. vulgaris* accessions and their respective codes collected from Ethiopia (IBC).

Number	Accessions name	Regional state	Zone (woreda)	Accessions code	Latitude	Longitude	Altitude
1	241739	SNNP	North Omo	A	06-52-59-N	37-47-56-E	1715.00
2	241737	SNNP	Hadiya	B	07-31-51-N	38-01-94-E	1740.00
3	211362	Benishangul	Metekel (Dangure)	C	11-41-15-N	35-37-19-E	1000.00
4	211331	Somali	Shinile	D	09-28-33-N	41-01-49-E	1850.00
5	211327	Oromiya	Harerge	E	09-05-00-N	40-45-00-E	1850.00
6	208703	Oromiya	West Wellega	F	08-32-26-N	34-49-18-E	1846.00
7	211356	Benishangul	Metekel (Dibate)	G	10-21-32-N	36-19-30-E	1250.00
8	208702	Oromiya	West Wellega	H	08-31-17-N	34-44-09-E	1880.00
9	208698	Oromiya	West Wellega	I	08-30-59-N	34-46-28-E	1880.00
10	208695	Oromiya	West Wellega	J	08-32-00-N	34-46-52-E	1880.00
11	211329	Oromiya	Harerge	K	09-02-00-N	40-44-00-E	1870.00
12	211361	Benishangul	Metekel (Dangure)	L	11-32-59-N	36-37-19-E	1300.00

it has been developed into many different forms through selective breeding by local farmers. About 30% of the current world production comes from Mexico, Central and South America, and significant quantities are grown in Asia and Africa (Ibarra-Perez et al., 1997; Burle et al., 2010). In Ethiopia, common beans are concentrated in the dry and warmer parts of the country mainly along the Rift Valley (for example Alemu Demelash and Bekele Adam, Ethiopia, unpublished paper). They are adapted to the low and mid altitude areas at an altitudinal range of 900-2100 m.a.s.l. Besides, common beans grow in areas with annual rainfall range of 200 to 600 mm (IFPRI, 2010). Common bean is largely a self-pollinated plant though cross pollination is possible (Ibarra-Perez et al., 1997). The seeds are non-endospermic and vary greatly in size and color from the small black wild type to the large white, brown, red and spotted types (Cobley and Steele, 1976). It shows variation in growth habits from determinate bush to indeterminate extreme climbing types. The bushy type is the most predominant bean grown in Africa (Buruchara, 2007). Nutritionally, common bean contains high protein content, a good source of energy and also provides folic acid, dietary fibre and complex carbohydrates (Edje et al., 1980; Sousa and Delgado, 1993; Svetleva et al., 2006). For the poor, it plays a strategic role in alleviating malnutrition and for other health related functions. Even though it is an important crop as a food security, production of the crop is inconsistent due to biotic and abiotic stresses. There was no genetic diversity study on Ethiopian *P. vulgaris*, particularly using molecular markers. In this study, we assess the genetic diversity of common bean accessions collected from different parts of Ethiopia. Common bean was considered for this research because of its economic importance in the country serving as a cash crop for smallholder farmers. It can be cultivated at times of irregular rainfall and previous reports also indicated that it can grow in the lowlands of Ethiopia where soils are relatively affected by salt (Alemu and Seifu,

2003). The aim of this study was therefore to investigate the genetic diversity of *P. vulgaris* accessions collected from Ethiopia using ISSR markers on accessions collected from different parts of Ethiopia. This will provide information on the overall genetic variability of *P. vulgaris* accessions which may assist in the identification and selection of the genetic materials for conservation for different regions of Ethiopia.

## MATERIALS AND METHODS

This study was conducted from September 2011 to August 2012 at Addis Ababa University, Ethiopia and the following materials and methods were employed.

### Plant materials

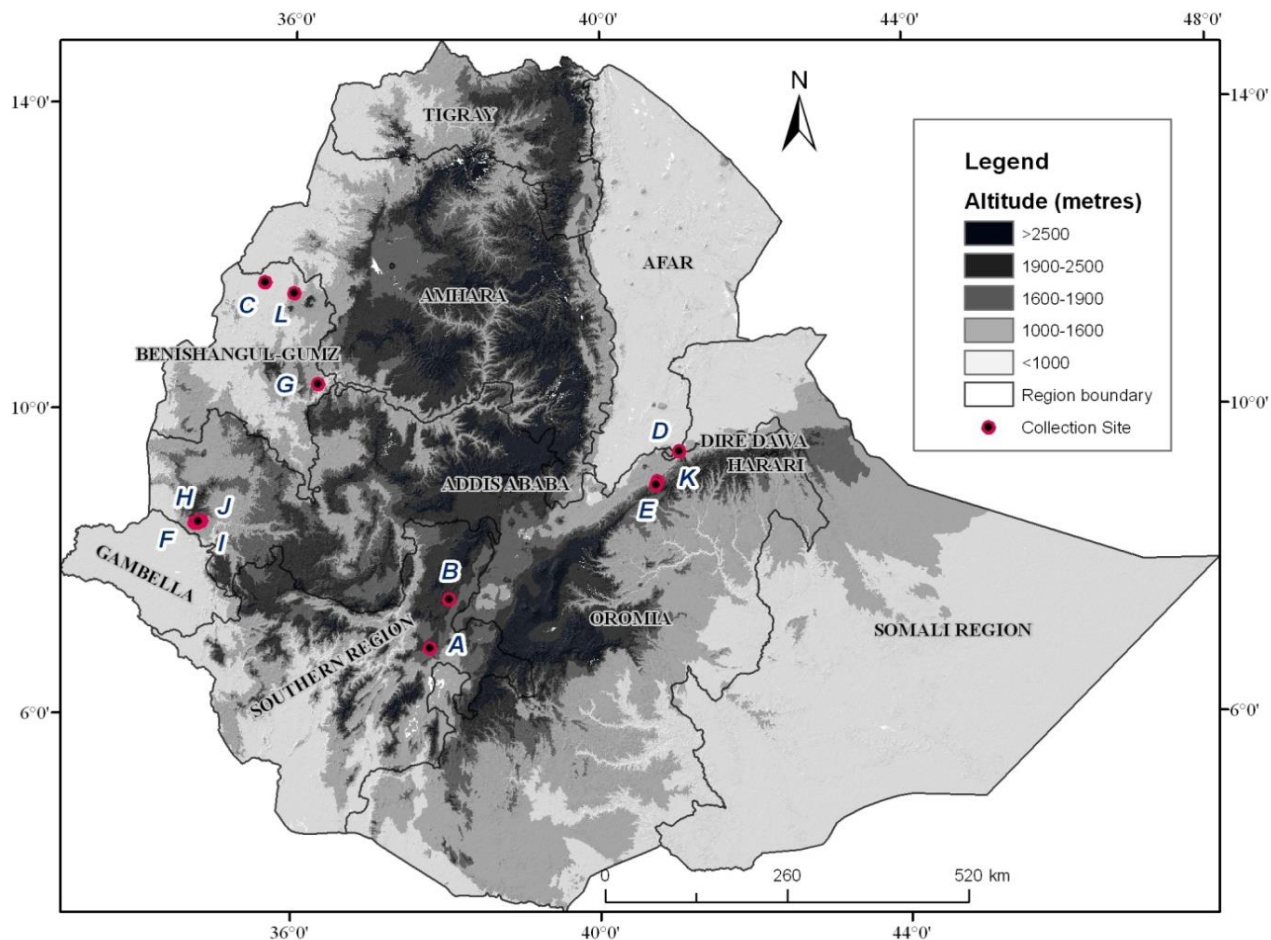
The common bean accessions were obtained from the Institute of Biodiversity and Conservation (IBC), Addis Ababa, Ethiopia. Each of the 12 landrace accessions was represented by eight individuals making the total number 96. The individual samples were sown in the greenhouse to obtain young leaves for DNA extraction. The accessions were collected from different lowland areas of Ethiopia (Table 1 and Figure 1).

### DNA extraction

About 300 mg young leaves from each representative plant sample were ground in liquid nitrogen and total template DNA was isolated by using Cetyl Trimethyl Ammonium Bromide (CTAB) method of Borsch et al. (2003) extraction protocol with slight modifications in the extraction fraction. Quality of the DNA was checked by electrophoresis of the samples on 1% (w/v) agarose gel and staining with ethidium bromide. Then, the template DNA samples were diluted with sterile double distilled water in 1:20 ratio, for PCR amplification.

### PCR amplification and electrophoresis

Nineteen ISSR primers (Sigma-Aldrich) were screened using



**Figure 1.** Map of Ethiopia showing sites where accession of *P. vulgaris* were collected. A = North Omo; B = Hadiya; C = Metekel (Dangure); D = Shinile; E = Harerge; F=West Wellega; G= Metekel (Dibate); H=West Wellega; I= West Wellega; J =West Wellega K= Harerge; L= Metekel (Dangure).

different individuals of common bean accessions, and then seven ISSR primers, 825, 827, 834, 836, 841,866 (Bold), that produced polymorphic and clear band pattern were selected for further study (Table 2). DNA amplification was carried out in a total volume of 25  $\mu$ l reaction mixture containing 2  $\mu$ l template DNA, 17.07  $\mu$ l of  $H_2O$ , 0.2  $\mu$ l of 100 mM with each dNTPs (Himedia, India), 2.5  $\mu$ l PCR buffer B (Himedia, India), 2.5  $\mu$ l of 25 mM  $MgCl_2$ , 0.5  $\mu$ l primer (20 pmol/ $\mu$ l) and 0.2  $\mu$ l of 5 U/ $\mu$ l firepol Taq DNA Polymerase (Solisbiodye, Estonia). Amplification was performed using Techne, Model FTC41H2D Thermal Cycler. ISSR amplification was carried out for a cycle of 5 min at 94°C, followed by 40 cycles of 30 s at 94°C, 1 min at 49/45°C, and 2 min at 72°C and a final cycle of 7 min at 72°C. The amplification products were resolved by electro-phoresis using 1.7% (w/v) agarose gel with 1 X TAE buffer. 10  $\mu$ l of each amplification product were loaded with 2  $\mu$ l of 6x loading dye. Gels were stained with ethidium bromide and band detection was performed using a BIO-RAD Gel Doc.

#### Data scoring and analysis

Each ISSR band was considered as an independent locus and polymorphic bands were scored as present (1) or absent (0) for all the 96 individual samples. Only clearly reproducible bands were scored and differences in band intensity were not considered. Data

analysis was conducted using only the polymorphic bands. Analysis of Molecular Variance (AMOVA) was used to calculate variation among and within accessions using Areliquin version 3.01 (Excoffier et al., 2006). NTSYS (Numerical Taxonomy and Multiware Analysis System) - pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek et al., 1999) software's were used to calculate Jaccard's similarity coefficient. The Unweighted Pair Group Method with Arithmetic mean (UPGMA) (Sneath and Sokal, 1973) was used to evaluate and compare individual samples and accessions, and generates dendrogram using Numerical taxonomy and multivariate analysis system (NTSYS)- pc version 2.02 (Rohlf, 2000). The Neighbor Joining (NJ) method (Saitou and Nei, 1987; Studier and Keppler, 1988) was used to compare individual genotypes and evaluate patterns of genotype clustering using Free Tree 0.9.1.50 Software (Pavlicek et al., 1999). A principal coordinate analysis was performed based on Jaccard (1908) for all individuals and a plot was generated using 3D coordinates.

## RESULTS

### ISSR primers and their banding patterns

The total number of scored bands varied from six for primer

**Table 2.** List of total primers, primer sequence and annealing temperatures used during the selection of polymorphic primer in *P. vulgaris* genetic analysis.

Primer code	Primer Sequence	Annealing temperature
CR 2	CACACACACACACAAG	49
809	AGAGAGAGAGAGAGAGG	49
810	GAGAGAGAGAGAGAGAT	45
812	GAGAGAGAGAGAGAGAA	45
818	CACACACACACACAAG	45
824	TCTCTCTCTCTCTCG	45
825	ACACACACACACACT	49
827	ACACACACACACACAG	49
834	AGAGAGAGAGAGAGAGYT	49
836	AGAGAGAGAGAGAGAGYA	49
841	GAGAGAGAGAGAGAGAYC	49
857	ACACACACACACACACYG	49
860	TGTGTGTGTGTGTGTGRA	49
866	CTCCTCCTCCTCCTCCTC	49
872	GATAGATAGATAGATA	45
873	GACAGACAGACAGACA	45
878	GGATGGATGGATGGAT	45
880	GGAGAGGAGAGGAGA	45
881	GGGTGGGGTGGGGTG	45

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

825 to 13 and 12 for primers 836 and CR-2 respectively (Table 2). The remaining primers, 827, 834, 841 and 866, showed eight, 11, ten and nine polymorphic bands, respectively, with a mean of 9.85 bands per primer and the size of the bands ranged from 100 bp to 800 bp. Figure 2 shows the amplification pattern of primer 836. All the primers showed 100% polymorphism. Among the 12 accessions, the highest gene diversity was obtained for Shinile ( $H = 0.29$ ); while Metekel (L) was the least diverse ( $H = 0.10$ ). In addition, similar values in gene diversity were found for North Omo ( $H = 0.28$ ) and Metekel (C) ( $H = 0.26$ ). The overall gene diversity for the total accession was 0.35 (Table 3). Relatively, similar gene diversity patterns were also observed in Shannon Diversity Index (I) (Table 3).

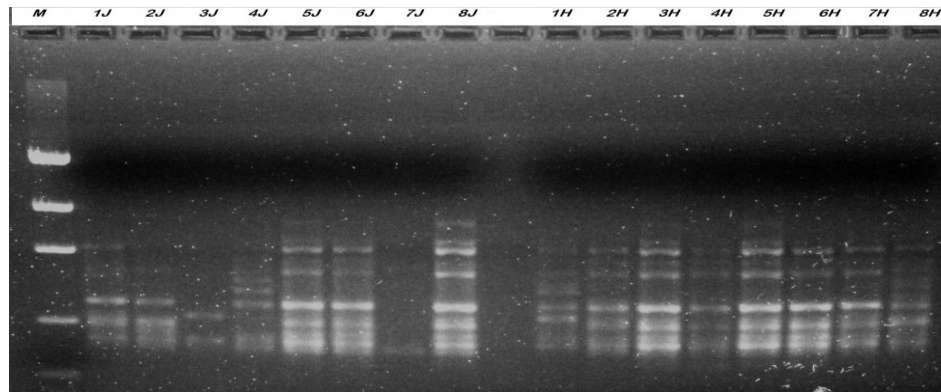
### Analysis of molecular variance (AMOVA)

Analysis of molecular variance on 12 accessions (with out grouping samples by region or geographic location) revealed that highest percentage of variation is attributed to variation within accessions (62.55%). Variation among accession accounted for the 37.45% of total variation. Generally, the result of AMOVA revealed patterns of genetic diversity and it supports the larger genetic diversity found

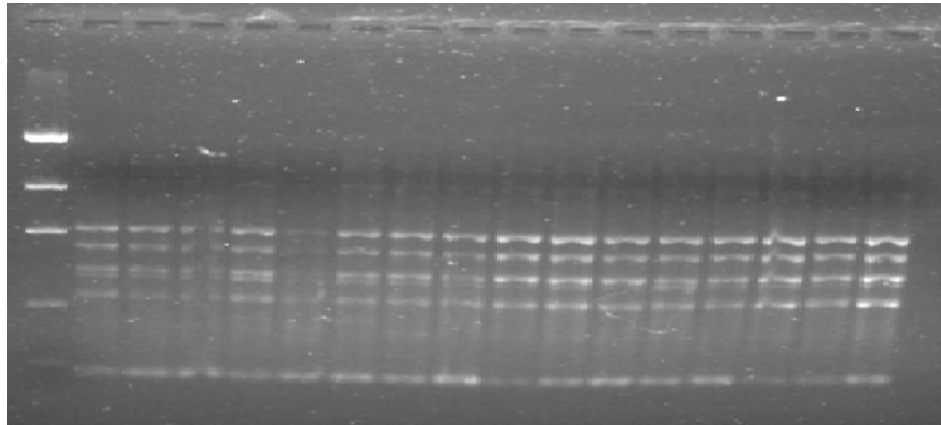
within the accessions but low genetic diversity was found among accessions (Table 5).

### Cluster analysis

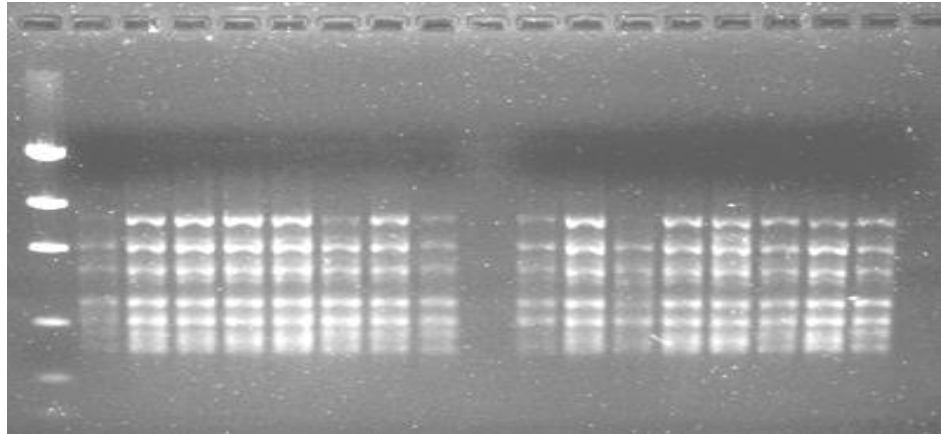
NJ tree showed that there were three major distinct clusters and two subclusters within the third major cluster (Figure 3). In some cases, individuals from each accession tended to form their own cluster; while few individuals from each accession were distributed all over the tree. Among the twelve accessions, Metekel (L) (major cluster I), North Omo (A) (major cluster II) and Harerge (E) (major cluster III, sub-cluster I) showed a relatively clear grouping UPGMA based analysis resulting in the separation of the twelve common bean accessions into two main clusters. Accession from Harerge (K) was an outlier whereas, the rest 11 accessions together grouped into clusters II (Figure 4). Jaccard similarity coefficient based pair wise comparisons showed that Harerge (E) and West Wellega (F) have a similarity coefficient value of 0.53; whereas Harerge (K) with accessions of Hadiya, Shinile and West Wellega (F) showed a distant relation with similarity coefficient of 0.28, 0.29 and 0.29, respectively (Table 6).



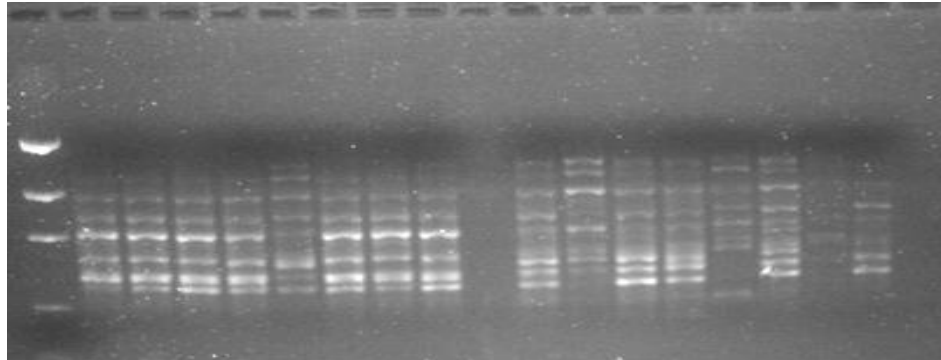
Primers 834



Primers 836



Primer CR-2



**Figure 2.** 12 ISSR fingerprints generated using primer 836, 834, CR-2. J1- J8, H1-H8 are both accessions from *W.Wellega*.

**Table 3.** Number of scorable bands (NSB), number of polymorphic loci (NPL), percent polymorphism (PP), genetic diversity (H), and standard deviation (SD) and Shannon diversity index (I) for each primer.

Primers	NSB	NPL	PP	H±SD	I±SD
825	6	6	100	0.23± 0.13	0.38± 0.16
827	8	8	100	0.32± 0.12	0.12± 0.16
834	11	11	100	0.34± 0.12	0.52± 0.14
836	13	13	100	0.39± 0.10	0.57± 0.12
841	10	10	100	0.31± 0.14	0.48± 0.17
866	9	9	100	0.44± 0.10	0.62± 0.12
CR-2	12	12	100	0.40± 0.08	0.58± 0.09
Average	9.85	9.85	100	0.35±0.11	0.47±0.14
Over all	69	69	100	0.35±0.12	0.53± 0.15

**Table 4.** Number of polymorphic loci (NPL), percent polymorphism (PP), and genetic diversity (H), Shannon Index (I) and standard deviation (SD) for all accessions.

Accessions	NPL	PP (%)	H±SD	I±SD
North Omo (A)	49	71.01	0.28 ± 0.20	0.41± 0.28
Hadiya (B)	39	56.52	0.21 ± 0.21	0.31± 0.30
Metekel (Dangure)(C)	49	71.01	0.26± 0.19	0.39± 0.27
Shinile (D)	51	73.91	0.29± 0.20	0.43± 0.28
Harerge (E)	27	39.13	0.16± 0.21	0.24± 0.30
W. Wellega (F)	30	43.48	0.18± 0.22	0.27± 0.31
Metekel (Dibate) (G)	29	42.03	0.18± 0.22	0.26± 0.31
W. Wellega (H)	44	63.77	0.24± 0.20	0.36± 0.29
W. Wellega (I)	48	69.57	0.24± 0.19	0.36± 0.27
W. wellega (J)	49	71.01	0.23± 0.19	0.35± 0.26
Harerge (K)	32	46.38	0.17± 0.20	0.25± 0.29
Metekel (Dangure) (L)	19	27.54	0.10± 0.19	0.15± 0.27

**Table 5.** Analysis of molecular variance (AMOVA) among accessions of *P. vulgaris* without grouping the populations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Significance
Among accessions	11	287.719	2.70480	37.45	0.00
Within accessions	84	379.500	4.51786	62.55	0.00
Total	95	667.219	7.22266	100	

Moreover, individual based UPGMA clustering of an overall analysis showed a strong clustering of individuals with respect to their accessions, like the two individuals of Metekel (G and L), where as some individuals of Hadiya(B) were intermixed with Harerge (E) (Figure 5). This result is similar to that of individual based NJ analysis.

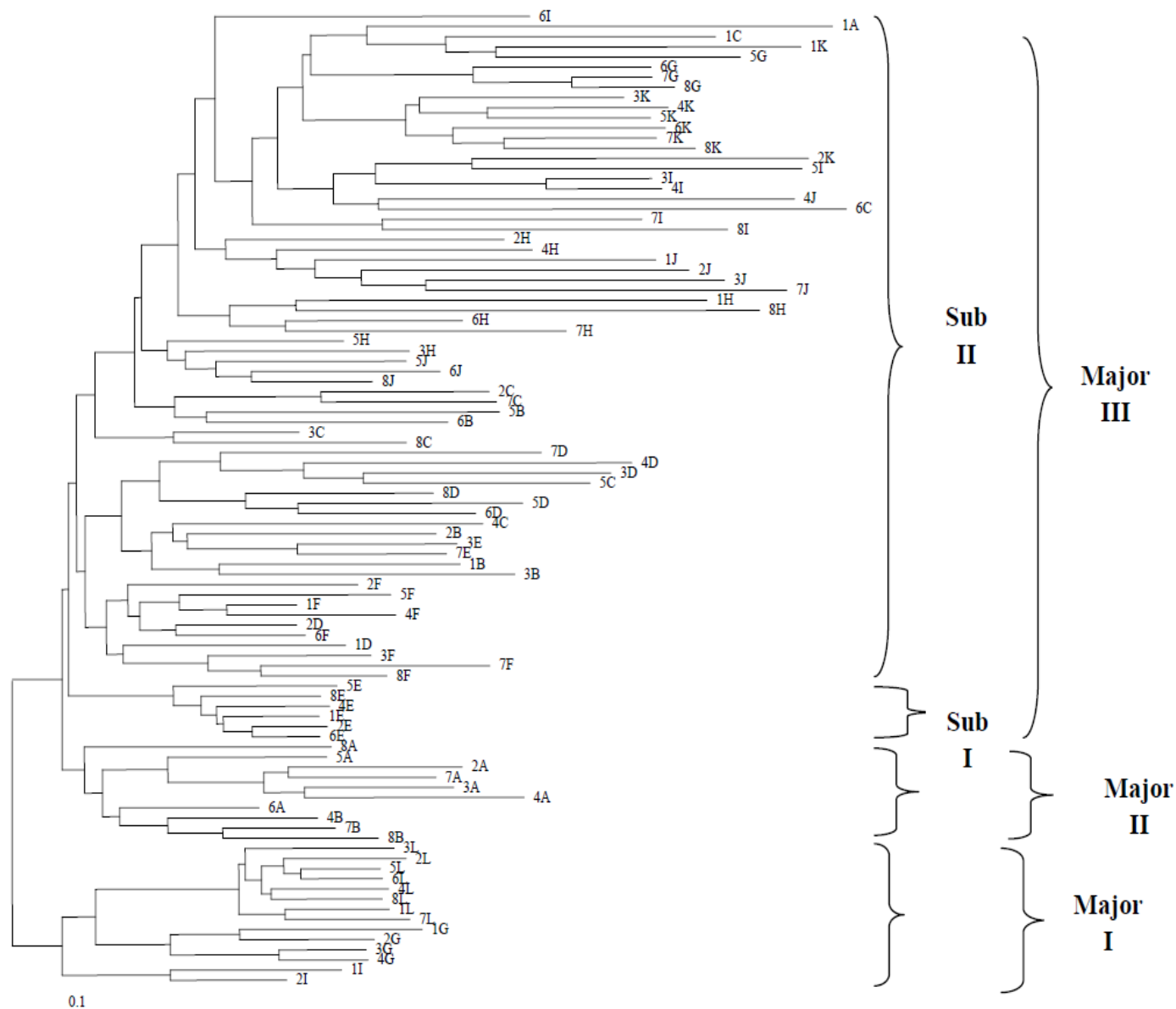
### Principal coordinate analysis (PCoA)

The first three coordinates of the PCoA having Eigen values of 4.95, 3.41 and 2.25 with a variance of 12.22, 8.41 and 5.56%, respectively, were used to reveal the

grouping of individuals (Figure 6). Most individuals from Metekel (L) tended to form their own grouping. The remaining individuals from each accession did not show a clear assembly rather they were inter-mixed with each other. Moreover, mixed individuals did not show separate group within each other (Figure 6).

### DISCUSSION

In this study, all the diversity parameters verify that there is medium to high gene diversity in accessions of *P. vulgaris*. Accessions from Shinile, North Omo, Metekel (C) and

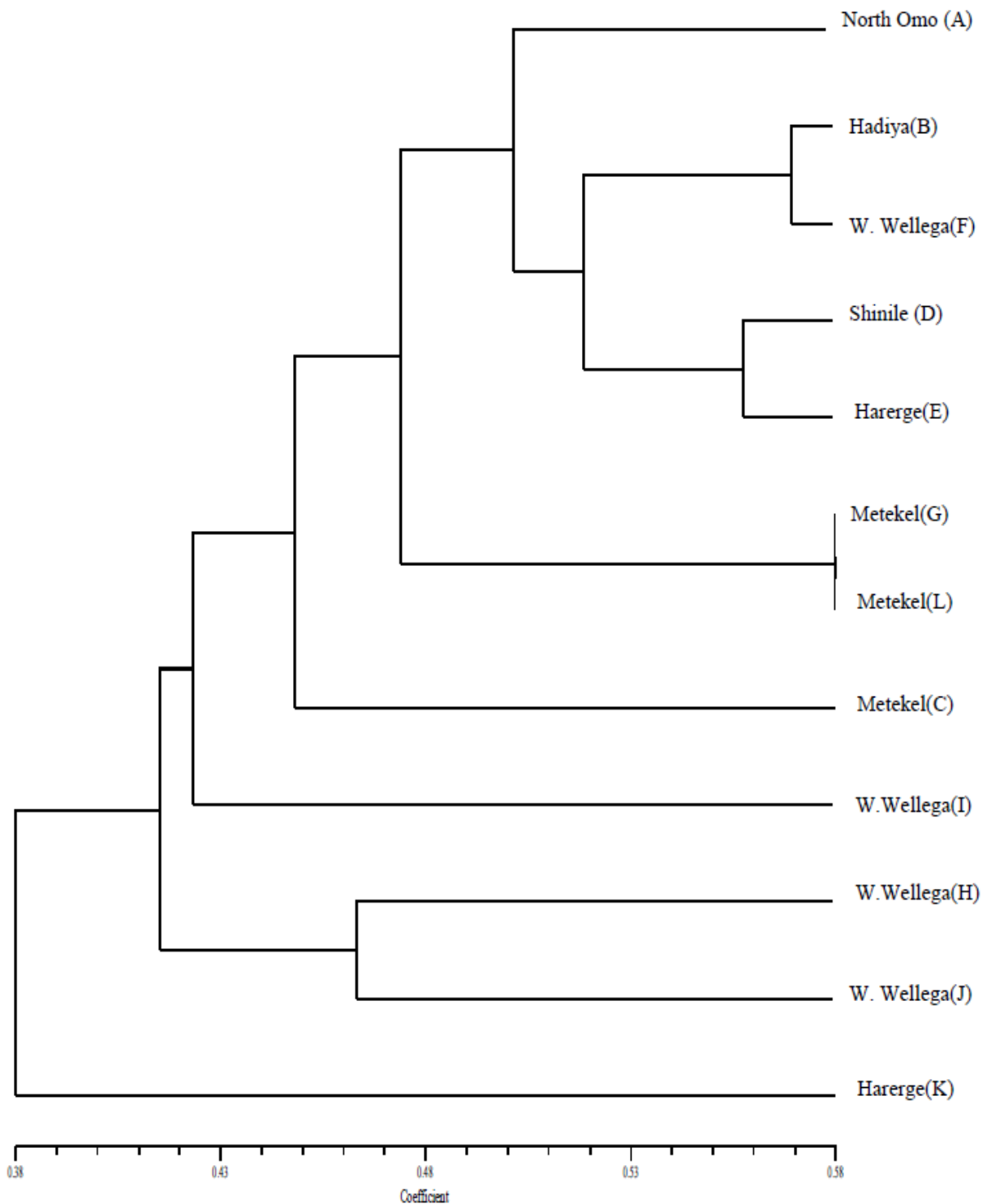


**Figure 3.** Neighbor-joining analysis of 96 individuals. A = North Omo; B = Hadiya; C = Metekel (Dangure); D = Shinile; E = Harerge; F=West Wellega; G= Metekel (Dibate); H=West Wellega; I= West Wellega; J =West Wellega K= Harerge; L= Metekel (Dangure).

West Wellega (H) having 0.29, 0.28, 0.26 and 0.24 gene diversity, respectively, were indeed high. Similarly, the result from Shannon diversity index illustrates that individuals from Shinile were the most diverse ( $I=0.43$ ). The overall total Nei's gene diversity and Shannon's diversity index were high, 0.35 and 0.53, respectively. This result is in agreement with data obtained from the study of other bean species from tropical, subtropical or warm temperate regions that have been recently studied with ISSR markers, such as *Vicia faba* (Abdel-Razzak et al., 2012), and *Dalbergia cochinchinensis* (Hien and Phong, 2012). Our result is also in line with the reports of Sadeghi and Cheghamirza (2012) that accessions of *P.*

*vulgaris* showed high genetic diversity using ISSR markers.

AMOVA showed that there is higher genetic variation within accessions (62.55%) than among accessions (37.45%). Cruz et al. (2004), studying wild accessions of *P.vulgaris* L. from Mexico using ISSR markers, reported similar results, 55 to 60% and 40 to 45% of genetic diversity, within and among accession, respectively. A number of reasons may contribute for the high levels of genetic diversity within accessions. These could be high seed exchange among community, human activities during harvesting and marketing and presence of large accession size. Hence, these may lead to the intermixing



**Figure 4.** UPGMA based dendrogram for 12 *P. vulgaris* accessions collected from different areas of Ethiopia (D, E), (G, L) and (H, J) show clustering. W.Wellega=West wellega.

of accessions. In general, in breeding species with more or less continuously distributed accessions, the proportion of internal variation is often above 70%; while below 30%

is attributed to inter accession difference (Hamrick et al., 1992).

The UPGMA analysis showed that each accession formed



**Table 6.** Pairwise Jaccard similarity coefficient based comparisons among 12 accessions of *P. vulgaris*.

Accession	North Omo(A)	Hadiya(B)	Metekel (Dangure)(C)	Shinile (D)	Harerge(E)	* W. wellega (F)	Metekel(G)	W. wellega(H)	W. wellega(I)	W.wellega(J)	Harerge(K)	Metekel(L)
A												
B	0.485											
C	0.409	0.418										
D	0.417	0.434	0.436									
E	0.503	0.5	0.443	0.526								
F	0.454	0.527	0.402	0.480	0.531							
G	0.375	0.410	0.414	0.336	0.414	0.413						
H	0.400	0.436	0.363	0.370	0.463	0.435	0.372					
I	0.390	0.382	0.336	0.385	0.424	0.404	0.408	0.394				
J	0.408	0.365	0.370	0.392	0.424	0.377	0.297	0.437	0.36			
K	0.421	0.284	0.392	0.299	0.336	0.296	0.418	0.343	0.385	0.357		
L	0.488	0.437	0.388	0.402	0.527	0.515	0.512	0.406	0.42	0.356	0.367	

\* W. Wellega =West Wellega.

a discrete cluster based on accessions from nearby localities, for example, Shinile and Harerge (E), accessions from Metekel (G, L) and the two accessions from West Wellega (H, J). This clustering may be formed because of seed movement between the areas because of geographical and cultural factors. On the otherhand, accessions from different localities may tend to form their own clustering like Hadiya and W.Wellega (F). This indicates that there may be massive seed movements in distant geographical areas. This movement is associated with response to recurrent drought donation by relief agencies such as NGOs and governmental organizations as well as during marketing. The separation and formation of a cluster between accessions may suggest that accessions may have been isolated from each other for a longer period in time and as a result there was limited gene flow due to long distance. Genetic variability is useful for heterotic patterns in hybrid breeding and for relating the observed pattern with presence of certain economically important traits. Such information can be used to design effective germplasm conservation and for setting germplasm collection task as well as to guess or predict the risk of genetic erosion in certain areas. So, understanding the genetic diversity of *P.vulgaris* will be valuable for further crop improvement through breeding programs and for taking appropriate conservation efforts.

PCoA analysis showed similar result to UPGMA except most individuals from Metekel (L) tended to form their own grouping. The remaining individuals from each accession did not show a clear assembly rather they were inter-mixed with each other. The reason for these inter mixing could be explained by gene flow from one area to the other due to various factors, for example marketing.

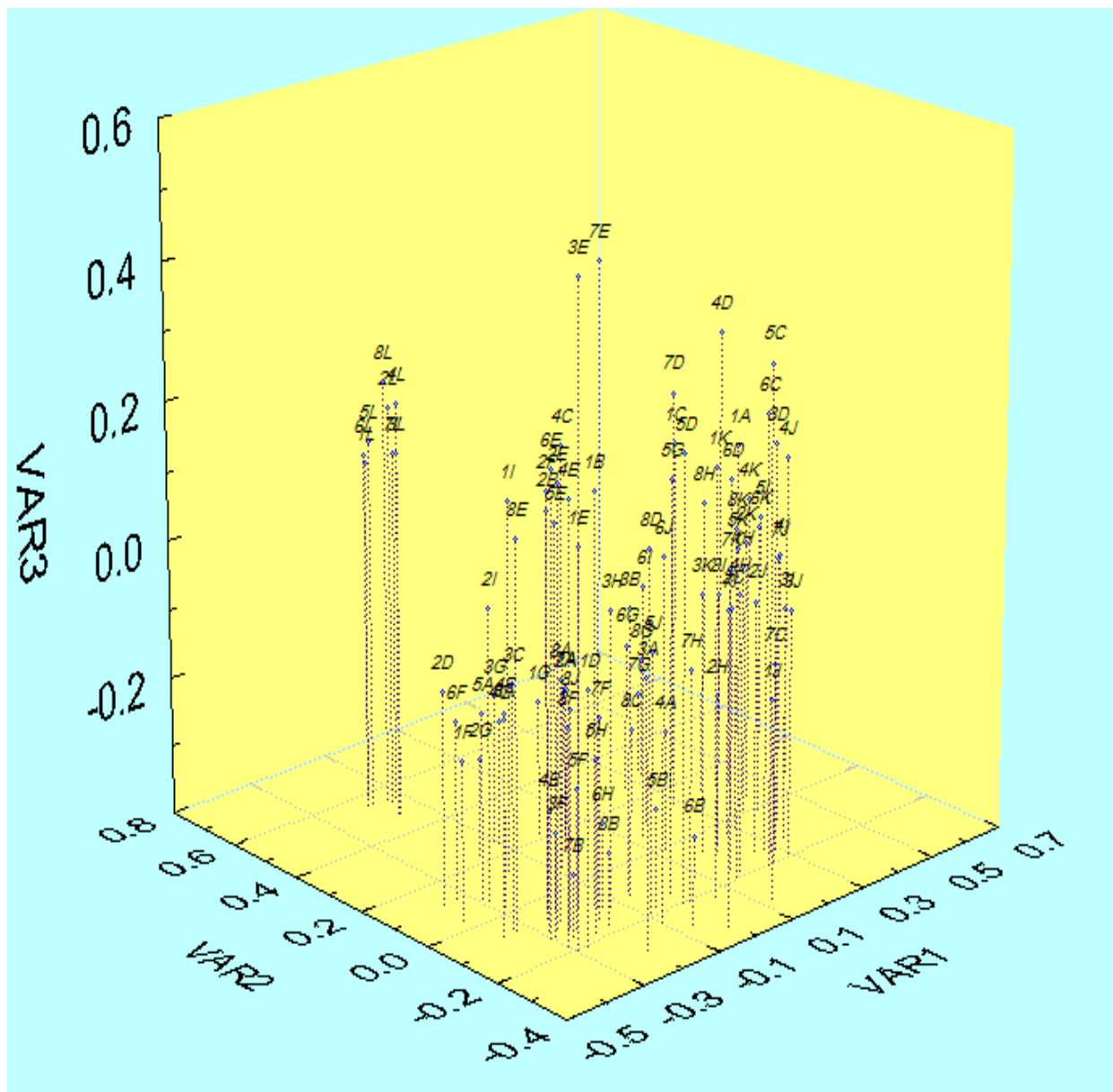
## Conclusion

The present study shows that the existence of wide range of genetic diversity in common bean accessions of Ethiopia which mostly resides within accessions, and this indicated that different regions harbor high levels of diversity. This is an important landmark both for improvement and conservation programs. Although, the study was based on a limited number of markers, this observation should be taken in to account in planning future conservation and research programs of the species. Furthermore, the wider molecular variability observed represents a good indication for breeding programs. This is an encouraging result for further collection activities so as to capture more variability from other agroecologies of the country. The current common bean collection in the Ethiopian gene bank contains predominantly samples from South, East and West parts of the country. Thus, it would be useful to increase representative samples from Northern parts of the country to capture the maximum diversity. Furthermore, the present study showed accessions from Shinile and North Omo sites exhibited higher genetic diversity than accessions from Harerge (E). These areas may have a conducive environment for better adaptation of the plant.

## ACKNOWLEDGEMENTS

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**Figure 6.** Three dimensional representation of principal coordinate analysis of genetic relationships among 96 individuals of twelve *P. vulgaris* accessions. W.Wellega=West wellega; A =North Omo; B= Hadiya; C= Metekel; D=Shinile; E=Harerge; F=West wellega; G= Metekel; H=West wellega; I= West wellega; J=West wellega; K= Harerge; L= Metekel.

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## Full Length Research Paper

## Molecular characterization of *Trichoderma* sp. isolated from rhizospheric soils of Uttar Pradesh (India) based on microsatellite profiles

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The objectives of this research were to characterize isolates of *Trichoderma* collected from rhizospheres of chickpea, pigeonpea and lentil crop from different places of Uttar Pradesh, India, using microsatellite-primed polymerase chain reaction (MP-PCR) and ribosomal DNA (rDNA) sequence analysis and to combine these results with morphological characteristics for classification. Thirty isolates of *Trichoderma* sp. obtained from rhizosphere soil of plantation crops, and agricultural fields of UP region were studied using inter-simple sequence repeat (ISSR) and Internal transcribed spacer- polymerase chain reaction (ITS-PCR). The genetic relatedness among 15 isolates of *Trichoderma* sp. was analyzed with six microsatellite primers. ISSR profiles showed 83.7% genetic diversity among the isolates with the formation of four clusters. Analysis of dendrogram revealed that similarity coefficient ranged from 0.27 to 0.95. ITS-PCR of rDNA region with ITS1 and ITS4 primers produced 600 bp products in all isolates. This result presented the identification patterns of *Trichoderma* isolates.

**Key words:** *Trichoderma* sp., genetic diversity, polymerase chain reaction (PCR), molecular marker, microsatellite.

### INTRODUCTION

Soil microorganisms influence ecosystems by contributing to plant nutrition (Alan et al., 1998), plant health (Bruns et al., 1991), soil structure (Castle et al., 1998) and soil fertility. It has been widely recognized, particularly in the last two decades, that majority of harsh environments are inhabited by surprisingly diverse microbial communities. Bacteria, actinomycetes and fungi are three major groups of soil inhabiting microorganisms.

An estimated 1,500,000 species of fungi exist in the

world (Anu et al., 2010).

*Trichoderma*, commonly available in soil and root ecosystems has gained immense importance since the last few decades due to its biological control ability against several plant pathogens (Elad and Chet, 1983). Antagonistic microorganisms, such as *Trichoderma*, reduce growth, survival or infections caused by pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions and enzyme secretion. In addition,

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the release of biocontrol agents into the environment has created a demand for the development of methods to monitor their presence or absence in soil (Giller et al., 1997). Therefore, monitoring population dynamics in soil is of much importance. Previous methods employed to identify strains of *Trichoderma* spp. in soil samples have included the use of dilution plates on selective media (Lieckfeldt et al., 1999). However, this method does not distinguish between indigenous strains and artificially introduced ones (Knudsen et al., 1996). The *Trichoderma* isolates were differentiated by mycelia growth rate and colony appearance, as well as microscopic morphological features, including phialides and phialospores (Hibbett, 1992). These can also be distinguished by randomly amplified polymorphic DNA (ISSR)-PCR, restriction fragment length polymorphisms in mitochondrial DNA and ribosomal DNA and sequence analysis of ribosomal DNA (Mukherjee et al., 2013; Kubicek and Harman, 1998; Bryan et al., 1995). The use of molecular phylogenetic markers has refined *Trichoderma* taxonomy significantly, and phylogenetic analysis of the large number of *Trichoderma* species is still a field of active ongoing research. Microsatellites, which are also known as short tandem repeats or simple sequence length polymorphisms, are stretches of tandem mono-, di-, tri-, and tetranucleotide repeats of varying lengths (Sagar et al., 2011). Such sequences are widely dispersed in eukaryotic genomes including those of fungi; they are also present but less frequent in prokaryotic genomes.

## MATERIALS AND METHODS

### Isolation and Identification of *Trichoderma*

*Trichoderma* isolates were originally isolated from soil collected from rhizospheres of chickpea, pigeonpea and lentil crop from different places of Uttar Pradesh, India, and *Trichoderma* isolates were isolated on Potato Dextrose Agar medium by following serial dilution plate technique. They were cultured on PDA 25°C for seven days. After an incubation period, colonies were purified and determined to be *Trichoderma* species and confirmed using *Trichoderma* morphological key. The identity of the purified bioagents was then confirmed by ITCC, Division of Plant Pathology IARI, New Delhi-12. Single-spore isolates of 30 *Trichoderma* isolates were cultured in Erlenmeyer flasks (250 ml) containing 100 ml potato dextrose broth at 25°C for seven days. Mycelia were harvested by filtration through whatman filter paper. Samples were frozen in liquid nitrogen and ground to fine powder using a mortar and pestle (Shahid et al., 2014).

### Genomic DNA extraction from *Trichoderma* Isolates

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. The mycelia were incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 100 mM NaCl and 2% SDS, for 1 h at 60°C followed by centrifugation at 12,000 rpm for 15 min (Shahid et al., 2014). The supernatant was then extracted with equal volume of water saturated phenol and further centrifuged at 12,000 rpm for 10 min; the aqueous phase was further extracted with equal volume of phenol: chloroform: isoamyl

alcohol (25:24:1) and centrifuge at 12,000 rpm for 15 min; the aqueous phase was then transferred in a fresh tube and the DNA was precipitated with chilled ethanol (100%) (Yao et al., 1992). DNA was pelleted by centrifuging at 12000 rpm for 15 min and washed in 70% ethanol by centrifugation. The pellets were air dried and suspended in TE buffer (pH 8.0).

### Qualitative and quantitative estimation of DNA

The extraction of total genomic DNA from the *Trichoderma* isolates as per the above procedure was followed by RNAase treatment. Genomic DNA was re suspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse (60 µg). After incubation the sample was reextracted with PCI (phenol: chloroform: isoamyl-alcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both and isolates of *Trichoderma* were taken up for ITS-PCR spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

### PCR Amplification of its region of *Trichoderma* Isolates

Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase chain reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25% bromophenol blue, 40% w/v sucrose in water and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination with horizontal electrophoresis.

### ISSR of *Trichoderma* Isolates

For ISSR, six microsatellite primers that is, A-1; A-2; A-3; A-4; A-5 and A-6 were selected (Table 1). PCR was programmed with an initial denaturing at 94°C for 4 min followed by 35cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 70°C for 90 s and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25% bromophenol blue, 40% w/v sucrose in water and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination by horizontal electrophoresis (Venkateswarlu et al., 2008).

### Scoring and data analysis

The image of the gel electrophoresis was documented through Bio-Profil Bio-1D gel documentation system and analysis software. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed *In silico* into similarity matrix using Numerical Taxonomy System Biostatistics, (NTSYSpc version 2.11W) (Muthumeenakshi et al, 1994). The SIMQUAL program was used to calculate the Jaccard's coefficients. The ISSR patterns of each isolate was evaluated, assigning character state "1" to indicate the presence of band in the gel and "0" for its absence in the gel.

Thus a data matrix was created which was used to calculate the

**Table 1.** The nucleotide sequence used for ITS and *Trichoderma* PCR.

Primer Name	Sequence( 5'-3')	Mer	TM	GC (%)
<b>ITS-Primers pairs</b>				
T/ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57
T/ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45
<b>ISSR primers</b>				
A-1	5'YC (TG) <sub>7</sub> T3'	17	49.77	47
A -2	5'(GA) <sub>9</sub> AC3'	20	53.70	55
A-3	5'(GA) <sub>9</sub> T3'	20	58.01	47
A-4	5'(GA) <sub>8</sub> AC3'	18	56.35	40
A-5	5'(AG) <sub>8</sub> AC3'	18	60.17	50
A-6	5'(AG) <sub>8</sub> AT3'	18	60.26	47

**Table. 2** Isolates of *Trichoderma* spp.

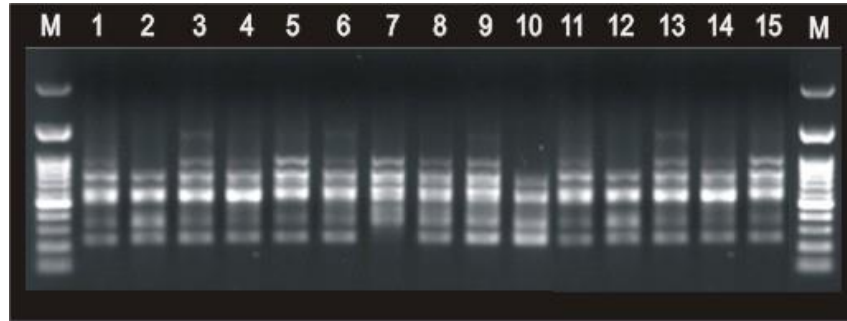
S/N	ITCC number New Delhi	IARI,	Culture number	Reference number	Source	Fungus identified
T1	ITCC-7437/09		21PP	01	Kausambi	<i>Trichoderma longibrachiatum</i>
T2	ITCC-7438/09		31PP	02	Allahabad	<i>Trichoderma longibrachiatum</i>
T3	ITCC-7439/09		81PP	03	Mirzapur	<i>Trichoderma longibrachiatum</i>
T4	ITCC-7440/09		100PP	04	Sonbhadra	<i>Trichoderma longibrachiatum</i>
T5	ITCC-7441/09		120PP	05	Bhadohi	<i>Trichoderma longibrachiatum</i>
T6	ITCC-7442/09		06 CP	06	Sultanpur	<i>Trichoderma atroviride</i>
T7	ITCC-7443/09		24CP	07	Sitapur	<i>Trichoderma atroviride</i>
T8	ITCC-7444/09		28CP	08	Barabanki	<i>Trichoderma longibrachiatum</i>
T9	ITCC-7445/09		71L	09	Hardoi	<i>Trichoderma atroviride</i>
T10	ITCC-7446/09		115L	10	Bahraich	<i>Trichoderma atroviride</i>
T11	ITCC-7447/09		52L	11	Unnao	<i>Trichoderma atroviride</i>
T12	ITCC-7448/09		75PP	12	Auriya	<i>Trichoderma atroviride</i>
T13	ITCC-7449/09		126PP	13	Kanpur Dehat	<i>Trichoderma atroviride</i>
T14	ITCC-7450/09		5 CP	14	Kanpur Nagar	<i>Trichoderma longibrachiatum</i>
T15	ITCC-7451/09		105PP	15	Etawah	<i>Trichoderma atroviride</i>

Jaccard similarity coefficient for each pair wise comparison. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc (Knudsen et al., 1996).

## RESULTS AND DISCUSSION

Thirty isolates were obtained using the *Trichoderma* selective medium from the rhizosphere soil (Table 2). The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Muthumeenakshi et al., 1994). They also occur in multiple copies with up to 200 copies per haploid genome (Ospina-Giraldo et al., 1999; Ospina-Giraldo et al., 1998) arranged in tandem repeats with each repeat

consisting of the 18S small subunit (SSU), the 5.8S and the 28S large subunit (LSU) genes. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Rohlf, 1993). In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies. In the present study, we focused on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *Trichoderma* spp. ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 primers. These results are in accordance with several workers who observed the amplified rDNA fragment of approximately 500 to 600 bp by ITS-PCR in *Trichoderma* (Sagar et al., 2011; Seaby, 1996). The ITS PCR has helped to detect polymorphism at ITS region of rDNA among the *Trichoderma* isolates (Figure 1). Products of size in the range of 600 bp were



**Figure 1.** PCR amplification of ISSR (A1 marker) of *Trichoderma* species (Lane 1-15). Lane M, Low range DNA Marker (1 kb); Lane 1, ITCC,7437/21PP; lane 2, ITCC,7438/31PP; Lane 3, ITCC,7439/81PP; Lane 4, ITCC,7440/100PP; Lane 5, ITCC,7441/120PP; lane 6, ITCC,7442/06 CP; lane 7, ITCC,7443/24CP; lane 8, ITCC,7444/28CP; Lane9, ITCC,7445/71L; lane 10, ITCC,7446/115L; Lane 11, ITCC,7447/52L; lane 12, ITCC,7448/75PP; lane 13, ITCC,7449/126PP; lane 14, ITCC,7450/5 CP; lane 15, ITCC,7451/105PP.



**Figure 2.** PCR amplification of ISSR (A2 marker) of *Trichoderma* species (Lane 1-15). Lane M, Low range DNA Marker (1 kb); Lane 1, ITCC,7437/21PP; lane 2, ITCC,7438/31PP; Lane 3, ITCC,7439/81PP; Lane 4, ITCC,7440/100PP; Lane 5, ITCC,7441/120PP; lane 6, ITCC,7442/06 CP; lane 7, ITCC,7443/24CP; lane 8, ITCC,7444/28CP; Lane9, ITCC,7445/71L; lane 10, ITCC,7446/115L; Lane 11, ITCC,7447/52L; lane 12, ITCC,7448/75PP; lane 13, ITCC,7449/126PP; lane 14, ITCC,7450/5 CP; lane 15, ITCC,7451/105PP.

produced by Mukherjee et al. (2002) who studied the identification and genetic diversity analysis.

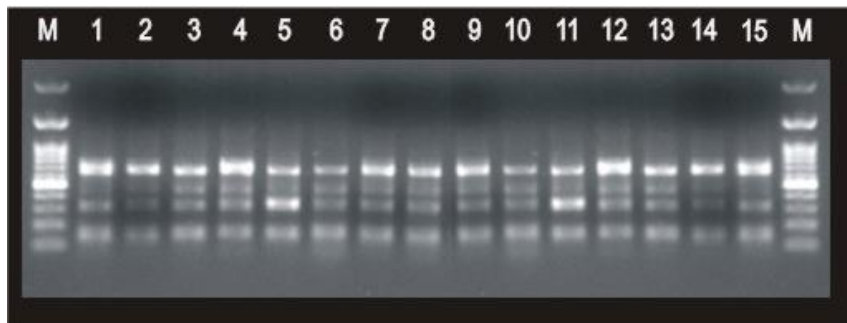
The genetic relatedness among 30 isolates of *Trichoderma* sp. and were analyzed by six microsatellite primers A-1 (Figure 1) give four polymorphic loci; A-2 (Figure 2) give six polymorphic loci; A-3 (Figure 3); give 9 polymorphic loci A-4 (Figure 4); give three polymorphic loci A-5 (Figure 5) give four polymorphic loci (Smith and Goodman, 1999). All amplified products with the primers had shown polymorphic and distinguishable banding patterns which indicate the 83.7% genetic diversity of *Trichoderma* isolates. A total of 31 reproducible and 26 scorable polymorphic bands ranging from approximately 100 to 2000 bp were generated with six primers among the 15 *Trichoderma* isolates (Table 3). ISSR profiles showed that primer A-2 and A3 scored highest bands which ranged between 100 to 2000 bp. Relationships among the isolates was evaluated by cluster analysis of

the data based on the similarity matrix (Figure 6). The dendrogram was generated UPGMA using NTSYSpc software. Analysis of dendrogram revealed that similarity coefficient ranged from 0.27 to 0.95. Based on the results obtained all the 15 isolates can be grouped into four main clusters. First cluster represents five *Trichoderma* isolates, second contains three, third cluster contain three and finally fourth cluster contain four isolates, respectively. Shahid et al. (2014) also revealed that *Trichoderma* sp. also showed the robust polymorphism which were collected from different geographical locations of Uttar Pradesh, India (Yao et al., 2010; Wright and Upadhyaya, 1998).

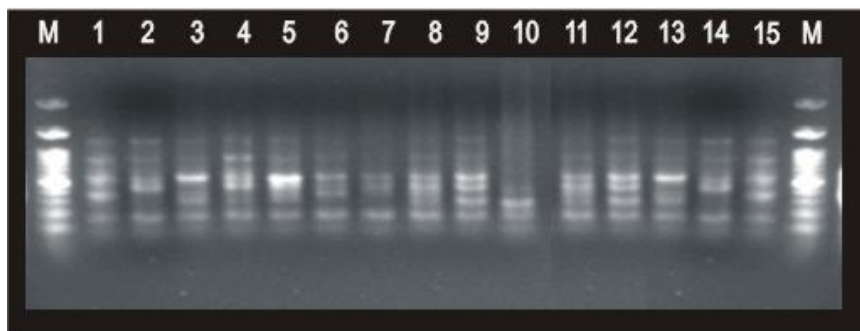
## Conclusion

Preliminary studies indicates that the *Trichoderma* isolates

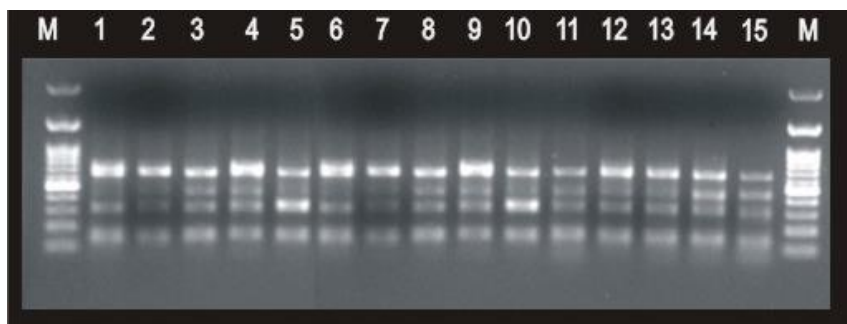




**Figure 3.** PCR amplification of ISSR (A3 marker) of *Trichoderma* species (Lane 1-15). Lane M, Low range DNA Marker (1 kb); Lane 1, ITCC,7437/21PP; lane 2, ITCC,7438/31PP; Lane 3, ITCC,7439/81PP; Lane 4, ITCC,7440/100PP; Lane 5, ITCC,7441/120PP; lane 6, ITCC,7442/06 CP; lane 7, ITCC,7443/24CP; lane 8, ITCC,7444/28CP; Lane9, ITCC,7445/71L; lane 10, ITCC,7446/115L; Lane 11, ITCC,7447/52L; lane 12, ITCC,7448/75PP; lane 13, ITCC,7449/126PP; lane 14, ITCC,7450/5 CP; lane 15, ITCC,7451/105PP.



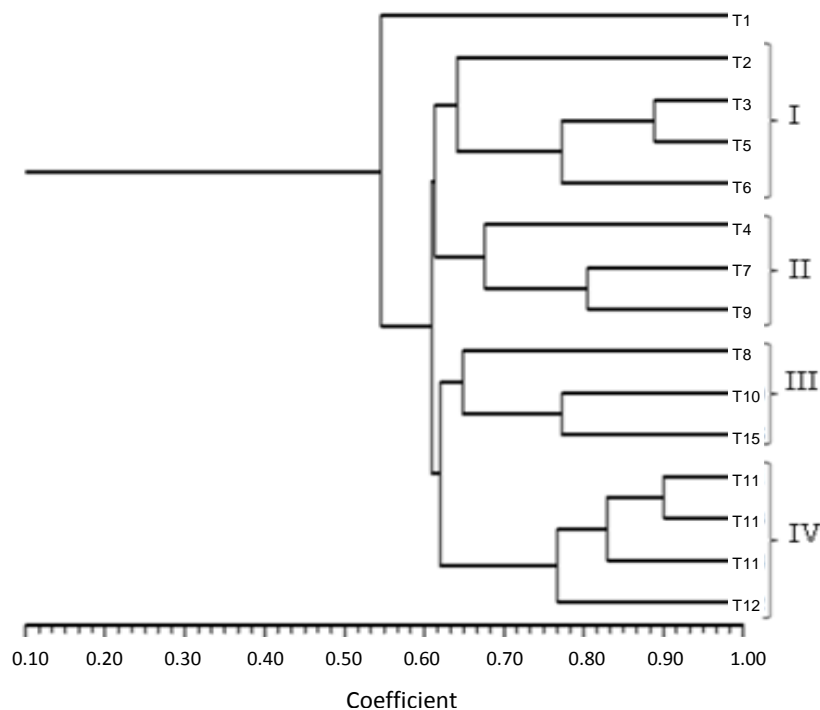
**Figure 4.** PCR amplification of ISSR (A4 marker) of *Trichoderma* species (Lane 1-15). Lane M, Low range DNA Marker (1 kb); Lane 1, ITCC,7437/21PP; lane 2, ITCC,7438/31PP; Lane 3, ITCC,7439/81PP; Lane 4, ITCC,7440/100PP; Lane 5, ITCC,7441/120PP; lane 6, ITCC,7442/06 CP; lane 7, ITCC,7443/24CP; lane 8, ITCC,7444/28CP; Lane9, ITCC,7445/71L; lane 10, ITCC,7446/115L; Lane 11, ITCC,7447/52L; lane 12, ITCC,7448/75PP; lane 13, ITCC,7449/126PP; lane 14, ITCC,7450/5 CP; lane 15, ITCC,7451/105PP.



**Figure 5.** PCR amplification of ISSR (A5 marker) of *Trichoderma* species (Lane 1-15). Lane M, Low range DNA Marker (1 kb); Lane 1, ITCC,7437/21PP; lane 2, ITCC,7438/31PP; Lane 3, ITCC,7439/81PP; Lane 4, ITCC,7440/100PP; Lane 5, ITCC,7441/120PP; lane 6, ITCC,7442/06 CP; lane 7, ITCC,7443/24CP; lane 8, ITCC,7444/28CP; Lane9, ITCC,7445/71L; lane 10, ITCC,7446/115L; Lane 11, ITCC,7447/52L; lane 12, ITCC,7448/75PP; lane 13, ITCC,7449/126PP; lane 14, ITCC,7450/5 CP; lane 15, ITCC,7451/105PP.

**Table 3.** Analysis of the polymorphism obtained with ISSR markers in 15 *Trichoderma* sp.

S/N	Primers	Total loci	Polymorphic loci	Polymorphism (%)
A-1	5'(GA) <sub>9</sub> AC3'	6	4	67
A -2	5'(GA) <sub>9</sub> T3'	6	6	100
A-3	5'(GA) <sub>8</sub> AC3'	9	9	100
A-4	5'(AG) <sub>8</sub> AC3'	5	3	60
A-5	5'(AG) <sub>8</sub> AT3'	5	4	80
Total		31	26	83.87

**Figure 6.** ISSR based phylogenetic trees.

had very good diversity and there are strong possibility to get the isolate specific primers that will be utilized for identifying the particular *Trichoderma* isolates with good biological potential from the field isolates without carrying out the cumbersome bioassay again

### Conflict of Interests

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

### ACKNOWLEDGEMENT

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## Full Length Research Paper

## Evaluation of different methods to overcome *in vitro* seed dormancy from yellow passion fruit

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Seeds from yellow passion fruit (*Passiflora edulis* Sims) present dormancy imposed by the seed-coat. The present study aimed to evaluate some methods to overcome dormancy of seeds from *P. edulis* grown under *in vitro* conditions. The experimental design was completely randomized in factorial scheme (15 scarification methods × 2 substrates: PlantMax<sup>®</sup> and MS medium), constituting 30 treatments with 10 replicates. The following treatments were established: Control (untreated seeds); seeds soaked in water at room temperature for 12, 24 and 48 h; seeds soaked in hot water at 80°C for 1, 2.5 and 5 min; chemical scarification with sulphuric acid (98%) for 1, 5, and 10 min; immersion of the seeds in absolute ethanol for 5, 10 and 25 min; physical scarification with wood sandpaper (no. 125); and total seed coat removal with a Gavin<sup>®</sup> mini-vise. After each treatment, the seeds were inoculated *in vitro* in two sterile substrates (MS medium or PlantMax<sup>®</sup>). The results show that the best treatment to overcome dormancy of *P. edulis* seeds was total removal of seed coat with Garvin<sup>®</sup> mini-vise. This treatment yielded 100% seed germination under *in vitro* conditions either on PlantMax<sup>®</sup> or MS medium. Approximately 150 seeds per hour could be decoated according to the operator's skills. However, in some cases, there was physical damage to the zygotic embryo. Seed treated with sulphuric acid for 1 min also showed promising results (average 0.107). Seeds germinated within seven days after being treated with sulphuric acid and inoculated *in vitro* in PlantMax<sup>®</sup> sterile substrate. Moreover, poor germination rates were achieved when seeds were treated with sulphuric acid, followed by inoculation *in vitro* onto MS medium (0.0711). All seeds germinated on *in vitro* PlantMax<sup>®</sup> sterile substrate and presented an epinastic phenotype, possibly due the ethylene biosynthesis *in vitro*.

**Key words:** *Passiflora*, dormancy, seed germination.

### INTRODUCTION

The yellow passion fruit (*Passiflora edulis* Sims.) is the most important and widely cultivated species of *Passiflora* genus, producing fruits for both fresh market and juice industry. Brazil is one of the major worldwide producers of yellow passion fruit, with a cultivated area of approximately 50,795 ha devoted to this crop (IBGE,

2009). This species can be propagated quite readily by seeds, cuttings and grafting onto seedling rootstocks (Alexandre et al., 2009a, b). Plants of the yellow form are almost exclusively seed-propagated, and seeds germinate within approximately 2 to 3 weeks (Vieira and Carneiro, 2005), although present dormancy imposed by

the seed-coat when cultivated *in vitro*.

Dormancy is referred to as the physiological state in which viable seeds fail to germinate when provided with water and adequate environmental conditions favorable for germination. Dormancy is considered as a strategy to avoid germination under certain conditions where seedling survival is likely to be low (Ellis et al., 1985a; Schmidt, 2000). Seed dormancy has been reported for different *Passiflora* species, example, *P. edulis* (Morley-Bunker, 1980; Hall et al., 2000; Alexandre et al., 2004), *P. incarnata* L. (Wehtje et al., 1985), *P. mollissima* (La Rosa, 1984), and *P. nitida* Kunth (Passos et al., 2004). According to Ellis et al. (1985b), *Passiflora* species possess non-endospermic seeds, meaning seeds with only residual or no endosperm, but with mature embryos. Their seeds have a hard coat with a semi-permeable inner layer. Despite ready uptake of water by the seeds during the imbibition, they might contain strong inhibitors which do not allow the embryo growth. Some of these inhibitors are located in the thin residual endosperm layer surrounding the embryo or within the embryo cotyledons. The inhibitors are blocked from leaching by the semi-permeable membranous testa incorporated in the coverings, and once excised, embryos germinate promptly. In summary, *Passiflora* species have exogenous dormancy (Morley-Bunker, 1980), which is probably due to the combination of mechanical and chemical dormancy.

Several studies have demonstrated that *in vitro* regeneration in yellow passion fruit has been achieved from pre-existing meristems located in shoot apices or nodal segments (Drew, 1991; Faria and Segura, 1997; Reis et al., 2003), from adventitious buds developed from leaf discs (Dornelas and Vieira, 1994; Appezzato-da-Glória et al., 1999), from the hypocotyl (Reis et al., 2003; Rêgo et al., 2011) and from internodal segments (Biasi et al., 2000), all derived from seedlings germinated *in vitro*. However, a basic problem related to *in vitro* growth of Passifloraceae species is the seed germination, which still presents technical difficulties. In fact, the great difficulty found for the *in vitro* culture establishment of yellow passion fruit is related to unsuccessful germination of the seeds, even when it was applied any of the already published protocols (Kantharajah and Dodds, 1990). According to Morley-Bunker (1980), seeds from yellow passion fruit present dormancy imposed by seed-coat tegument. Hall et al. (2000), suggested that only after the total removal of the seed coat the dormancy is overcome; scarification of the seeds may work, but it is technically difficult to execute, since the mature passion fruit seeds

are small and hard.

Various methodologies used to overcome seed dormancy were published for different species: chemical scarification with sulphuric acid in *Cotinus coggygria* Scop (Olmez et al., 2009), *Tamarindus indica* (Muhammad and Amusa, 2003), and *Parkia biglobosa* (Aliero, 2004); and chemical scarification using ethylic alcohol in *Euphorbia heterophylla* L. (Kern et al., 2009). In addition, for the seeds of *Passiflora* species, the induction of germination has been done by using gibberellic acid (Morley-Bunker, 1980; Passos et al., 2004), hot water (Oliveira et al., 2010), water at room temperature (Pinto et al., 2010), scarification with wood sandpaper (Oliveira et al., 2010; Lombardi et al., 2007), and seed coat fracture or fermenting seed with cytase (Morley-Bunker, 1980). The objective of this study was to develop an efficient, simple, rapid, and reliable method to overcome the lack *in vitro* germination of passion fruit seeds.

## MATERIALS AND METHODS

The experiment was conducted in Tissue Culture Laboratory at the Federal University of Roraima, Roraima Brazilian State. Three hundred seeds from the same fruit of yellow passion fruit cultivar, Yellow Giant, were collected from a commercial plantation in county of Boa Vista, Roraima, Brazil. Seeds were surface-sterilized and then submitted to chemical and physical scarification methods, before placing them for *in vitro* germination.

### Scarification methods to overcome *in vitro* seed dormancy

The treatments applied to overcome *in vitro* seed dormancy as shown in Table 1 include: Control (non treated seed); hot water (80°C for 1, 2.5 and 5 min); absolute ethanol (for 5, 10 and 15 min); sulphuric acid 98% (for 1, 5 and 10 min); scarification with wood sandpaper (no. 125); and total seed coat removal utilizing a Garvin mini-vise® (jaw width = 1 inch) (Figure 1). The scarification was made before the seeds' surface sterilization, as recommended by Kantharajah and Dodds (1990) and d'Utra Vaz et al. (1993). After the total removal of the seed coat, seeds were submitted for the surface sterilization process.

### Germination media and growth conditions

Seeds were germinated *in vitro* in a sterile commercial pot mix (Plantmax® (Eucatex, SP, Brazil) and MS (Murashige and Skoog, 1962) medium. For surface sterilization, the seeds were soaked into a dilute commercial bleach solution (0.7% hypochlorite) for 20 min, followed by three rinses with sterile distilled water. For *in vitro* germination, the seeds were transferred to test tubes (150 × 15 mm) containing either 10 ml MS medium, supplemented with 3%

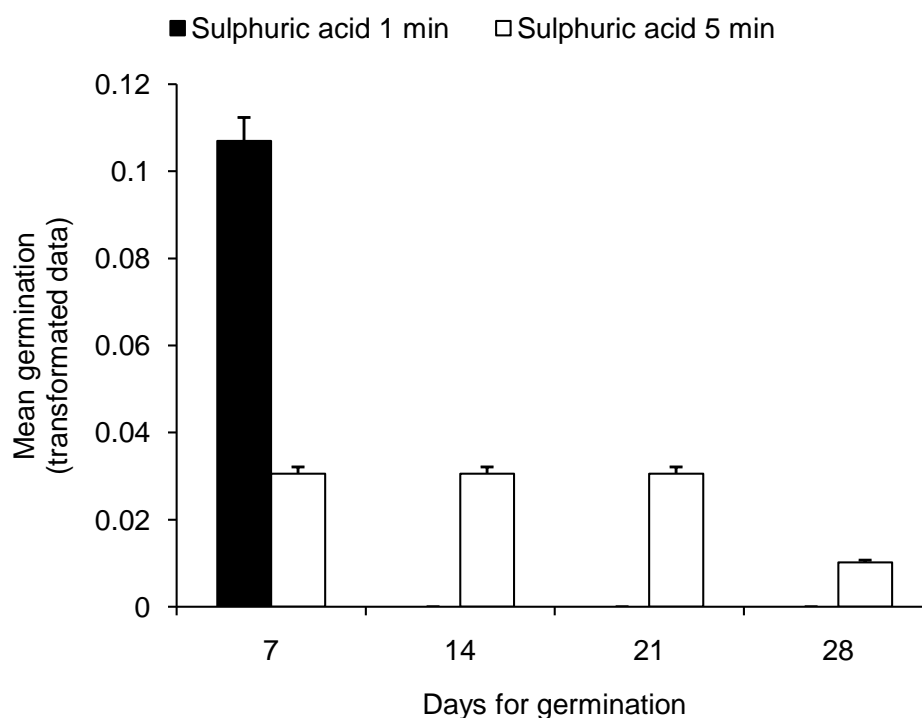
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**Abbreviations:** MS, Murashige and Skoog (1962); B5, Gamborg medium (1968); BA, 6-benzyladenine; Kin, kinetin; IAA, indole-3-acetic acid.

**Table 1.** Relation of the different scarification methods for overcoming *in vitro* seed dormancy from yellow passion fruit (*Passiflora edulis* Sims.)

Treatment	Methods for break seed dormancy
T1	Seed with intact coat (control)
T2	Soaking in water at room temperature for 12 h
T3	Soaking in water at room temperature for 24 h
T4	Soaking in water at room temperature for 48 h
T5	Soaking in hot water at 80°C for 1 min
T6	Soaking in hot water at 80°C for 2.5 min
T7	Soaking in hot water at 80°C for 5 min
T8	Soaking in sulphuric acid for 1 min
T9	Soaking in sulphuric acid for 5 min
T10	Soaking in sulphuric acid for 10min
T11	Soaking in ethilic alcohol for 5 min
T12	Soaking in ethilic alcohol for 10 min
T13	Soaking in ethilic alcohol for 25 min
T14	Seed scarifications with wood sandpaper (no. 125)
T15	Total removal of seed coat with mini-vise

**Figure 1.** *In vitro* germination dynamics of *Passiflora edulis* Sims., as affected by different time of chemical scarification using sulphuric acid at 98% (for 1 and 5 min).

sucrose, 0.8% agar (Sigma-Aldrich, St. Louis, MO) and 0.01% inositol or to a dish containing 2.65 cm<sup>3</sup> Plantmax<sup>®</sup> substrate. The pH of the medium was adjusted to 5.8 before been autoclaved (121°C, 1.1 atm, 20 min). The tubes were sealed with one layer of PVC film (Goodyear, Brazil). The cultures were incubated at 27 °C, in the dark to favor hypocotyl etiolation. After 20 days of incubation, the tubes containing germinated seeds (hypocotyl approximately 12 cm high) were transferred to a growth chamber with a day and night

cycle of 16 and 8 h for 20 days, respectively. Eight to ten explants were collected from each *in vitro* grown plantlet.

#### Statistical analysis

The experiment was arranged in entirely randomized design in factorial scheme (15 × 2). Fifteen treatments were combined with

**Table 2.** Variance analysis of the eight treatments to overcome *in vitro* seed germination and following seed inoculation in two substrates: PlantMax® and Murashige and Skoog medium (1962).

Source of Variation	df	Square mean	F		
Scarification (Scar)	7	0.00487	18.76937**	-	-
Substrates (Sub)	1	0.00658	25.32513**	-	-
Scar x Sub	7	0.00152	5.83453**	-	-
Scar /Sub	14	0.0032	12.30195	P = 0.0	-
Scar /PlantMax®	1	0.02045	0.00292	11.24919	P = 0.0
Scar /MS medium	2	0.02424	0.00346	13.33187	P = 0.0
Sub/ Scar	8	0.00215	8.27086	P= 0.00002	-
Sub/ SA. 1 min	1	0.00645	0.00645	24.8528	P = 0.0
Sub/ SA. 5 min	2	0.00645	0.00645	24.8528	P = 0.0
Sub/ SA. 10 min	3	0.00118	0.00118	4.55972	P = 0.03442
Sub/ EA. 5 min	4	0.00013	0.00013	0.51656	P = 100.0
Sub/ EA. 10 min	5	0.00053	0.00053	2.03445	P = 0.15593
Sub/ EA. 25 min	6	0.00118	0.00118	4.55972	P = 0.03442
Sub/ SP	7	0.0012	0.0012	4.6311	P = 0.03306
Sub/ Mini-vise	8	0	0	0	P = 100.0
Error	144	0.00026			
Total	159				
Average	0.088				
CV(%)	18.66				

SA, Sulphuric acid; EA, ethylic alcohol; SP, sandpaper.

**Table 3.** Comparison of means between different treatments to overcome *in vitro* seed germination from yellow passion fruit, inoculated in PlantMax® substrate and Murashige and Skoog (1962) medium, respectively.

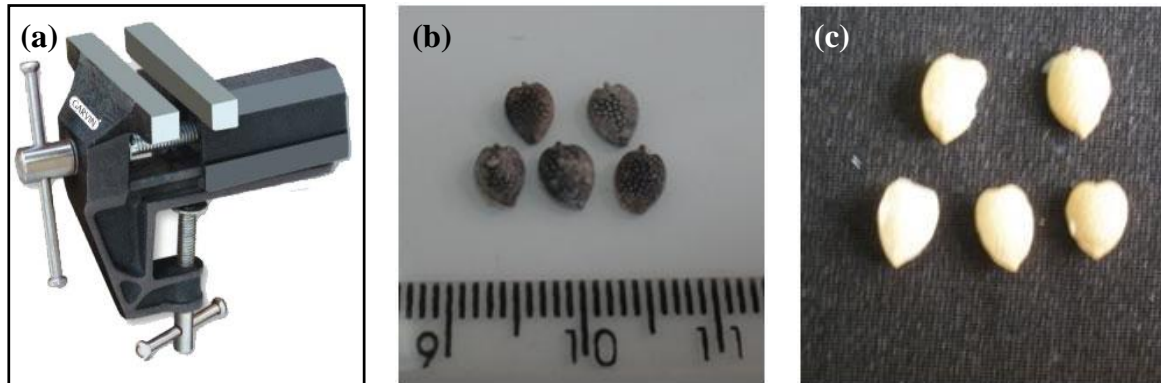
Treatment	Mean and percentage (%) of seed germination in two substrates	
	PlantMax®	MS medium
Soaking in sulphuric acid for 1 min	0.107(70) <sup>Aab</sup>	0.0711(0) <sup>Bb</sup>
Soaking in sulphuric acid for 5 min	0.107(70) <sup>Aab</sup>	0.0711(0) <sup>Bb</sup>
Soaking in sulphuric acid for 10min	0.086(30) <sup>Abc</sup>	0.0711(0) <sup>Bb</sup>
Soaking in ethylic alcohol for 5 min	0.076 (10) <sup>Ac</sup>	0.0711(0) <sup>Ab</sup>
Soaking in ethylic alcohol for 10 min	0.081 (20) <sup>Ac</sup>	0.0711(0) <sup>Ab</sup>
Soaking in ethylic alcohol for 25min	0.086(30) <sup>Abc</sup>	0.0711(0) <sup>Bb</sup>
Manual seed scarification with wood sandpaper	0.0762(40) <sup>Bc</sup>	0.0918(10) <sup>Ab</sup>
Total removal of seed coat with a mini-vise	0.1225(100) <sup>Aab</sup>	0.1225(100) <sup>Aa</sup>

two types of substrates for *in vitro* seed germination. Each treatment was composed by 10 replicates (one seed per tube). The data were transformed by  $\arcsin [(x + 0.5)/n]^{1/2}$  before variance analysis and compared by F-test at significance level of  $P \leq 0.05$ . The averages were compared by Tukey's test at the same significance level. The analyses were made using the software Genes (Cruz, 2006).

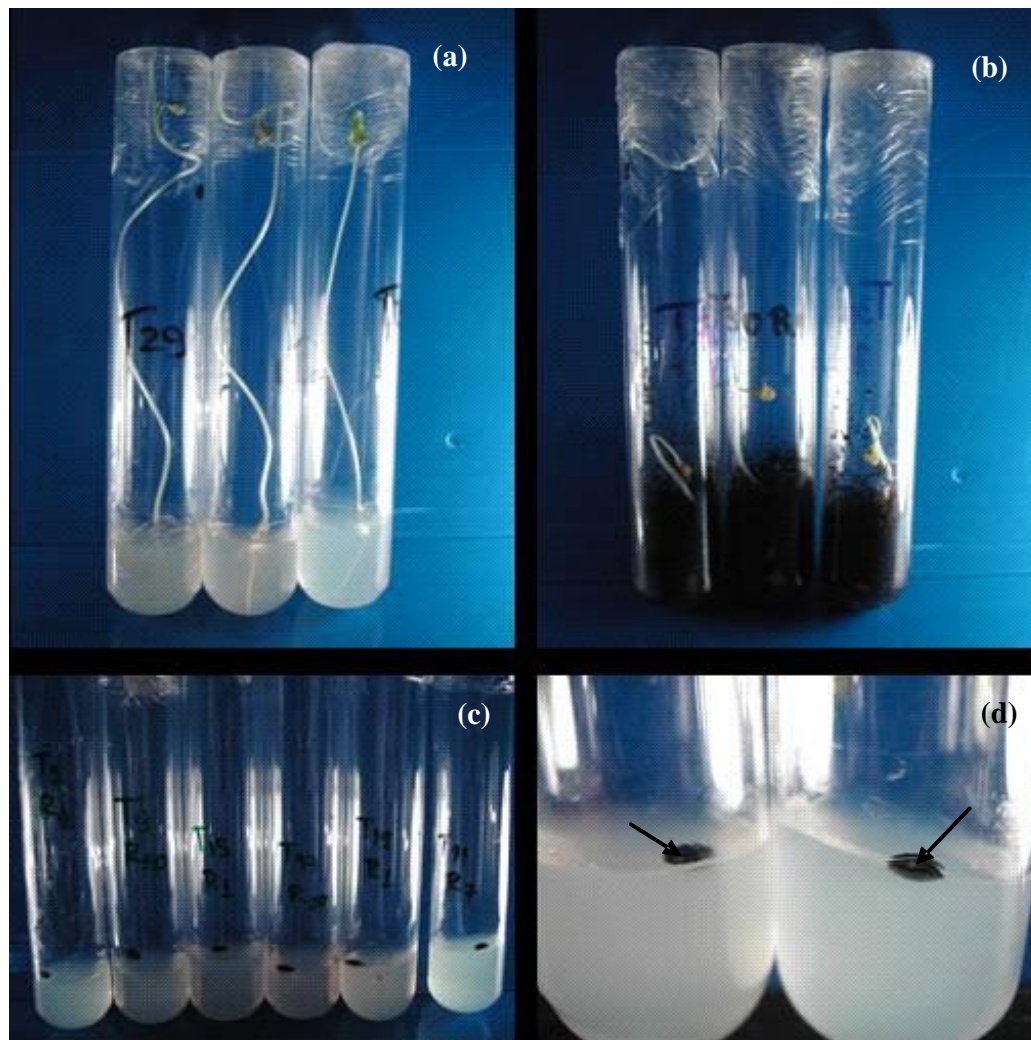
## RESULTS

The experimental results revealed that soaking *P. edulis* seeds in water at room temperature, or hot water at 80°C for different periods of time prevented germination, similar to results observed for the control treatment

(untreated seed) (Figure 3c). From the 15 scarification methods utilized in this study (Table 1) attempting to overcome seed dormancy, only ten were able to induce seed germination, followed by inoculation in both sterile PlantMax® substrate and MS medium without the presence of any plant growth regulator (Tables 2 and 3). The raw data from the 10 treatments were transformed by arcsin and then submitted to variance analysis (Table 2). There were significant differences among the tested scarification methods, substrates, and by the interaction between the scarification methods x substrates. When we unfolded the scarification methods into the substrates, significant differences were also observed, indicating different responses among scarification methods used in



**Figure 2.** Physical scarification of the seed from yellow passion fruit (*P. edulis* Sims.) (a). Garvin® Mini-vise table with integrated clamp (jaw width = 1 inch). (b) and (c). Detail of intact and decoated seeds using mini-vise.



**Figure 3.** Overcoming *in vitro* seed dormancy of yellow passion fruit (*P. edulis* Sims.). (a) Etiolated plantlets derived from seeds germinated in MS medium 21 days after total removal of the seed coat. (b) Plantlets derived from seeds germinated in PlantMax® substrate 21 days, displaying epinastic phenotype. (c) Non-germinated seeds; from left to right: Control (untreated seeds), soaked in water at room temperature (for 12, 24 and 48 h), and in hot water at 80°C (for 1, 2.5 and 5 min). (d) Detail of non-germinated seeds after scarification with wood sandpaper (number 125).



each substrate (Table 2). However, when unfolding substrates into scarification methods, there were significant differences only for seeds that were chemically scarified with sulphuric acid 98% (1, 5 and 10 min), absolute ethanol (25 min) or scarified with wood sandpaper (Figure 3d). On the other hand, there were no significant differences if the seeds were scarified by soaking in ethanol (for 5 and 10 min) or by total removal of the seed coat.

The comparisons among the treatments (Table 3) show that there were significant differences between scarification method for each substrate (PlantMax<sup>®</sup> and MS medium), and the best result to overcoming the *in vitro* seed dormancy was the total removal of the seed coat with mini-vise, resulting in 100% germination under sterile conditions (Figure 3a and b). The second better treatment was chemical scarification with sulphuric acid 98% (for 1 or 5 min). Seeds soaked in sulphuric acid (98%) for 1 min followed by the transfer to PlantMax sterile substrate induced faster germination compared to 5 min treatment. After seven days after seed inoculation, the seeds treated with sulphuric acid for 1 min reached 70% germination, whereas in the seeds soaked in sulphuric acid for 5 min had germination rate ranging from 7 to 28 days after inoculation (Figure 1). The lowest germination rates were achieved when seeds were immersed into sulphuric acid 98% for 10 min (Table 3). Prolonged submersion in sulphuric acid was rather harmful for the germination and further emergence; hence this procedure should be avoided in seeds of *P. edulis*.

Low germination rates were also observed when chemical scarification (sulphuric acid or absolute ethanol) was followed by inoculation in MS medium. The chemical scarification produced better results when scarified seeds were inoculated into PlantMax<sup>®</sup> substrate. All seeds chemically or physically scarified developed a pronounced hook located just below the shoot apex when inoculated on *in vitro* PlantMax<sup>®</sup> substrate (Figure 4e to g). This response resembled to an epinastic phenotype as observed in "triple response to ethylene".

## DISCUSSION

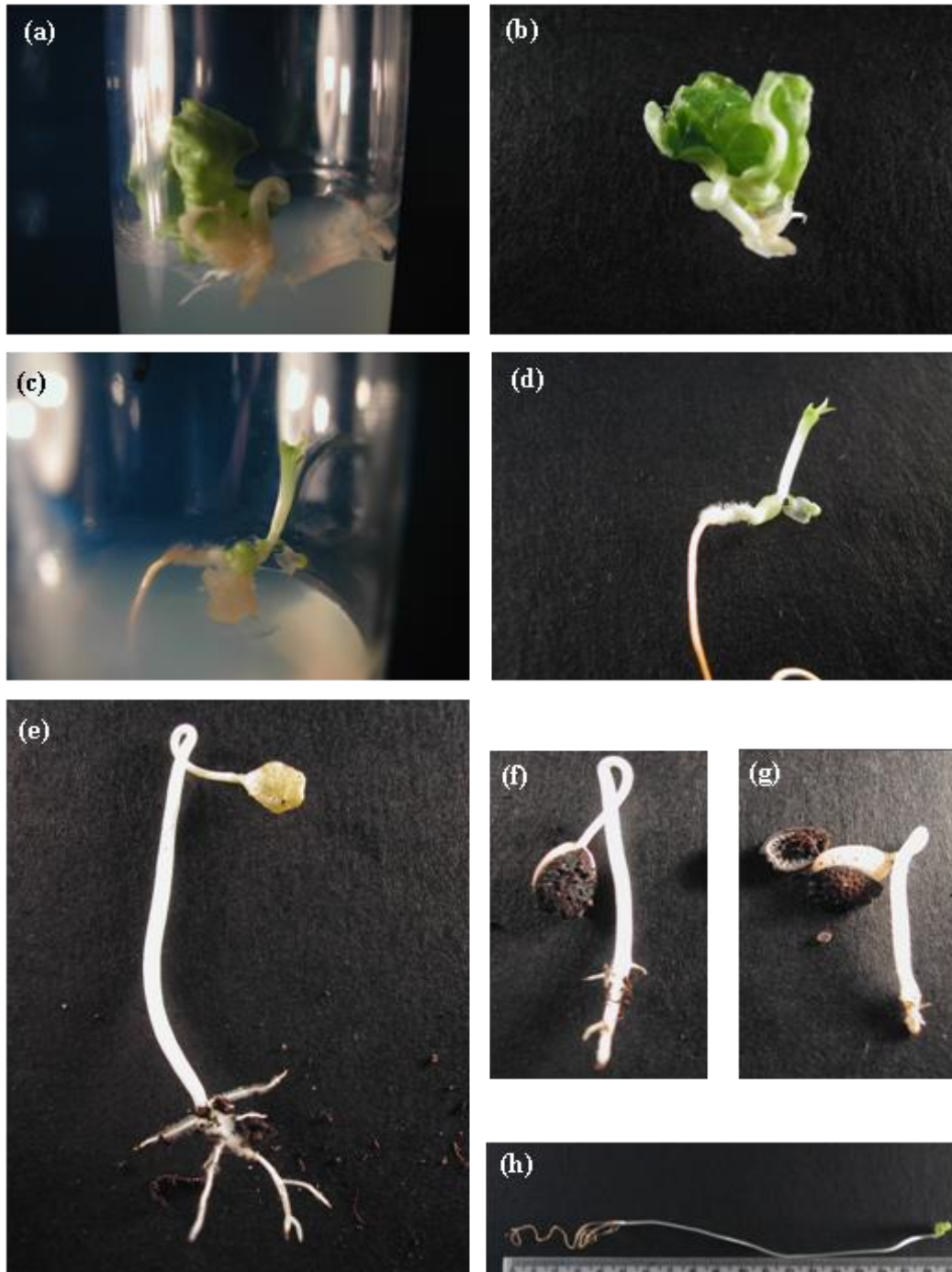
Under *in vitro* growing conditions, seeds of yellow passion fruit presented a high degree of dormancy as found in the control treatment which did not germinate in both PlantMax<sup>®</sup> substrate and MS medium. Similar results were described by Morley-Bunker (1980) working with *Passiflora* species (but mainly *P. edulis*) from South Africa, Ghana, Hawaii, Malawi, and Kenya. The author verified that seeds germinated better when mechanically fractured and kept under alternated temperature regimes of 12 /12 h at 20/30°C, rather than constant at 30°C. Scarifying the seed coats with sandpaper or by fermenting seeds with cytase for 24 h did not promote

germination. Likewise, the incorporation of gibberellic acid to the germination medium with agar also did not improve germination. Germination of fractured seeds sown in sand bed was even slower than that in agar. Previous works dealing with the difficulties of *in vitro* seed germination of yellow passion fruit were reported elsewhere by Manders et al. (1994) and Hall et al. (2000).

Furthermore, lack of germination was also observed when seeds were soaked into water at room temperature for 12, 24 or 48 h, followed by inoculation in both PlantMax<sup>®</sup> and MS-based medium. Morley-Bunker (1980) reported that when passion fruit seeds were soaked in water at room temperature for 24, 48, and 72 h, no germination was recorded. Likewise, no improvement on germination occurred when seeds of *Passiflora mollissima* (Kunth) L.H. Bailey, *Passiflora tricuspis* Mast. and *Passiflora nov* sp. was soaked for 24 h in water at room temperature (Delanoy et al., 2006). Conversely, Ellis et al. (1985b) claimed that the treatment for 48 h improved the germination percentage only for *P. mollissima*, when compared to the control.

The use of hot water (80°C) for different times (1, 2.5 and 5.0 min) did not stimulate seed germination. Conflicting results were reported by Oliveira et al. (2010) working with seed dormancy in *P. cincinnata* Mast. The best results to overcome seed dormancy were achieved by drying seeds under shade conditions, followed by treatment with hot water at 50°C or alternatively, seeds should be dried under shade followed by scarification. Soaking the seeds in hot water may lead to the loosening of the seed coat wall, allowing water to permeate into the tissues, leading to physiological changes promoting embryo growth (Agboola and Adedire, 1998; Sabongari, 2001; Aliero, 2004). Treatment with hot water was also efficient to reduce seed dormancy in some species of the genus *Cassia* (Skerman, 1977; Rodrigues et al., 1990). Moreover, when the seeds were treated with sulphuric acid (98%) followed by inoculation of the seeds in MS medium, a poor germination rate took place. However, when the seeds were scarified and transferred to PlantMax<sup>®</sup> substrate, variable levels of germination occurred (Table 3). Excellent results using sulfuric acid were also obtained for some Leguminosae species (Olmez et al., 2009, Muhammad and Amusa, 2003, Aliero, 2004), but no previous records were found for the genus *Passiflora*.

Chemical scarification by soaking in absolute ethanol for different times (5, 10 and 25 min) also led to variable levels of seed germination (Table 3). No significant differences were observed between the exposure for 5 or 10 min in absolute ethanol, followed by the inoculation in both MS medium or PlantMax<sup>®</sup> (Table 2 and 3), being the lowest germination percentages observed among the treatments. By increasing the exposure time in alcohol to 25 min remarkably improved germination rate was obtained after transferring to PlantMax<sup>®</sup> substrate sterile, but still a poor germination response when seeds were



**Figure 4.** Different physical damages in seedlings from yellow passion fruit (*P. edulis* Sims.) caused by mini-vise. (a) to (c) Detail of triple response (epinastic phenotype) after total removal seed coat with mini-vise. (f) and (g), Seedlings germinated after chemical scarification with sulphuric acid (98%). Note that seed coat yet is present. (h) Seedlings germinated in MS medium, after total removal seed coat, measuring approximately 19 cm 21 days old.

transferred onto MS medium (Table 3). Kern et al. (2010) compared the effects of methanol, ethanol, propanol and acetaldehyde on germination and growth of *E. heterophylla*. Ethanol at concentrations ranging from 0.25 to 1.5% showed a dose-dependent inhibition of germina-

tion and growth of *E. heterophylla*. Measurements of respiration and alcohol dehydrogenase activities during seed water uptake and initial seedling growth revealed that ethanol induced an extended effect under hypoxic conditions in the growing tissues. These effects were

caused by alcohol dehydrogenase activity, which is required for the conversion of ethanol to acetaldehyde, though plays a role in the ethanol-induced injuries.

Scarified seeds from yellow passion fruit of both chemical and physical methods, followed by transfer to PlantMax<sup>®</sup> displayed epinastic phenotype (Figure 3e to g), possibly due to *in vitro* ethylene biosynthesis. Similar results were described for the first time by Stewart and Freebairn (1969), linking this phenotype to ethylene biosynthesis during germination of lettuce and tomato seeds. According to Guzman and Ecker (1990), ethylene concentrations above 0.1  $\mu\text{L.L}^{-1}$  induce changes in the growth pattern of seedlings by reducing the rate of elongation and increasing lateral expansion, which leads to swelling of the region below the hook. These effects of ethylene are common shoot culture of several dicots species, also the case of passion fruit, whose climacteric rise of respiration would be expressed at vegetative tissue level as already reported (Reis et al., 2003). Yellow passion fruit is indeed very sensitive to ethylene, which affects the shoot apex development, rhizogenesis, axillary shoot development from nodal segments, and adventitious shoot regeneration from hypocotyl explants (Faria and Segura, 1997; Naik and Chand, 2003; Reis et al., 2003; Trevisan and Mendes, 2005; Mensuali-Sodi et al., 2007).

Etiolated dicot seedlings are usually characterized by a pronounced hook located just behind the shoot apex. This shape facilitates the penetration of the seedlings through the soil, protecting the tender apical meristem (Taiz and Zeiger, 2010). Like epinasty, hook formation and maintenance result from ethylene-induced asymmetric growth. The closed shape of the hook is consequence of the faster elongation of the outer side of the stem compared with inner side. However, when the hook is exposed to light, it opens because the elongation rate of the inner side increases, equalizing the growth rates on both sides (Guzman and Ecker, 1990). Possibly the pronounced hook present in yellow passion fruit *in vitro* cultivated is associated with the fact that passion fruit produces ethylene at high rates (Ludford, 1995), and the possible accumulation of ethylene within tissue culture flasks need to be taken into account. Perhaps the use of ventilated culture vessels is an alternative to modify vessel atmosphere and improve gas exchange, improving the seed germination *in vitro* (Trevisan and Mendes, 2005). In this sense, further is needed to evaluate the utilization of the use of vented lids for seed germination of passion fruit, which can be applied to enhance elimination of ethylene in PlantMax<sup>®</sup> substrate during seed germination.

The physical scarification methods, like wood sandpaper and total removal seed coat with mini-vise showed different results. The former produced poor germination of the seeds from yellow passion fruit, in both MS medium and PlantMax<sup>®</sup> substrate. Morley-Bunker (1980) also stated that this method is not efficient to overcome

seed dormancy imposed by seed coat in Passifloraceae. Contrarily, Kuhne (1968), Ellis et al. (1985b), and Morton (1987) working with *P. edulis* Sims., reported that scarification with sandpaper improved germination. It is important to observe that seed germination was carried out under *ex vitro* conditions. On the other hand, the total removal seed coat with a mini-vise (Tables 2 and 3) was the best scarification method to overcome seed dormancy in both substrates (PlantMax<sup>®</sup> and MS medium), where 100% of the seed germinated (Figure 2a and b). This indicates that seed dormancy in yellow passion fruit is imposed by seed coat as described by Morley-Bunker (1980) and Hall et al. (2000). This scarification method (mini-vise) is easy to execute, cheap and also is not dangerous to the handler. Furthermore, it is possible to produce seedlings measuring approximately 18 cm length, at 30 days after inoculation *in vitro* MS medium, producing around at 15 explants (1 cm length) (Figure 3a and h).

Whole decoated seeds germinated, including those that had damaged embryos (Figure 3a to d). The seed coat from yellow passion fruit is very hard and possibly the main consequence is the interference with water uptake, mechanical restraint to radicle protrusion, interference with gas exchange and supply of inhibitors to the embryo (Morley-Bunker, 1980). In conclusion, the results confirm that *P. edulis* Sims seeds showed physical dormancy, which can be easily overcome by total removal of the seed coat with a mini-vise.

## Conflict of Interests

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

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Full Length Research Paper

## Canonical correlations in elephant grass for energy purposes

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Elephant grass has the potential to be used as a source for energy production. Besides dry matter yield, other characteristics related to biomass quality are important. The canonic correlation analysis is a multivariate statistical procedure that allows for discovering characteristic associations among groups. The objective of this study was to evaluate the canonic correlation existing between the groups of agronomic traits and biomass traits in elephant-grass genotypes aiming to identify possible associations between the groups of variables in order to use this information in breeding programs of elephant grass for energy. The experiment was conducted at Colégio Estadual Agrícola Antônio Sarlo, located in Campos dos Goytacazes, RJ, Brazil. The evaluation cuts were made in June 2009 and February 2011; the first and third evaluation cuts, respectively. In the first cut, the following morpho-agronomic traits were evaluated: dry matter yield, percentage of dry matter, number of tillers, plant height, stem diameter, percentages of ash, cellulose, lignin, acid detergent fiber and crude protein and calorific value. In the third cut, in addition to the morpho-agronomic traits assessed in the first cut, the leaf-blade width and percentages of ash, cellulose, lignin, acid detergent fiber and neutral detergent fiber were also evaluated. The experimental design was of randomized blocks with 40 genotypes. Variance analysis and canonic correlation were performed between the morpho-agronomic and biomass-quality traits for the cuts isolated. A positive correlation was found between the percentage of dry matter and the fiber content and the plant height and number of tillers and the percentage of crude protein.

**Key words:** Bioenergy, multivariate, *Pennisetum purpureum*.

### INTRODUCTION

There is great expectation on the possible economic benefits from the clean development mechanism projects

coming from the use of renewable sources in the agricultural sector (Boddey et al., 2008). Elephant grass (*Pennisetum*

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*purpureum* Schum.) has been used for decades as a source of nutrients in the animal diet, and millions of tons of this material are produced every year (Cleef et al., 2013). In addition to this purpose, the biomass originated from elephant grass has the potential to be used in the production of biofuels in the form of charcoal, alcohol, methane, or even for direct combustion (Lee et al., 2010).

Elephant grass is an abundant, fast growing plant with significant potential as a renewable energy source and for conversion to higher calorific value fuels (Strezov et al., 2008). According to Morais et al. (2009), this culture is highly efficient in the fixation of atmospheric CO<sub>2</sub>, and it can produce over 60 mg ha per year. There is only a small response of biomass production to the additions of urea fertilizer (Morais et al., 2013) and accumulates N derived from Biological nitrogen fixation (BNF) (Videira et al., 2012). With this, large amounts of dry matter can be produced with a small amount of fertilizer N.

Besides the production of dry matter, biomass quality characteristics are important to evaluate. The energy capacity of wood in Eucalyptus can be influenced both by its chemical composition and lignin and extractives contents (Santos et al., 2011). According to Paterlini et al. (2013), elephant grass has low fiber content and high extractives contents in the leaves, having good properties for its energy use.

The analysis of canonic correlation is a multivariate statistical procedure that allows for verifying the linear multi-dimensional relationships between two sets of variables (Costa et al., 2011). The association between traits is of great importance for plant breeding, because the selection practiced in a certain trait may cause changes in another (Marzban et al., 2014). This technique has been used in elephant grass (Cunha et al., 2011) to analyse the basal tillers density and plant height which are responsible for production of dry matter of evaluated clones.

Based on the above information, the objective of this study was to evaluate the canonic correlation between the groups of agronomic traits and biomass traits in elephant-grass (*Pennisetum purpureum* Schum.) genotypes aiming to identify the associations and the inter-dependence between the studied sets of variables order to use this information in breeding programs of elephant grass for energy.

## MATERIALS AND METHODS

### Cultivation conditions and genetic materials

The experiment was conducted at Colégio Estadual Agrícola Antônio Sarlo, in Campos dos Goytacazes/RJ, Brazil, located at 21°44' S latitude, 41°18' W longitude and 12 m elevation. The soil is characterized as a terrace soil, classified as a Dystrophic Ultisol (FAO, 1997). Planting was performed with stems positioned with their base in contact with the apex of the next plant, in 10 cm deep furrows, in March 2008. The plot-leveling cut was made 90 days after planting. The evaluation periods were considered from June

2008 to June 2009 and April 2010 to February 2011 for the first and third evaluation cuts, respectively.

During planting, 100 kg ha<sup>-1</sup> P<sub>2</sub>O<sub>5</sub> (Single superphosphate) were used. After plot leveling, topdressing was performed again, with 25 kg ha<sup>-1</sup> N (ammonium sulfate) and 25 kg ha<sup>-1</sup> K<sub>2</sub>O (potassium chloride). The experimental design was of randomized blocks with 40 treatments (genotypes). Each 4.5 m<sup>2</sup> plot was composed of a 3.0 m row, with 1.5 m spacing between rows. During the cut, 1.5 m was considered in each plot for evaluation and 0.75 m of each side of the row was considered the border, totaling a floor area of 2.25 m<sup>2</sup>.

The elephant-grass genotypes utilized were: Elefante da Colômbia, Mercker, Três Rios, Mercker Santa Rita, Pusa Napier N 2, Gigante de Pinda, Napier N 2, Mercker S. E. A, Taiwan A-148, Porto Rico 534-B, Albano, Híbrido Gigante Colômbia, Pusa Gigante Napier, Costa Rica, Cubano Pinda, Mercker Pinda, Mercker Pinda México, Mercker 86 México, Taiwan A-144, Napier S.E.A., Taiwan A-143, Elefante de Pinda, Mineiro, Mole de Volta Grande, Napier, Teresópolis, Taiwan A-46, Duro de Volta Grande, Mercker Comum Pinda, Cameroon - Piracicaba, Taiwan A-121, P241 Piracicaba, IAC-Campinas, Elefante Cacheira Itapemirim, Roxo, Guaçu/L.Z.2, Cuba-116, King Grass, Roxo Botucatu, and Vruckwona Africano.

### Evaluated traits

In the first cut, the following morpho-agronomic traits were evaluated: dry matter yield, percentage of dry matter, number of tillers per linear meter, plant height and stem diameter. The biomass-quality traits assessed in the first cut were: percentages of ash, cellulose, lignin, acid detergent fiber and crude protein, and calorific value. In the third cut, in addition to the morpho-agronomic traits assessed in the first cut, the leaf blade width was also evaluated. The biomass-quality traits evaluated in the first cut were: percentages of ash, cellulose, lignin, acid detergent fiber and neutral detergent fiber.

The variable dry matter yield (DMY), in mg. ha<sup>-1</sup>, was obtained from the conversion of fresh matter production by the percentage of dry matter. To obtain the percentage of dry matter (%DM), a tiller (leaves and stem) was collected for drying in an oven at 65°C for 72 h until constant weight was reached (ADS - air-dried sample); the dried material was ground in a Wiley mill (1 mm sieve) and conditioned in a plastic bottle. Next, the samples were once again dried in an oven at 105°C for 12 h (ODS - oven-dried sample). Soon after, the %DM was estimated as the product between ADS and ODS. The plant height (HGT) was measured from the soil until the curvature of the last expanded leaf, expressed in m; stem diameter (SD), expressed in cm, was measured at 10 cm above the soil, with a caliper ruler; the number of tillers was measured per linear meter (NT); and the leaf blade width (LW), at the first fully expanded leaf, expressed in cm.

The analyses of the percentages of crude protein (CP), acid detergent fiber (%ADF) and neutral detergent fiber (%NDF) were carried out as described by Pomeranz and Meloan (1994). The percentage of lignin was determined from the ADF (cellulose, lignin, cutin, minerals, silica and ADIN) using the reagent sulfuric acid (72%); the percentage of ash (%ASH) was determined as the difference in weight after analysis of determination of the %LIG; the percentage of cellulose (%CEL) was determined as the difference in weight between %ADF and %LIG, as described by Pomeranz and Meloan (1994); and the calorific value (CFV), expressed as kcal. g<sup>-1</sup>, by using 1 g of sample, determined using the adiabatic calorimeter IKA model C-200.

### Statistical analysis

Variance analysis and canonic correlation were performed for the

**Table 1.** Estimates of the mean squares of genotype and residue mean and coefficient of variation of 11 traits of 40 genotypes of elephant grass in the first evaluation cut.

Variable	Mean square		Mean	CV(%)
	Genotype	Residue		
DMY	66.70*	21.90	20.37	22.96
%DM	64.89*	9.56	37.15	8.32
NT	617.40*	59.22	44.14	17.43
HGT	0.24*	0.11	3.33	10.26
SD	9.38*	2.62	11.80	13.73
%ASH	1.86*	0.71	3.11	27.21
%CEL	4.95 <sup>ns</sup>	3.56	34.86	5.41
%LIG	29.30 <sup>ns</sup>	20.52	47.38	9.56
%ADF	4.92 <sup>†</sup>	1.35	8.92	13.02
%CP	0.83 <sup>ns</sup>	0.65	3.65	22.16
CFV	3848.38 <sup>ns</sup>	2649.56	3898.24	1.32

DMY, dry matter yield (t. ha<sup>-1</sup>); %DM, percentage of dry matter; NT, number of tillers per linear meter; HGT, plant height (m); SD, stem diameter (mm); %ASH, percentage of ash; %CEL, percentage of cellulose; %LIG, percentage of lignin; %ADF, percentage of acid detergent fiber; %CP, percentage of crude protein; CFV, calorific value (kcal. g<sup>-1</sup>); CV, coefficient of variation. \*significant at 1% probability; <sup>ns</sup> = not significant at 1% by the F test.

cuts isolated. Canonic correlation analyses were conducted to check the existing associations between a first group of morphological traits (dependent variables Y) with the second group formed by the biomass-quality traits (according to the independent variables X). All statistical analyses were conducted on the Genes computer software (Cruz, 2013).

## RESULTS AND DISCUSSION

### Variance analysis

In the first evaluation cut, there were significant differences at 1% probability among the genotypes of elephant grass with regard to the variables dry matter yield (DMY), percentage of dry matter (%DM), number of tillers per linear meter (NT), plant height (PHT), stem diameter (SD), percentage of ash (%ASH) and percentage of acid detergent fiber (%ADF). In the case of percentage of cellulose (%CEL), percentage of lignin (%LIG), percentage of crude protein (%CP) and calorific value (CFV), there were no significant differences at 5% probability among the evaluated genotypes. The observed dry matter yield was 20.37 t. ha<sup>-1</sup> (Table 1). Morais et al. (2009), evaluating genotypes of elephant grass during the growth period of seven months, observed mean values of 18.9 t. ha<sup>-1</sup>, which is close to that found in the first evaluation cut of the present study. The average number of tillers was 11.8 mm (Table 1).

At the third cut, there was significance for all the evaluated traits, except for plant height (HGT) and percentage of ash (%ASH). The traits dry matter yield (DMY), percentage of dry matter (%DM), number of tillers per linear meter (NT), stem diameter (SD), leaf blade width (LW), percentage of cellulose (%CEL), percentage of neutral

detergent fiber (%NDF) and percentage of lignin (%LIG) presented significance at 1% probability. The average stem diameter found in the third cut (17.29 mm) was higher than that found by Ferreira et al. (2013) whose highest value was 15.40 mm in the genotype Itambé I-1.20, not used in this experiment (Table 2).

The percentage of NDF corresponds to the sum of the lignin, cellulose and hemicellulose contents in the plant tissue. As the %NDF is increased, the biomass for energy purposes also increases. The average %NDF observed was 76.38 (Table 2). These values are above the 57.1 and 59% found by Flores et al. (2012) in groups of unfertilized elephant grass and the same grass fertilized with 100 kg. ha<sup>-1</sup> of nitrogen, respectively. Another advantageous result found in this study concerned %ASH, because low ash contents are desirable for energy uses. Paterlini et al. (2013) found an average %ASH of 6.15%. In another study, conducted by Flores et al. (2013), the lowest value found for this trait was 4.9% in plants at 180 days after sprouting, which is higher than 4.38% (Table 2).

### Canonical correlation

The first, second and third canonic correlations were, at the level of 5% probability, significantly different from zero at the first evaluation cut (Table 3). Thus, it is concluded that the groups are not independent and that the inter-group associations can be established. Hence, only these three correlations are of interest in this study, because these were the canonic pairs that maximized the relationship between the primary components of the morpho-agronomic and biomass-quality traits in the

**Table 2.** Estimates of the mean squares of the genotype and residue mean and coefficient of variation of eleven traits of forty genotypes of elephant grass in the third evaluation cut.

Variable <sup>1/</sup>	Mean square		Mean	CV (%)
	Genotype	Residue		
DMY	196.49*	62.63	36.96	21.41
%DM	26.90*	7.28	34.28	7.87
NT	187.71*	70.08	32.30	25.91
HGT	0.073**	0.03	2.70	7.06
SD	15.87*	5.07	17.29	13.03
LW	71.99*	17.12	39.54	10.46
%ASH	0.78**	0.44	4.38	15.19
%CEL	4.83*	1.42	38.50	3.10
%ADF	10.07*	3.51	47.37	3.95
%NDF	8.49*	2.23	76.38	1.95
%LIG	2.15*	0.47	7.79	8.82

DMY, dry matter yield (t. ha<sup>-1</sup>); %DM, percentage of dry matter; NT, number of tillers per linear meter; HGT, plant height (m); SD, stem diameter (mm); %ASH, percentage of ash; %CEL, percentage of cellulose; %LIG, percentage of lignin; %ADF, percentage of acid detergent fiber; %CP, percentage of crude protein; CFV, calorific value (kcal. g<sup>-1</sup>); CV, coefficient of variation. \*significant at 1% probability; \*\* = not significant at 1% by the F test.

**Table 3.** Canonic correlations estimated between agronomic traits (group I) and biomass quality (group II) in elephant grass in the first evaluation cut.

Variable	Canonical pairs				
	1 <sup>o</sup>	2 <sup>o</sup>	3 <sup>o</sup>	4 <sup>o</sup>	5 <sup>o</sup>
DMY	0.17426	0.00121	0.12068	0.21066	0.95430
%DM	-0.45237	-0.71747	0.20645	0.31041	0.37632
NT	0.78062	-0.55633	0.23500	-0.02711	0.15866
HGT	-0.50184	0.34644	0.65748	0.07227	0.43661
SD	-0.17376	0.73871	0.04741	0.57699	0.29825
%ASH	-0.00652	-0.17461	-0.31509	0.44914	0.53672
%CEL	0.10556	-0.53751	0.64803	-0.48569	0.19730
%LIG	-0.10467	-0.40174	-0.12661	-0.47119	0.74066
%ADF	-0.33047	-0.56879	-0.45071	0.00103	-0.13921
%CP	0.91105	0.13390	-0.28606	-0.06577	-0.23554
CFV	-0.20598	-0.30631	-0.16856	-0.84628	-0.33063
R	0.69813	0.5614	0.1338	0.2936	0.3731
α (%)	0.0	0.0	1.47	10.53	41.65

DMY, dry matter yield (t. ha<sup>-1</sup>); %DM, percentage of dry matter; NT, number of tillers per linear meter; HGT, plant height (m); SD, stem diameter (mm); %ASH, percentage of ash; %CEL, percentage of cellulose; %LIG, percentage of lignin; %ADF, percentage of acid detergent fiber; %CP percentage of crude protein; CFV, calorific value (kcal. g<sup>-1</sup>); R, correlation; α, significance level.

elephant grass.

The association made in the first canonic pair is between the traits HGT and %CP. The results indicate that %CP decreases as HGT increased. For the same canonic pair it was observed that between the traits NT and %CP, as the NT increased, %CP elevated. Regarding the third canonic pair, it can be observed that as the %DM decreases, the %CEL and %ADF also reduced. Observing the associations of the third pair, as

the HGT increased, %CEL also increased (Table 3), because as the plant ages, with an increase in HGT and %DM, the plant cell wall thickens and lignifies, especially due to the proportion of stems in the harvested material (Xie et al., 2011). According to Braga et al. (2014), elephant grass genotypes due to having lower ash requires less energy to thermal conversion process to break links hemicelulose and cellulose than rice husk for the production of bio-oil by pyrolysis.



**Table 4.** Canonic correlations estimated between agronomic components (group I) and biomass quality (group II) in elephant grass at the third evaluation cut.

Variable	Canonical pairs				
	1 <sup>o</sup>	2 <sup>o</sup>	3 <sup>o</sup>	4 <sup>o</sup>	5 <sup>o</sup>
DMY	0.1842	-0.18283	0.73418	0.40409	-0.17855
%DM	0.60212	0.68645	0.16150	-0.08567	0.17938
NT	0.67795	0.31069	-0.00244	0.59179	-0.28928
HGT	0.19719	0.01362	0.04583	-0.18788	-0.18954
SD	-0.35403	-0.35300	-0.50891	0.25258	-0.64921
LW	-0.78153	0.29916	0.34309	0.05606	-0.30385
%ASH	-0.13972	0.08914	0.68986	0.00338	0.70471
%CEL	0.67303	0.44269	0.29361	0.20102	-0.47375
%ADF	0.86729	0.2622	-0.19036	0.27878	-0.25516
%NDF	0.68488	0.64475	0.07453	0.18679	-0.27349
%LIG	0.84655	0.20363	-0.31169	-0.33215	-0.18551
R	0.6989	0.5588	0.4647	0.0699	0.3885
$\alpha$ (%)	0.0	0.0	0.27	5.55	83.59

DMY, dry matter yield (t. ha<sup>-1</sup>); %DM, percentage of dry matter; NT, number of tillers per linear meter; HGT, plant height (m); SD, stem diameter (mm); LW, leaf blade width (mm); %ASH, percentage of ash; %CEL, percentage of cellulose; %ADF, percentage of acid detergent fiber; %NDF, percentage of neutral detergent fiber; %LIG, percentage of lignin; R, correlation;  $\alpha$ , significance level.

In the third cut, the first, second and third canonic pairs were not independent; that is, they obtained significance at 1% probability. The associations that can be established in the first canonic pair are between the morpho-agronomic traits %DM, NT and LW and the biomass-quality traits %CEL, %ADF, %NDF and %LIG. Based on the obtained results, when %DM and NT increase, there was an elevation in %CEL, %ADF, %NDF and %LIG. With regards to LW and the same biomass-quality traits, the effect is inversely correlated: when LW is reduced, %CEL, %ADF, %NDF and %LIG increased (Table 4).

Similarly to what was observed in the first canonic pair, in the second, the increase in %DM leads to augmentations in %CEL and %NDF (Table 4). Evaluating the energy value of elephant grass at different regrowth ages, Silva et al. (2007) observed an increase in %DM and %NDF as the regrowth days advanced. This increase in %NDF is important for the elephant grass, because it indicates potential for energy production.

With regard to the third canonic pair, an association can be observed between the morpho-agronomic traits DMY and SD and %ASH. As the DMY increases and SD decreases, %ASH increased (Table 4). Flores et al. (2013), evaluating elephant-grass genotypes under different nitrogen doses and cut ages on a Latosol, observed that, both in leaf and stem, the cut age (greater accumulation of dry matter) decreased the ash contents in the elephant grass that is an opposite result to that found herein. According to Flores et al. (2013), the ash contents are considered high, reaching 10.3% at the

dose of 77.5 kg. ha<sup>-1</sup> of N, and according to the authors of that study this can be due to the good availability of nutrients of the soil utilized, promoting the better nutrition of the elephant grass.

## Conclusions

The percentage of dry matter in elephant-grass genotypes is positively correlated with the fiber content (%ADF and %NDF). The association between these morpho-agronomic and biomass-quality traits is a criterion of paramount importance in the selection of elephant-grass genotypes for energy production.

Plant height and number of tillers are positively correlated with the percentage of crude protein. The percentage of ash is negatively correlated with the stem diameter and positively correlated with the dry matter yield.

## Conflict of Interests

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

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## Full Length Research Paper

## Evaluation of somatic embryogenesis and plant regeneration in tissue culture of ten sorghum (*Sorghum bicolor* L.) genotypes

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Optimization of tissue culture conditions for *Sorghum bicolor* L. through somatic embryogenesis from immature embryos is important for the genetic manipulation and improvement of this agronomically valuable crop. In an attempt to develop a successfully reproducible *in vitro* regeneration protocol for a group of diverse sorghum genotypes, 10 sorghum lines including locally adapted and commercially important elite genotypes were assessed for their regeneration potential on different culture media-containing adequate growth regulators combinations. The maximum response of embryogenic callus induction was obtained from explants cultured on Murashige and Skoog (MS) medium supplemented with 1.5 mgL<sup>-1</sup> of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.7 mgL<sup>-1</sup> L-proline. The addition of kinetin to the MS-based culture media had a negative effect on the formation of embryogenic calli. The results reveal that embryogenic callus formation and regeneration were highly genotype dependent. The line LG3 revealed the highest mean number of embryogenic callus (47.5 ± 6.0%) across the media tested. On the other hand, SPP462 was the least responsive (13.4 ± 3.3%) to embryogenic callus induction. The regeneration percentage of the different genotypes ranged from zero to 22.1%. The lines LG3, LG4, Dorado, SPGM94021 and SPMD94001 succeeded to form shoots on the three tested regeneration media. Nevertheless, the genotypes LG8 and TX2794 produced shoots on only two out of the three media. Three lines failed to regenerate on any of the tested media. Adding 0.5 mgL<sup>-1</sup> naphthalene acetic acid and 0.5 mgL<sup>-1</sup> of indole-3-butyric acid (IBA) did not enhance the root induction. Regenerated shoots developed into normal mature plants. Regeneration of sorghum genotypes could be improved through the use of different auxins and cytokinins in callus induction and shoot formation media. The auxin, 2,4-D was critical for the induction of embryogenic calli. However, the addition of the cytokinin (kinetin) adversely affected the formation of embryogenic callus. On the other hand, the shoot induction was more influenced by the addition of indole-3-acetic acid (IAA), 6-benzylaminopurine (BA) and thidiazuron (TDZ).

**Key words:** Sorghum, immature embryos, callus induction, regeneration, auxin, cytokinin.

### INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench], a tropical plant belonging to the family Poaceae, is one of the most

significant crops in Asia, Africa and Latin America. *Sorghum* is the fifth most cultivated and consumed grain

in the world after maize, rice, wheat and barley (FAO STAT, 2010). The crop is well adapted to tropical and subtropical areas throughout the world. In addition to its principal uses as flour, in the preparation of porridge and unleavened bread, sorghum species are sources of fiber, fuel and secondary products and are also used in the alcohol industry (sweet sorghum) as they contain high amounts of starch. In addition, sorghum grain has enhanced protein quality that could contribute significantly to the nutritional value of the diets of people and livestock (Ignacimuthu and Premkumar, 2014). Sorghum has been considered as one of the most difficult plant species to manipulate through tissue culture (Manjula et al., 2000; Chandrakanth et al., 2002; Hagio, 2002; Harshavardhan et al., 2002; Jeoung et al., 2002; Visarada et al., 2003; Kishore et al., 2006; Gupta et al., 2006; Maheswari et al., 2006). Protocols have been established for *in vitro* plant regeneration of sorghum from different types of explants such as immature embryos (Brar et al., 1979; Sairam et al., 2000; Nguyen et al., 2007; Grootboom et al., 2008; Muhumuza and Okori, 2013), immature inflorescences (Boyes and Vasil, 1984; Eapen and George, 1990; Zarif et al., 2013) shoot tips (Bhaskaran et al., 1988; Bhaskaran and Smith, 1988, 1989; Nahdi and de Wet, 1995; Seetharama et al., 2000; Kingsley and Ignacimuthu, 2014), leaf base (Mishra and Khurana, 2003), leaf segments (Pola and Mani, 2006) and also from cultured mesophyll protoplasts (Seetharama and Sairam, 1997; Sairam et al., 1999). However, the frequency of plant regeneration reported so far does not seem to be high enough. Rapid and highly uniform regeneration system with high regeneration efficiency is a prerequisite for successful genetic transformation.

Most of the studies were carried out on previously assessed, tissue culture amendable sorghum genotypes. In the present study, 10 sorghum genotypes have been selected on the basis that they represent genotypes of diverse origins and backgrounds. The criteria of selection were based on the importance of these genotypes for commercial hybrids formation. Our study was carried out with the main objective to identify a suitable tissue culture medium for callus induction and to determine the ability of these genotypes for plant regeneration through somatic embryogenesis. Establishing a reliable regeneration protocol will pave the way for successful use of immature zygotic embryos as target tissues for DNA delivery of important genes, to produce genetically modified sorghum

lines with enhanced characteristics adapted with the climate changes and thus, cover the needs for food and feed.

## MATERIALS AND METHODS

### Sorghum genotypes and preparation of explants

Seeds of 10 elite sorghum genotypes (*S. bicolor* L.) of locally adapted and commercially important elite germplasms of different origins were obtained from the Sorghum Department, Field Crops Research Institute, ARC, Egypt. Sorghum genotypes included: three local lines (that is, LG3, LG4 and LG8), one cultivar "Dorado" from central America and 6 lines with exotic background (that is SPP462, SPGM94021 and SPMD94001 from ICRISAT, TX2794 and TX631 from USA and Keyman from Zimbabwe).

Sorghum seeds were sown in the field at equal intervals (every three weeks). The plants were carefully cultivated. Their panicles were harvested after 9 to 11 days since pollination, or when immature embryos were 1.0 to 2.0 mm in length. Immature embryos were aseptically isolated from freshly harvested sorghum panicles or sorghum heads stored at +4°C for no longer than two days prior to isolation. Panicles were surface sterilized by submerging each head in 50% Clorox bleach (5.25% sodium hypochlorite) supplemented with two drops of Tween-20, with constant stirring for 10 min. The panicles were subsequently rinsed three times with sterile distilled water and allowed to air dry in a laminar flow hood. Individual seeds were picked from the heads and subsequently sterilized in 70% ethanol for 1 min followed by one rinse with sterile distilled water and 20 min in 50% Clorox followed by three rinses with sterile distilled water. The seeds were then placed in an open Petri plate and allowed to briefly air dry in a laminar flow hood. The immature embryos were excised and used as explants.

### Callus induction media

Aseptically excised immature embryos were cultured with scutellum side up on seven callus induction media (Table 1). All the media were MS-based (Murashige and Skoog, 1962) except medium C13 which was an N6-based medium (Chu et al., 1975). The main difference among the six media (C12, C13, C14, C15, C16 and C17) was in the concentrations of 2,4-D and kinetin. In contrast, medium C11 was supplemented with 0.5 mg L<sup>-1</sup> nicotinic acid, 0.5 mg L<sup>-1</sup> pyridoxine-HCl, 10 mg L<sup>-1</sup> thiamine-HCl, 1.5 mg L<sup>-1</sup> 2,4-D, 10 mg L<sup>-1</sup> ascorbic acid and 2% sucrose. All callus induction media were supplemented with 0.5 g L<sup>-1</sup> 2-(N-morpholino) ethanesulfonic acid (MES), 10 g L<sup>-1</sup> polyvinylpyrrolidone (PVP) and 3% sucrose. Immature zygotic embryos were cultured for six weeks on callus induction media, and sub-cultured biweekly onto fresh media. Intervals between subcultures were reduced to one week when excess phenolic secretions were found. All callus cultures were maintained in dark at 25°C.

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**Abbreviations:** ABA, Abscisic acid; ANOVA, analysis of variance; BA, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; MES, 2-(N-morpholino) ethanesulfonic acid; MS, Murashige and Skoog; NAA, α-naphthaleneacetic acid; PVP, polyvinylpyrrolidone; TDZ, thidiazuron.

**Table 1.** Callus induction media used in optimization of regeneration conditions. (amounts of ingredients per 1 liter medium).

Ingredient	Callus induction media						
	CI1	CI2	CI3	CI4	CI5	CI6	CI7
MS salts with vitamins	-	4.4 g	-	4.4 g	4.4 g	4.4 g	4.4 g
MS salts without vitamins	4.4 g	-	-	-	-	-	-
N6 salts with vitamins	-	-	4 g	-	-	-	-
Nicotinc acid	0.5 mg	-	-	-	-	-	-
Pyridoxine HCl	0.5 mg	-	-	-	-	-	-
Thiamine HCl	2.5 mg	-	-	-	-	-	-
2,4-D	1.5 mg	1.5 mg	1.5 mg	0.5 mg	2 mg	1 mg	0.5 mg
L-proline	0.7 g	0.7 g	0.7 g	0.7	0.7 g	0.7 g	0.7
Kinetin	-	-	-	0.5 mg	0.1 mg	0.2 mg	1 mg
Myo inositol	0.1 g	-	-	-	-	-	-
MES	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g
PVP	10 g	10 g	10 g	10 g	10 g	10 g	10 g
Ascorbic acid	10 mg	-	-	-	-	-	-
Sucrose	2%	3%	3%	3%	3%	3%	3%

**Table 2.** Media used for the regeneration of sorghum lines (amounts of ingredients per 1 liter medium).

Ingredient	Shoot induction media			Rooting media	
	R <sub>0</sub>	STR1M1	STR1M4	Rooting <sub>0</sub>	Rooting <sub>1</sub>
MS salts with vitamins		4.4 g	4.4 g	2.2	2.2
MS salts without vitamins	4.4 g	-	-		
Benzyl adenine	-	-	2 mg		
Kinetin	-	1.5 mg	-		
Myoinositol	0.1 g	-	-		
L-proline	0.7 g	0.7 g	0.7 g		
Nicotinic acid	0.5 mg	-	-		
Pyridoxine HCl	0.5 mg	-	-		
ThimanineHCl	10 mg	-	-		
Sucrose	6%	3%	3%	2%	2%
PVP	10 g	10 g	10 g		
IAA	1 mg	-	0.5 mg		
IBA				0.5 mg	-
NAA				0.5 mg	-
Zeatin	0.5 mg	-	-		
TDZ	0.1 mg	-	-		
ABA	0.25 mg	-	-		

### Shoot regeneration and root formation

Three different shoot induction media and two rooting media (Table 2) were used for the all tested sorghum genotypes. All tissue culture media were adjusted to pH 5.8 and solidified with 0.3% phytigel.

Six weeks later, embryogenic calli were cultured for two to six weeks on shoot induction media. Cultures were incubated at 25 ± 2°C with a 16 h photoperiod. Light was provided by cool white fluorescent lamps at a photon flux density of 30 μmol m<sup>-2</sup> s<sup>-1</sup>.

Regenerated shoots were transferred onto two different rooting media (Table 2) for root development.

### Acclimatization and plant recovery

The regenerated plantlets were acclimatized in the greenhouse in an aquarium containing a modified Hoagland solution as recommended by Johnson et al. (1957). Healthy rooted plantlets were then transferred from Hoagland solution to pots containing a

mixture of peatmoss: soil: sand (1:1:1) under 16 h photoperiod in greenhouse adjusted at 22-25°C and 50% humidity.

### Data collection and statistical analysis

For callus induction, each experiment was repeated three times, each consisting of 30-70 immature embryos. After six weeks on callus induction media, all embryogenic calli were equally distributed among the three regeneration media and the regeneration frequency was calculated as percentage of callus producing at least one shoot. Statistical analysis was performed according to Silva and Azevedo (2009) using ASSISTAT software, version 7.7. Experiments were designed as factorial experiments in a completely randomized design with three repetitions. Means of percentage were compared by t-test at a level of 5% of probability. The values of the standard errors were obtained using MSTAT-C software, version 4.

## RESULTS AND DISCUSSION

Somatic embryogenesis and plant regeneration of sorghum have been significantly improved by screening the response of different genotypes on different growth regulators containing media. The effects of different media components on sorghum regeneration have been determined. The identification of regenerable sorghum genotypes other than those commonly used in the previous studies has been achieved.

### Embryogenic callus induction

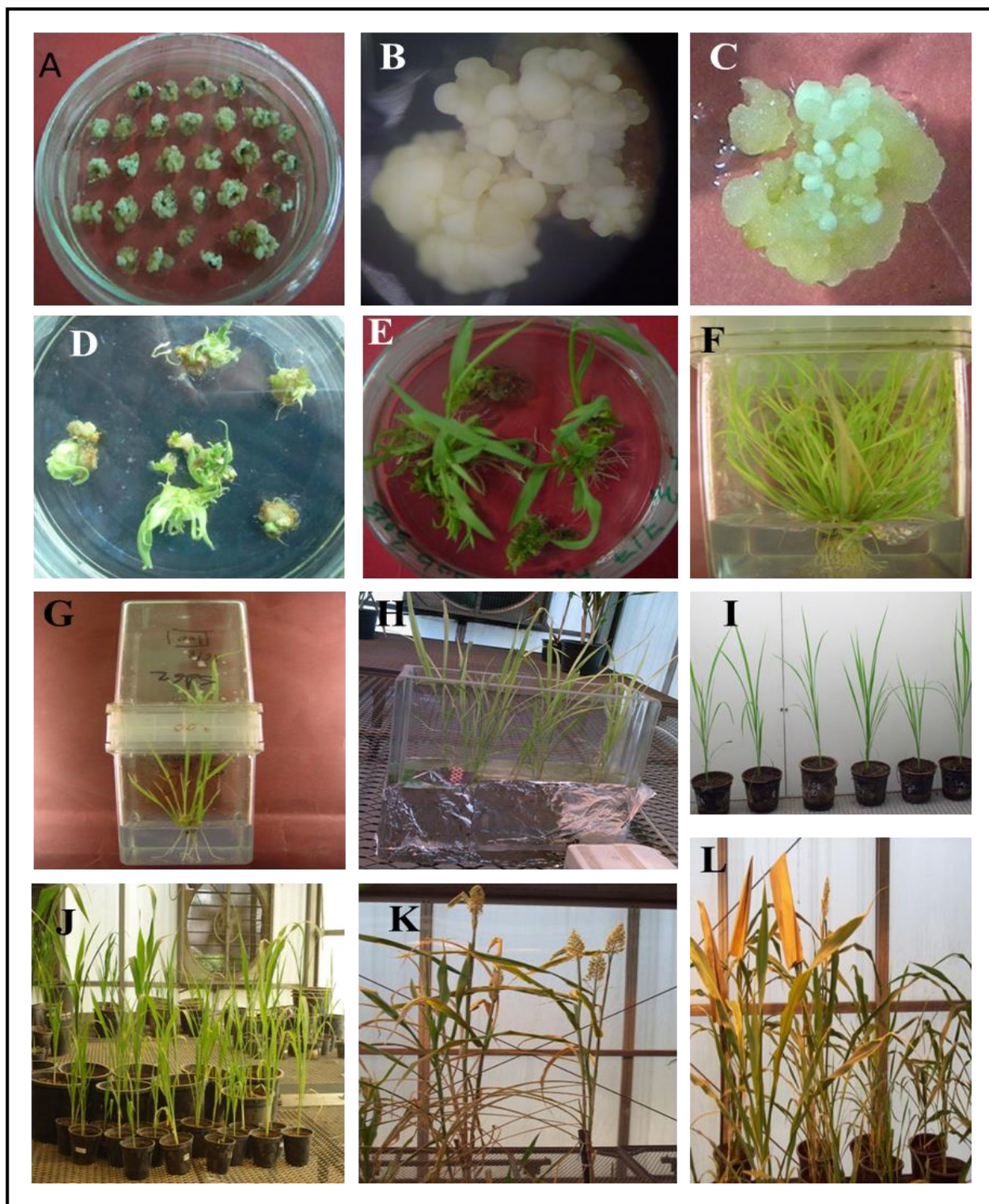
Sorghum has been suggested as one of the most recalcitrant plant species for regeneration and transformation (Zhao et al., 2000). Sorghum tissue culture recalcitrance is mainly due to genotype dependence, phenolic production, lacking of regeneration in long term *in vitro* culture, low frequency and prolonged phase of somatic embryos conversion into plantlets (Maheswari et al., 2006; Jogeswar et al., 2007; Raghuvamshi and Birch, 2010). Nowadays, crop agronomical and quality characteristics amelioration could not be achieved if without efficient regeneration and transformation systems. In the present study, we investigated callus initiation and regeneration potential of 10 sorghum genotypes on different nutrient media. Callus initiation was observed after few days of culturing on callus induction media showing two types of embryogenic calli: white compact embryogenic callus and creamy to yellowish embryogenic callus (Figure 1).

### Effect of callus induction media

Seven different media were investigated for their potential to induce embryogenic callus from the 10 sorghum genotypes. Out of the seven investigated callus induction

media, two media, that is, CI4 and CI7, failed to induce any embryogenic calli from any genotype under investigation. The results (Table 3) reveal that the media CI1, CI2 and CI3 had higher potentiality to induce embryogenic calli from the 10 genotypes as compared to media CI5 and CI6. The mean numbers of calli induced from the ten sorghum genotypes on media CI1, CI2 and CI3 were insignificantly different, that is 42.4, 41.3 and 40.8%, respectively although, they were significantly different from their respective values for media CI5 and CI6 (16.8 and 12.9%, respectively). The main difference between these two groups of media was that media CI5 and CI6 contain kinetin (0.1 and 0.2 mgL<sup>-1</sup>, respectively), while medium CI1, CI2 and CI3 were devoid of kinetin. This could suggest that the addition of kinetin to the medium exerts a suppressing effect on the induction of embryogenic calli. Moreover, the results reveal that this suppressing effect was accentuated by increasing the concentration of kinetin in the callus induction medium as the mean numbers of calli on medium CI5 (0.1 mgL<sup>-1</sup> kinetin) was higher than on medium CI6 (0.2 mgL<sup>-1</sup>). Meanwhile, media CI4 (0.5 mgL<sup>-1</sup> kinetin) and CI7 (1 mgL<sup>-1</sup> kinetin) failed to produce any embryogenic calli. The negative effect of kinetin on callus culture is in consistence with the findings of Mastellar and Holden (1970), Wernicke and Brettell (1982), Zhao et al. (2010) and Muhumuza and Okori (2013) in sorghum. Similar effect of kinetin was noted by Lazar et al. (1983) and Rashid et al. (2009) in wheat. In addition, Li et al. (2009) reported that caryopses of indiagrass cultured on media supplemented with 2,4-D alone generally outperformed those cultured on media supplemented with both 2,4-D and kinetin for embryogenic callus induction. In contrast, the results of Arulselvi and Krishnaveni (2009) revealed that increasing the level of kinetin to a concentration of 0.5 mgL<sup>-1</sup> in the I6 medium than in MS medium increased the frequency of embryogenic calli. This controversy could be attributed to the modification made in the I6 medium. Similarly, Pola et al. (2008) reported that the addition of kinetin to MS medium supported and improved frequency of embryogenesis.

The results (Table 3) also reveal that the mean number of embryogenic calli produced from different sorghum genotypes on media CI1, CI2 and CI3 were not significantly different. These three media, although contain the same concentration of 2,4-D (1.5 mgL<sup>-1</sup>) and L-proline, they have different constituents. These results point out the importance of the auxin, 2,4-D as a main component of the embryogenic callus induction media. In accordance with our results, Rueb et al. (1994), Vikrant and Rashid (2003), Jogeswar et al. (2007) and Zhao et al. (2010) reported that the auxin 2,4-D is critical in the induction of primary calli and embryogenic calli in monocotyledon plants. In this respect, Muhumuza and Okori (2013) pointed out that the combined effects of kinetin and 2,4-D in callus induction medium, highly



**Figure 1.** Steps for callus induction and regeneration of sorghum. **(A)** A plate of embryogenic calli. **(B)** White compact embryogenic callus. **(C)** Yellowish white embryogenic callus. **(D)** Shoot induction from embryogenic calli on regeneration media. **(E)** Shoot and root formation on shooting media. **(F)** Rooting with multiple shoots per callus. **(G)** Rooting with few shoots per callus. **(H)** Regenerated sorghum plantlets acclimatized in Hoagland solution in aquarium. **(I)** Regenerated sorghum plantlets acclimatized in growth chambers. **(J)** Regenerated plants in the greenhouse. **(K and L)** Mature regenerated plants in greenhouse.

**Table 3.** Influence of media composition on embryogenic callus induction in primary cultures of immature embryos of different sorghum lines.

Genotype	Type <sup>b</sup>	Percentage of embryogenic callus <sup>a</sup>					
		C11	C12	C13	C15	C16	Mean
LG3	L	73.2 ± 7.2 <sup>aA</sup>	63.6 ± 6.9 <sup>aAB</sup>	54.3 ± 4.7 <sup>bB</sup>	17.0 ± 2.7 <sup>abcdC</sup>	29.6 ± 4.8 <sup>aC</sup>	47.5 ± 6.0 <sup>A</sup>
LG4	L	59.6 ± 11.5 <sup>abA</sup>	43.2 ± 7.4 <sup>bcdeA</sup>	51.6 ± 6.5 <sup>bcA</sup>	22.0 ± 3.0 <sup>abcB</sup>	19.1 ± 7.6 <sup>abcB</sup>	39.1 ± 5.2 <sup>B</sup>
LG8	L	50.9 ± 4.0 <sup>bA</sup>	36.8 ± 5.0 <sup>cdefAB</sup>	21.9 ± 6.3 <sup>deBC</sup>	13.6 ± 1.2 <sup>abcdC</sup>	17.2 ± 0.4 <sup>abcC</sup>	28.1 ± 4.0 <sup>B</sup>
Dorado	C	54.2 ± 7.5 <sup>bA</sup>	53.5 ± 4.0 <sup>abA</sup>	50.0 ± 12.3 <sup>bcA</sup>	13.0 ± 5.0 <sup>bcdB</sup>	7.0 ± 0.8 <sup>bcdB</sup>	35.5 ± 6.2 <sup>BC</sup>
Keyman	E	30.2 ± 5.1 <sup>cB</sup>	34.5 ± 5.8 <sup>defB</sup>	83.4 ± 5.1 <sup>aA</sup>	25.6 ± 3.9 <sup>abB</sup>	19.5 ± 0.9 <sup>abcB</sup>	38.6 ± 6.3 <sup>B</sup>
SPP462	E	21.5 ± 5.9 <sup>cAB</sup>	30.2 ± 5.1 <sup>efA</sup>	12.1 ± 1.7 <sup>deBC</sup>	3.3 ± 1.9 <sup>dC</sup>	0.0 ± 0.0 <sup>dC</sup>	13.4 ± 3.3 <sup>G</sup>
TX2794	E	15.1 ± 4.3 <sup>cAB</sup>	26.4 ± 0.9 <sup>fA</sup>	22.7 ± 1.3 <sup>deAB</sup>	7.3 ± 2.1 <sup>cdB</sup>	8.8 ± 0.6 <sup>bcdB</sup>	16.1 ± 2.1 <sup>FG</sup>
SPGM94021	E	50.3 ± 5.1 <sup>bA</sup>	52.6 ± 10.7 <sup>abcA</sup>	60.7 ± 2.6 <sup>bA</sup>	17.0 ± 6.9 <sup>abcdB</sup>	4.1 ± 3.5 <sup>cdB</sup>	36.9 ± 6.4 <sup>BC</sup>
SPMD94001	E	19.5 ± 3.1 <sup>cA</sup>	23.0 ± 3.6 <sup>fA</sup>	15.0 ± 4.6 <sup>eA</sup>	29.6 ± 3.0 <sup>aA</sup>	23.4 ± 3.5 <sup>abA</sup>	22.1 ± 1.8 <sup>EF</sup>
TX631	E	50.1 ± 12.0 <sup>bA</sup>	49.6 ± 10.7 <sup>abcdA</sup>	36.3 ± 5.4 <sup>cdA</sup>	19.3 ± 5.8 <sup>abcdB</sup>	0.0 ± 0.0 <sup>dC</sup>	31.0 ± 5.9 <sup>CD</sup>
Mean		42.4 ± 3.9 <sup>A</sup>	41.3 ± 2.9 <sup>A</sup>	40.8 ± 4.3 <sup>A</sup>	16.8 ± 1.7 <sup>B</sup>	12.9 ± 2.0 <sup>B</sup>	30.8 ± 1.7

<sup>a</sup>Each value is the mean percentage of three individual experiments. Numbers (mean ± S.E.) with different letters within the same line (uppercase) and column (lowercase) differ significantly at  $\alpha=0.05$ . <sup>b</sup>L, local germplasm; E, elite line; C, cultivar.

significantly affected callus growth. The addition of kinetin hormone in the callus induction medium at low levels of 2,4-D significantly reduced the callus induction frequency of sorghum. Seemingly, higher 2,4-D levels exhibited inhibitory effects. Moreover, the addition of L-proline to the medium has been reported to enhance the frequency of embryogenic callus formation in cereals (Armstrong and Green, 1985; Perez et al., 1993; Rao et al., 1995; El-Itriby et al., 2003). Furthermore, the present results reveal that the N6-based medium (C13) did not exert significant effect on callus induction from nine out of the 10 assayed genotypes, as compared to the MS-based media (C11 and C12) (Table 3).

#### Effect of the sorghum genotype on callus induction

For each genotype, an average of 150 immature embryos in three replicates was used as explants for the experiments of callus induction. As shown in the results (Table 3), callus induction was found to be highly variable and genotype dependent. The line LG3 revealed the highest average number of calli (47.5 ± 6.0%) across the media tested. This average number was significantly higher than its corresponding value for all the other genotypes. The mean number of calli induced by the different sorghum genotypes differed significantly with the lowest frequency of induced calli (13.4 ± 3.3%) produced by SPP462. Previous investigations on sorghum tissue culture also revealed that callus induction and regeneration are highly genotype dependent (Thomas et al., 1977; Cai and Butler, 1990; Devi and Sticklen, 2001; Arulselvi and Krishnaveni, 2009; Muhumuza and Okori, 2013).

#### Interaction between callus induction media and sorghum genotypes

Results presented in Table 3 show that the influence of the media composition on the induction of embryogenic callus varied significantly among tested sorghum genotypes.

The genotypes LG3, LG4, LG8, Dorado and TX631 showed the highest average of callus induction on medium C11 which was the only MS-based medium supplemented with exogenous vitamins. In C11 medium, the concentrations of the nicotinic acid (0.5 mgL<sup>-1</sup>), pyridoxine (0.5 mgL<sup>-1</sup>) and myoinositol (0.1 mgL<sup>-1</sup>) were equal to their respective concentrations in the other tested medium supplemented with the MS vitamins. However, the concentration of thiamine was higher in medium C11 (10 mgL<sup>-1</sup>) as compared to 0.1 mgL<sup>-1</sup> in MS with vitamins. This reflects the positive effect of increasing the thiamine concentration in the medium of certain genotypes. Thiamine has been reported to exert diverse physiological functions in plants and serves as cofactor in enzymatic reactions, but plant cell requirements for vitamin concentration vary according to the plant species and type of culture (Goyer, 2010; Abrahamian and Kantharajah, 2011). Moreover, medium C11 had an additional 10 mgL<sup>-1</sup> ascorbic acid. Ascorbic acid acts in plants as an antioxidant in addition to its enzymatic cofactor activity, thus reducing phenolics secretion and improving regenerability. This finding is consistent with those of Abdelwahd et al. (2008) on *Vicia faba*.

The two genotypes SPP462 and TX2794 responded better on callus induction medium C12 containing MS with vitamins. Thus, revealing that excess thiamine and



**Table 4.** ANOVA for callus induction in sorghum genotypes.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F value
Genotypes (G)	9	16094.87260	1788.31918	17.1955**
Media (M)	4	25969.57493	6492.39373	62.4273**
Interaction (GXM)	36	17930.25973	498.06277	4.7891**
Treatment	49	59994.70727	1224.38178	11.7730**
Error	100	10399.92667	103.99927	

\*\*Significant at a level of 1% of probability ( $p < 0.01$ ).

ascorbic acid did not increase the capability of these genotypes to induce embryogenic calli.

The frequency of embryogenic calli of only genotype "Keyman" was significantly higher on medium CI3 (N6-based medium) than on the MS-based media. This result is in partial accordance with Sato et al. (2004). They did not observe any embryogenic callus on N6 medium with four sorghum tested lines. The variable responses of the different sorghum genotypes during callus induction and embryogenesis on MS and N6 media can be attributed to the different nitrogenous compounds in these two media. This is in consistence with the suggestion of Armstrong and Green (1985) with maize. Moreover, Hodges et al. (1986) pointed out that the form of nitrogen, which is different in N6 and MS media, may have a fundamental role in the expression of genes that control the embryogenic process.

The frequency of embryogenic calli of the genotype SPMD94001 was generally low on all the media tested. However, the highest frequency of calli in this genotype was induced by medium CI5 (29.6%) which is an MS-based medium supplemented by  $0.1 \text{ mgL}^{-1}$  kinetin. Conversely, the lowest frequency of calli was induced by the N6-based medium CI3. These result reveals the slightly positive effect of low level of kinetin on the callus induction ability of this genotype, which is in disagreement with Mastellar and Holden (1970), Wernicke and Brettell (1982), Zao et al. (2010) and Muhumuza and Okori (2013).

The analysis of variance (ANOVA) (Table 4) indicated that the response to callus initiation was influenced by genotype and callus induction medium independently and thus their interaction had a greater effect. In addition, media effect was higher than genotype effect (6462.39373, for media effect vs. 1788.31918, for genotype effect).

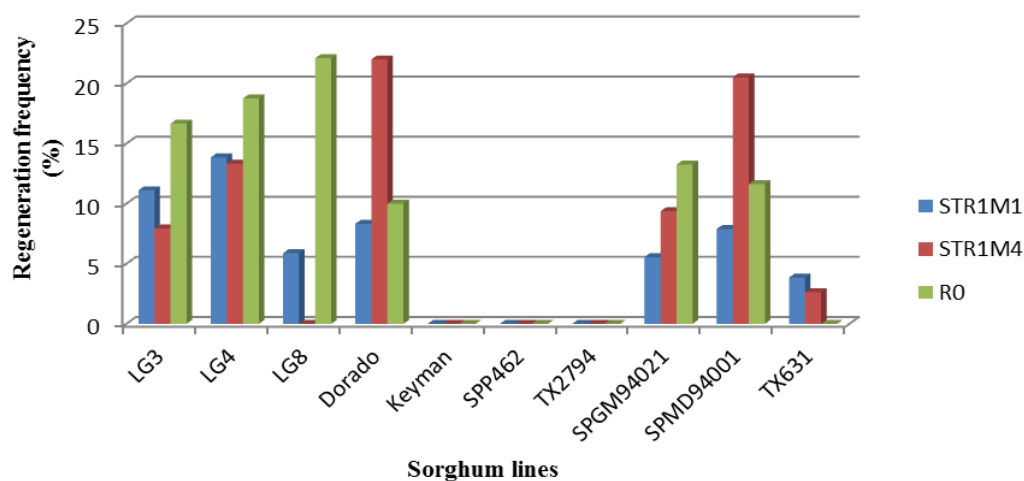
Secretion of phenolic compounds by callus tissues had a great effect on the embryogenic callus induction and regeneration frequency. Although 1% of PVP was added to all callus induction media, our observations indicate that some genotypes produced phenolic compounds in presence of PVP, which had negative effect on the formation of embryogenic callus as well as regeneration ability. To overcome this problem, frequent subcultures have been carried out in less than two weeks intervals.

Dorado and LG3 showed no secretion of phenolics. In contrast, all the other tested lines showed different degrees of phenolics, at least in one experiment or on a certain type of media. The genotypes LG8 and TX631 rarely secreted phenolic compounds. This was followed by SPGM94021 and SPMD94001 which showed weak phenolics secretion. The line LG4 revealed medium secretion, while the lines Keyman, SPP462 and TX2794 exhibited high degree of phenolic secretion.

Embryogenic callus cultures in cereal species were previously described and classified into two morphotypes, differing by morphology, growth rate and extent of embryo differentiation. These two types are: compact white embryogenic (type I) and friable embryogenic (type II) (Elkonin and Pakhomova, 2000). In maize and other cereals, type II embryogenic cultures have higher growth rate and higher regeneration frequency than the compact type I callus cultures (Armstrong and Green, 1985; El-Itriby et al., 2003). With different genotypes of sorghum, we observed the two types of embryogenic callus cultures. Unlike maize, both types of embryogenic callus were regenerable and in some cases, the white compact type I had higher frequencies of regeneration than type II embryogenic callus. In consistence with the present results, Grootboom et al. (2008) reported the formation of highly embryogenic totipotent type I callus in sorghum.

### Regeneration of the sorghum genotypes

The different sorghum genotypes exhibited different regeneration ability. Out of the 10 tested sorghum genotypes, only seven were successfully regenerated on at least two of the three examined regeneration media. The number of shoot per callus ranged from 2 to 17 shoots (Figure 1F and G). The calli of the genotypes Keyman, SPP462 and TX2794 failed to regenerate on any of the three regeneration media. Although these lines produced a high frequency of embryogenic calli with mean values of 38.7, 13.4 and 16.0%, respectively, across the seven tested media, these calli failed to develop any shoots. This could be attributed to the high level of phenolic compounds produced by these genotypes. Phenolic compounds secretion has been previously revealed by many authors in sorghum among



**Figure 2.** Effect of media composition on shoot formation of different sorghum lines.

other plant species to exert an inhibitory effect on callus regeneration (Oberthur et al., 1983; Dicko et al., 2006; Zhao et al., 2010).

### Interaction between regeneration media and sorghum genotypes

The effect of the three investigated regeneration media on the capability of the 10 sorghum genotypes to develop shoots is represented as a histogram (Figure 2). The regeneration percentage ranged from 0 to 22.1%. The genotypes LG3, LG4, Dorado, SPGM94021 and SPMD94001 succeeded to form shoots on the three tested media. Nevertheless, the genotypes LG8 and TX2794 produced shoots on only two out of the three media.

The local lines LG3, LG4 and LG8, in addition to the genotype SPGM94021 revealed the highest percentage of regenerated shoots on medium R0 (16.66, 18.75, 22.1 and 13.25%, respectively). The highest number of shoots formed by the genotypes Dorado and SPMD94001 was on medium STR1M4. Meanwhile, the genotype TX631 formed shoots on only two regeneration media (STR1M1 and STR1M4) with low frequency, although it was slightly higher on STR1M1 than on STR1M4.

The regeneration medium R0 in contrast to the other two regeneration media was supplemented with different growth regulators, IAA, zeatin, TDZ and abscisic acid (ABA). This might reflect the demand of the genotypes LG3, LG4, LG8 and SPGM94021 to these growth regulators for enhancing the process of shoot formation and development; while, shoot formation in Dorado and SPMD94001 is more influenced by the addition of BA and IAA (2 and 500 mgL<sup>-1</sup>, respectively). In this respect, different authors reported the use of different growth

regulators to improve the regeneration efficiency. Visarada et al. (2003) obtained efficient regeneration from immature embryo derived calli using 0.5 mgL<sup>-1</sup> BA + IBA. Rathus et al. (2004) revealed that a combination of 1 mgL<sup>-1</sup> IAA and 1 mgL<sup>-1</sup> zeatin gave the best results for shoot regeneration in sorghum. Correspondingly, Zhao et al. (2010) observed the best shoot induction when the explants were cultured on MS medium supplemented with 1 mgL<sup>-1</sup> IAA and 3 mgL<sup>-1</sup> BA and on MS medium supplemented with 2 mgL<sup>-1</sup> BA and 0.5 mgL<sup>-1</sup> kinetin.

The regenerated shoots were transferred to rooting media. Two rooting media that is "rooting 0" containing 0.5 mgL<sup>-1</sup> of each IBA and  $\alpha$ -naphthaleneacetic acid (NAA) and "rooting 1" free of growth regulators were examined. In all the tested genotypes, short, thick and poor rooting were observed on Rooting 0, while, long, strong and well develop roots were observed on rooting 1. Conversely, Rao and Kavikishore (1989) and Pola and Mani (2006) obtained good rooting on MS medium supplemented with NAA. Seemingly, Zhao et al. (2010) transferred the regenerated shoots onto MS medium supplemented with 3 mgL<sup>-1</sup> BA for rooting.

Some of the plantlets died when rooted plantlets were transferred directly to pots in greenhouse for acclimatization. This loss in the regenerated plantlets was significantly reduced when plantlets were transferred to an aquarium containing Hoagland solution (Figure 1H) before transferring to the pots and all of the survived plants reached maturity and found to be fertile (Figure 1K and L). Hydroponic culture were found to aid in plantlets acclimatization by providing a humid atmosphere, allowing the formation and ramification of new roots. In this respect, Pospíšilová et al. (1999) pointed out that the roots of the regenerated plantlets are fragile and susceptible to mechanical damage. For that reason, the plantlets may die shortly after transplanting into pots. Similarly,

Zapata et al. (2003) reported a significant increase in the number of leaves and roots in hydroponic system compared to control.

The results of the present investigation revealed different responses to *in vitro* culture and clear interactions of the sorghum genotypes with the different nutrient media. Thus, determination of the best genotype–medium combination is essential to obtain satisfactory regeneration frequency. This finding has been also reported by Assem (2001), Grootboom et al. (2008) and Arulselvi and Krishnaven (2009). Moreover, these results confirm that in sorghum, as in other cereals, *in vitro* response, that is the efficiency of callus induction and plant regeneration frequency are highly genotype dependent. This has been previously reported by different authors: in wheat (Yadava and Chawla, 2001), in maize (El-Itriby et al., 2003), in barley (Hussein et al., 2010) and in sorghum (Jogeswar et al., 2007; Grootboom et al., 2008).

In conclusion, most of the previous studies on *in vitro* culture of sorghum were on highly regenerable and tissue culture amenable genotypes, such as, P898012, BTX 430 and BTX 623 (Kishore et al., 2006; Girijashankar et al., 2007; Grootboom et al., 2008). Nevertheless, the present work investigated different Egyptian adapted sorghum genotypes of different origins and agronomical importance. These genotypes include tall, short, drought tolerant, drought susceptible, cultivated and tropical sorghum.

The genotypes tested showed a variation of genotypic responses to *in vitro* culture. From the obtained *in vitro* culture results, the genotypes of choice for transformation experiments would be in that order of priority, LG3 with media C11 and R0, SPMD94021 with media C13 and R0, LG4 with media C11 and R0, Dorado with media C11 and STR1M4 and LG8 with medium C11 or C12 and R0. These combinations display the best regeneration potential and should then increase the probability of producing transgenic plants using these lines.

### Conflict of Interests

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

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Full Length Research Paper

## Micropropagation of *Plectranthus edulis* (Vatke) Agnew from meristem culture

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***Plectranthus edulis* (Vatke) Agnew** is a tuber-bearing food crop in Ethiopia. However, its productivity is hampered by shortage of pathogen-free planting materials. Therefore, the objective of this study was to develop micropropagation protocol for this plant using meristem to produce clean planting materials. Meristems were collected from apical and axillary shoots from Holeta and Welayta areas and cultured on MS medium containing gibberellic acid (GA<sub>3</sub>) (1.0 mg l<sup>-1</sup>), α-naphthalene acetic acid (NAA) (0.1 mg l<sup>-1</sup>) in combination with 6-benzylaminopurine (BAP) (0.1, 0.5, 1.0, 2.0 and 5.0 mg l<sup>-1</sup>). Shoots were multiplied on MS medium containing 0.1, 0.5, or 1.0 mg l<sup>-1</sup> of BAP or 0.5, 1.0, 2.0 or 3.0 mg l<sup>-1</sup> of Kinetin alone or their combination with 0.05 or 0.1 mg l<sup>-1</sup> NAA. *In vitro* and *ex vitro* rooting was performed using different types of auxins followed by acclimatization. The highest percentage of shoots initiated from collected meristem at Holeta (73%) was obtained on MS medium containing 1.0 mg l<sup>-1</sup> BAP, 1.0 mg l<sup>-1</sup> GA<sub>3</sub> and 0.1 mg l<sup>-1</sup> NAA. The highest shoot number explants<sup>-1</sup> (7.2) was obtained on MS medium containing 1.0 mg l<sup>-1</sup> Kinetin and 0.1 mg l<sup>-1</sup> NAA, whereas the highest root number shoot<sup>-1</sup> (6.2) was obtained from *ex vitro*. All plants derived from Holeta and 96.7% of those from the Welayta survived after acclimatization. These results provided rapid and reproducible conditions for propagation of relatively pathogen-free planting material of this plant.

**Key words:** *In vitro* propagation, meristem culture, micropropagation, *Plectranthus edulis* (Vatke) Agnew, shoots multiplication.

### INTRODUCTION

*Plectranthus edulis* (Vatke) Agnew is also known as *Coleus edulis* and an indigenous root crop in Ethiopia. It serves an important part of the diet of the population (PGRC,

1996; IBC, 2005) and is widely used as a major source of tasty carbohydrates in many parts of Ethiopia (Taye et al., 2007). It is one of the major crops cultivated in Oromia

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**Abbreviations:** GA<sub>3</sub>, Gibberellic acid; NAA, α-naphthalene acetic acid; BAP, 6-benzylaminopurine; PGR, plant growth regulator; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid.

region (Sirika, 2011).

The genus *Plectranthus* has been distributed all over the tropical and subtropical regions of India, Pakistan, Sri Lanka, Tropical East Africa, Brazil and Egypt (Sunilkumar, 2005). *P. edulis* is one of the native tuber crops in Ethiopia (IBC, 2005) and is cultivated in the south and south western parts of Ethiopia whereas the wild species are found throughout the country (PGRC, 1996). It also serves as a medicinal plant and eating the cooked root avoids loss of appetite (Megersa, 2010).

The major constraints of production identified by Taye (2008) are the poor storability of the tubers and the shortage of seed tubers. Seed production of *P. edulis* is an important direction to emphasize for this crop, although the effect of seed quality on performance of the resultant crop is yet unknown. Alternatively, growers of *P. edulis* can continually acquire large numbers of disease-free planting stock from plants micropropagated from meristem explants.

Reports recently documented the in growing areas of *P. edulis* showed the occurrence of diseases (Taye, 2008). It is propagated vegetatively, and hence it is susceptible to diseases that are carried from one generation to the next through infected planting material. The systemic diseases of viruses, viroids, and mycoplasma and several bacteria are the most devastating in terms of yield loss for root and tuber crops (Bryan, 1983).

The application of plant tissue culture offers valuable ways to overcome many of the problems that are encountered in natural propagation. Meristem culture can be used alone or in combination with thermotherapy to improve the elimination rate of a number of viruses and bacteria (Kantha and Gamborg, 1975). Increased use of rapid multiplication techniques is enabling scientists to select and produce large amounts of pathogen-free material.

Meristem culture can even improve the technique by eliminating systemic pathogens present in the donor plant (Stepan-Sarkissian, 1990). Therefore, the objective of this study was to develop a micropropagation protocol for *P. edulis* using meristem as explant.

## MATERIALS AND METHODS

### Sterilization of explants

Seed tubers of *P. edulis* that were collected from Holeta (30 km west of Addis Ababa) and Welayta (330 km south west of Addis Ababa) were planted in greenhouse. Approximately 1.5 to 2.0 cm long apical and axillary shoots were collected from two-month-old greenhouse grown mother plants. The shoot explants were washed twice with tap water and detergent, and rinsed with double distilled water twice. Those explants were then surface sterilized with 70% alcohol for 30 s and rinsed three times with sterile double distilled water followed by sterilization by 0.5% sodium hypochlorite containing three drops of Tween 20 for 10 min. After sterilization, the explants were rinsed three times with sterile double distilled water.

### Meristem isolation and shoot induction

Leaves were removed and meristems of 0.2 to 0.5 mm diameter with one or two leaf primordia were isolated under a dissecting microscope using sterile forceps, scalpels and hypodermic needles.

The meristems were cultured on 90 mm diameter Petri dishes each containing 20 ml shoot induction medium. The shoot induction medium consisted of MS medium (Murashige and Skoog, 1962) containing different concentrations of BAP (0.1, 0.5, 1.0, 2.0 or 5.0 mg l<sup>-1</sup>) in combination with 0.1 mg l<sup>-1</sup> NAA, 1.0 mg l<sup>-1</sup> GA<sub>3</sub> and supplemented with 30 g l<sup>-1</sup> sucrose. Experimental design used in shoot induction was a three factor treatment structure in completely randomized design in which total of 5 combinations of three factors (five levels of the BAP, 0.1, 0.5, 1.0, 2.0, 5.0 mg l<sup>-1</sup> one level of NAA 0.1 mg l<sup>-1</sup> and one level of GA<sub>3</sub> 1.0 mg l<sup>-1</sup>) was observed. After the pH was adjusted to 5.8, the medium was autoclaved at 121°C for 15 min. Growth regulator-free medium was used as a control in these experiments.

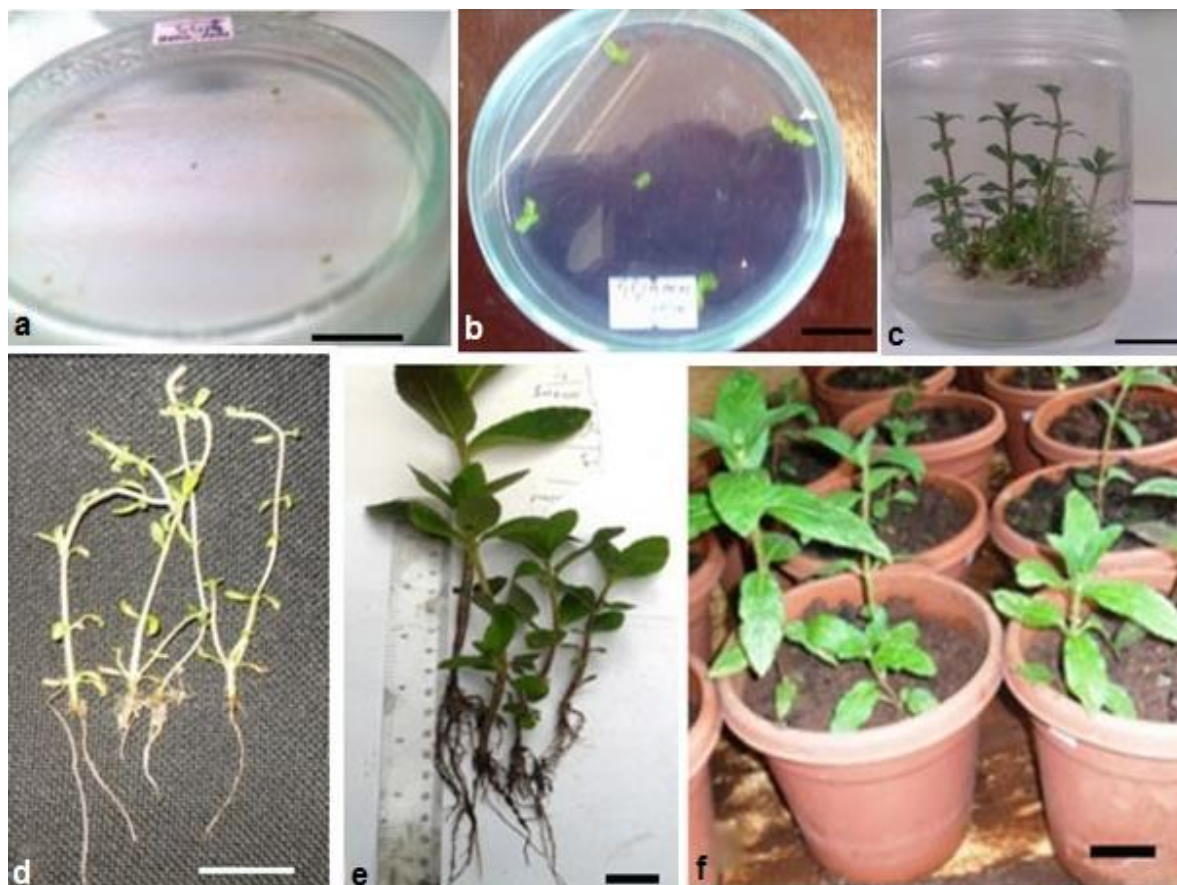
Five explants were cultured per Petri dish with six replications. Cultures were transferred to the same fresh medium every two weeks until shoots were initiated. The cultures were maintained at temperature of 25 ± 2°C under a light intensity of 20 μmol m<sup>-2</sup> s<sup>-1</sup> and a 16 h photoperiod provided by cool-white fluorescent lamps. The numbers of meristems induced to shoots were recorded. The experiment was then repeated once. Unless otherwise indicated, all cultures were maintained under these culture conditions.

### Shoot multiplication

Initiated shoots were cultured on shoot multiplication medium. Shoot multiplication medium consisted of MS medium with variable concentrations of BAP (0.1, 0.5, or 1.0 mg l<sup>-1</sup>) or Kinetin (0.5, 1.0, 2.0 or 3.0 mg l<sup>-1</sup>) alone or their combination with NAA (0.05 or 0.1 mg l<sup>-1</sup>). Growth regulator-free medium was used as a control. Shoots were cultured in Magenta GA-7 vessels containing 50 ml medium. For each treatment, a total of 30 explants were used. The treatment structure was considered as a completed two-factorial design with three levels of BAP (0.1, 0.5, and 1.0 mg l<sup>-1</sup>) alone or with either four levels of Kinetin (0.5, 1.0, 2.0 or 3.0 mg l<sup>-1</sup>) or two levels of NAA (0.05 and 0.1 mg l<sup>-1</sup>) augmented with a control (0 mg l<sup>-1</sup> BAP, 0 mg l<sup>-1</sup> Kinetin and 0 mg l<sup>-1</sup> NAA). The effects of different treatments were quantified on the basis of number of shoots explants<sup>-1</sup> for each treatment. Number and length of shoots explants<sup>-1</sup> were recorded after four weeks of culture. The whole experiment was repeated once.

### Rooting

Rooting was performed both *in vitro* and *ex vitro*. *In vitro* rooting was carried out in two ways: firstly, shoots were cultured on both full and half strength MS media containing 1.0 mg l<sup>-1</sup> NAA, IAA or IBA. Secondly, shoots were immersed into 5.0 mg l<sup>-1</sup> IBA for 5 min before transferring into growth regulator-free MS medium. Shoots were kept for a week in darkness and were then transferred to a 16-h photoperiod for three weeks. In *ex vitro* rooting, shoots were transferred to plastic pots filled with soil, compost, and sand in a 2:1:1 ratio respectively following immersion into 5.0 mg l<sup>-1</sup> IBA for 5 min and were then covered with polyethylene bags. The polyethylene bags were removed from the pots after a week under greenhouse condition. Experimental design used in rooting was a two factor treatment structure in completely randomized design in which five out of six combination of two factors (two levels of the MS salt half and full strength alone or with three levels of hormone 1.0 mg l<sup>-1</sup> NAA, 1.0 mg l<sup>-1</sup> IAA and 5.0 mg l<sup>-1</sup> IBA) together with one *ex vitro* rooting was observed. Rooting was evaluated in terms of rooting percentage, root number, and the mean root length after



**Figure 1.** Mersitem culture on MS medium containing 1.0 mg/l BAP in combination with 1.0 mg/l GA<sub>3</sub> and 0.1 mg/l NAA. **(a)** After one week. **(b)**. After 20 days. **(c)**. Shoots cultured on MS medium containing 1.0 mg/l BAP. **(d)**. Shoots rooted on half strength MS medium containing 1.0 mg/l IBA. **(e)**. Shoots rooted *ex vitro* by immersing in 5.0 mg/l IBA for 5 min: Bars = 2 cm. **(f)**. Plants after acclimatization: Bar = 5 cm.

one month of culturing.

#### Acclimatization

After four weeks in rooting medium, roots were washed thoroughly to remove residual medium and transferred to plastic pots containing a mixture of soil, compost and sand in a ratio of 2:1:1, respectively. Each pot was covered with a polyethylene bag and kept in the greenhouse. The polyethylene bags were removed after a week and the number of plants that survived in the greenhouse was recorded after a month. Furthermore, the pots were transferred to external environment and watered as necessary. The experimental design used in acclimatization phase was a one-factor treatment structure in a completely randomized design. The total number of plantlets transferred to pots was 240; each treatment represented by 60 plantlets (from *ex vitro* and *in vitro* rooted Holeta and Welayta origin).

#### Statistical analysis

Statistical analysis of quantitative data was carried out by the SPSS computer software version 16. Data were subjected to analysis of variance and variables that showed significant difference were compared by the LSD at 5% probability.

## RESULTS

### Shoot induction from meristem

The use of solid MS medium containing BAP, GA<sub>3</sub> and NAA resulted in the development of single or multiple shoots from a meristem. An earlier sign of growth from treated meristem was noticeable within seven days after culture of the explants (Figure 1a to c).

ANOVA results showed that cytokinin concentrations significantly affected percentages of shoot induction ( $p \leq 0.05$ ), although no statistically significant difference was seen between plants of the two collection areas in percentage of shoot induction. The highest percent shoot induction (73%) was achieved from MS medium containing 0.1 mg/l and 1.0 mg l<sup>-1</sup> BAP in combination with 1.0 mg l<sup>-1</sup> GA<sub>3</sub> and 0.1 mg l<sup>-1</sup> NAA (Table 1). Percentages of shoot induction in these treatments ranged from 52 to 73%. At higher concentrations of BAP (2.0 or 5.0 mg l<sup>-1</sup>) combined with 0.1 mg l<sup>-1</sup> NAA and 1.0 mg l<sup>-1</sup> GA<sub>3</sub> stunted adventitious shoots were observed.

**Table 1.** Effect of different concentrations of BAP combined with GA<sub>3</sub> and NAA on shoot induction from meristem explants of *P. edulis* after six weeks of culture.

Plant growth regulators			Percentage of shoot induction	
BAP (mg l <sup>-1</sup> )	GA <sub>3</sub> (mg l <sup>-1</sup> )	NAA (mg l <sup>-1</sup> )	Holeta	Welayta
0.0	0.0	0.0	10	13
0.1	1.0	0.1	73	60
0.5	1.0	0.1	53	57
1.0	1.0	0.1	72	65
2.0	1.0	0.1	53	58
5.0	1.0	0.1	58	52

**Table 2.** Percentage of shoots producing multiple shoots, number and length of shoots per explant that were cultured on MS medium containing different concentration of BAP, Kinetin and NAA.

PGR (mg/l)			Explants with multiple shoots (%)		Number of shoots explant <sup>-1</sup> ± SD		Shoot length (cm) ± SD	
BAP	Kinetin	NAA	Holeta	Welayta	Holeta	Welayta	Holeta	Welayta
0	0	0	16.6	0.0	1.2±0.4 <sup>a</sup>	1.0±0.0 <sup>a</sup>	7.2±1.5 <sup>a</sup>	7.3±1.4 <sup>a</sup>
0.1	0	0	73.3	76.7	2.1±0.8 <sup>bd</sup>	2.1±0.8 <sup>b</sup>	4.0±1.5 <sup>b-f</sup>	4.4±1.7 <sup>b-j</sup>
0.5	0	0	100	96.7	4.51±2.3 <sup>cg</sup>	4.1±1.3 <sup>c-g</sup>	2.8±1.5 <sup>c</sup>	3.0±1.6 <sup>c</sup>
1.0	0	0	100	100	4.9±1.6 <sup>cj</sup>	4.8±1.5 <sup>c-i</sup>	2.6±1.3 <sup>c</sup>	2.5±1.2 <sup>d</sup>
0.1	0	0.05	78.3	85	2.7±1.1 <sup>bdf</sup>	3.2±1.6 <sup>eg</sup>	3.3±2.1 <sup>c-g</sup>	4.1±2.5 <sup>bef</sup>
0.5	0	0.05	68.3	95	2.7±1.7 <sup>bdf</sup>	4.5±2.4 <sup>cdf</sup>	3.2±1.6 <sup>ceg</sup>	3.2±1.5 <sup>ci</sup>
1.0	0	0.05	100	100	5.8±2.0 <sup>ej</sup>	4.9±1.9 <sup>dfi</sup>	3.0±1.5 <sup>c</sup>	3.0±1.7 <sup>ci</sup>
0.1	0	0.1	75	86.7	3.1±1.4 <sup>dffh</sup>	3.2±1.7 <sup>eg</sup>	4.7±2.0 <sup>bd</sup>	4.3±2.1 <sup>be</sup>
0.5	0	0.1	100	100	4.6±1.6 <sup>cgj</sup>	4.0±1.5 <sup>cfg</sup>	3.3±1.1 <sup>ceg</sup>	3.2±1.5 <sup>ci</sup>
1.0	0	0.1	100	100	4.5±1.9 <sup>cg</sup>	5.2±2.4 <sup>d-j</sup>	3.3±1.6 <sup>ceg</sup>	3.2±1.7 <sup>ci</sup>
0	0.5	0	86.7	85	3.3±1.5 <sup>d-h</sup>	3.2±1.4 <sup>eg</sup>	4.2±1.9 <sup>b-g</sup>	4.2±1.9 <sup>bef</sup>
0	1.0	0	90	100	4.1±1.7 <sup>cg</sup>	4.7±1.4 <sup>c-i</sup>	4.3±1.8 <sup>bdf</sup>	4.2±1.9 <sup>bef</sup>
0	2.0	0	100	100	4.7±1.8 <sup>cgj</sup>	4.7±1.9 <sup>c-i</sup>	3.9±1.9 <sup>b-f</sup>	3.9±1.9 <sup>b-g</sup>
0	3.0	0	100	100	4.7±2.1 <sup>cgj</sup>	4.7±2.1 <sup>c-i</sup>	3.8±1.5 <sup>b-g</sup>	3.8±1.5 <sup>fg</sup>
0	0.5	0.05	86.7	93.3	3.5±1.8 <sup>fgh</sup>	3.7±1.7 <sup>ceg</sup>	4.6±2.1 <sup>bd</sup>	4.7±2.2 <sup>bhj</sup>
0	1.0	0.05	95	90	4.3±2.3 <sup>cg</sup>	4.0±2.3 <sup>cfg</sup>	4.6±1.9 <sup>bd</sup>	4.2±2.0 <sup>be</sup>
0	2.0	0.05	100	100	4.1±2.4 <sup>cg</sup>	3.9±2.3 <sup>c-g</sup>	4.1±2.1 <sup>b-g</sup>	4.3±2.4 <sup>beh</sup>
0	3.0	0.05	100	100	6.6±4.1 <sup>i</sup>	6.2±4.2 <sup>hj</sup>	4.0±1.7 <sup>bfg</sup>	4.3±1.8 <sup>be</sup>
0	0.5	0.1	100	98.3	5.7±1.3 <sup>ej</sup>	5.2±1.6 <sup>d-j</sup>	4.5±1.4 <sup>bd</sup>	4.6±1.7 <sup>bhj</sup>
0.0	1.0	0.1	100	93.3	7.2±2.1 <sup>i</sup>	5.9±2.8 <sup>d hij</sup>	2.9±0.9 <sup>c</sup>	3.3±1.4 <sup>ci</sup>
0.0	2.0	0.1	100	96.7	5.3±2.8 <sup>cej</sup>	4.6±2.7 <sup>cd fi</sup>	3.2±1.3 <sup>ce</sup>	3.3±1.4 <sup>ci</sup>
0.0	3.0	0.1	100	100	5.5±2.3 <sup>cej</sup>	5.1±1.6 <sup>d fi</sup>	3.5±1.1 <sup>cefg</sup>	4.7±1.6 <sup>bhj</sup>

Means followed by the same letter within a column were not significantly different at 5% probability.

### Shoot multiplication

Shoot number was highly influenced by concentration and type of the growth regulators. The highest number of shoots explant<sup>-1</sup> (7.2) was obtained on MS medium supplemented with 1.0 mg l<sup>-1</sup> Kinetin and 0.1 mg l<sup>-1</sup> NAA from shoots of Holeta origin. The highest number of shoots explant<sup>-1</sup> (6.2) of Welayta origin was obtained on MS medium containing 3.0 mg l<sup>-1</sup> Kinetin and 0.05 mg l<sup>-1</sup>

NAA (Table 2). Generally, the number of shoots explant<sup>-1</sup> was higher on media containing Kinetin than on those containing BAP. After 35 days of culture, most of shoots cultured on plant regulator (PGR) free medium (83.4% of Holeta and all explants of Welayta) produced only a single shoot with highest mean shoot length of 7.2 and 7.3 cm from Holeta and Welayta, respectively. The length of shoots was affected by sources of the plant, as well as types and concentrations of the growth regulators



**Table 3.** Effect of MS salt strength and types of auxin on rooting, number of roots shoot<sup>-1</sup> and root length of *P. edulis*.

Treatment		Rooted shoots (%)		Number of roots explant <sup>-1</sup> ± SD		Root length (cm) ± SD	
MS salt strength	Auxin (1.0 mg l <sup>-1</sup> )	Holeta	Welayta	Holeta	Welayta	Holeta	Welayta
Full strength	0	60	31.7	1.9±1.8 <sup>a</sup>	0.8±1.2 <sup>a</sup>	1.7±1.0 <sup>ac</sup>	1.0±0.7 <sup>ad</sup>
Full strength	NAA	30	35.0	0.6±1.1 <sup>bce</sup>	0.9±1.5 <sup>a</sup>	1.0±0.6 <sup>bc</sup>	0.9±0.3 <sup>ad</sup>
Full strength	IAA	10	33.3	0.2±0.5 <sup>bc</sup>	0.8±1.4 <sup>a</sup>	1.5±0.4 <sup>abc</sup>	1.5±0.5 <sup>bd</sup>
Full strength	IBA	95	61.7	3.2±1.5 <sup>d</sup>	1.61±1.5 <sup>b</sup>	1.6±1.0 <sup>ac</sup>	2.0±1.4 <sup>cd</sup>
Half strength	IAA	25	10	1.0±1.8 <sup>be</sup>	0.1±0.3 <sup>ce</sup>	1.6±0.5 <sup>ac</sup>	1.5±0.4 <sup>abc</sup>
Half strength	IBA	100	76.7	3.1±0.9 <sup>d</sup>	2.2±1.4 <sup>d</sup>	1.5±0.9 <sup>c</sup>	1.1±0.2 <sup>ad</sup>
*Half strength	0	38.3	16.7	0.7±0.9 <sup>bce</sup>	0.3±0.8 <sup>e</sup>	0.9±0.4 <sup>bc</sup>	0.9±0.2 <sup>ad</sup>
* <i>Ex vitro</i>	0	100	100	6.2±1.9 <sup>f</sup>	5.4±0.9 <sup>f</sup>	3.3±1.1 <sup>d</sup>	3.3±0.9 <sup>e</sup>

Plants of treatments indicated by asterisk, \*Half strength and \**Ex vitro* were grown on growth regulator free MS medium and potting mix respectively, but the shoots were dipped into 5.0 mg l<sup>-1</sup> IBA for 5 min before culturing on growth regulators free medium and potting mix. Means followed by the same letter within a column were not significantly different at 5% probability.

(Table 2). Shoots cultured on higher concentration of BAP (1.0 mg l<sup>-1</sup>) have significantly lower shoot length compared to explants cultured on medium containing a lower concentration of BAP (0.1 mg l<sup>-1</sup>).

Shoots cultured on medium containing 1.0 mg l<sup>-1</sup> BAP combined with 0.05 mg l<sup>-1</sup> NAA produced more shoots than those cultured on the same concentration of BAP alone. Mention-worthily among shoots cultured on PGR free MS medium, 75% of Holeta and 91.7% of Welayta showed spontaneous rooting. On the other hand, spontaneous rooting of 30 and 20% were exhibited by shoots of Holeta and Welayta origin, respectively, on MS medium containing 1.0 mg l<sup>-1</sup> Kinetin and 0.1 mg l<sup>-1</sup> NAA.

### Rooting and acclimatization

Rooting was observed three weeks after culturing the shoots on root induction medium, and most shoots developed roots by the fourth week (Figure 1d). The Holeta area plants had a better rooting response as compared to Welayta area plants in most cases (Table 3).

### Effect of MS salt strength and auxins on root induction

Shoots rooted better on half strength MS medium than full strength medium. The highest percentage of rooting (100%) was exhibited by shoots of Holeta origin when, only 76.7% of the shoots of Welayta origin produced roots on half strength MS medium containing 1.0 mg l<sup>-1</sup> IBA. In the same medium, shoots of Holeta origin produced a mean root number of 3.1 whereas those of Welayta origin produced 2.2 (Table 3).

*Ex vitro* exhibited 100% rooting and 6.2 and 5.4 mean roots shoot<sup>-1</sup> from Holeta and Welayta origin, respectively, and produced the longest mean root length of 3.3 cm in both cases. In *ex vitro* rooting, shoots of Holeta origin

showed a higher percentage of survival (90%) compared to shoots of Welayta origin which exhibited 76.7% survival. *In vitro* rooted shoots showed a better survival percentage during acclimatization than *ex vitro* rooted shoots. Rooting percentage, number of roots shoot<sup>-1</sup>, and root length were significantly different resulting from these treatments in both Holeta and Welayta origins. *Ex vitro* grown shoots showed well developed leaf and root structures (Figure 1e). After acclimatization of one month in greenhouse, 100% of the plants of Holeta origin and 96.7% of plants of Welayta origin survived (Figure 1f). All plants that were transferred to external environment while still in pots were survived after one month.

## DISCUSSION

### Shoot induction from meristem

MS medium containing 1.0 mg l<sup>-1</sup> GA<sub>3</sub> and 0.1 mg l<sup>-1</sup> NAA in combination with different concentrations of BAP exhibited direct shoot initiation from meristem explants. Similar results was observed in yams by Acedo (2006) using MS medium containing the growth regulators used in the present study, although the medium the author used was liquid. Other species like sweet potato and cassava also showed good response towards plant regeneration in MS medium in the presence of BAP combined with auxins as reported by various authors (Acedo, 2006; Wondimu et al., 2012). Dagnino et al. (1991) also reported that different cultivars of *Ipomoea batatas* responded differently to different concentrations of GA<sub>3</sub> yet it had no effect on growth of *Coracao alado* and promoted multiple shoot in 'Mae de Familia' cultivars.

Shoot development without tissue callusing was effected only when the three growth regulators were combined (Acedo, 2006). Armin et al. (2011) found that the best shoot induction response of purple colored sweet potato was obtained on MS medium supplemented with 1.0 mg l<sup>-1</sup>

BAP. Beyene et al. (2010) also reported the use of 0.5 or 1.0 mg l<sup>-1</sup> BAP in combination with 1.0 mg l<sup>-1</sup> GA<sub>3</sub> and 0.01 mg l<sup>-1</sup> NAA gave morphologically good looking shoots in cassava varieties. These findings were consistent with the results of the present study in which MS medium supplemented with 1.0 mg l<sup>-1</sup> BAP, 0.1 mg l<sup>-1</sup> NAA and 1.0 mg l<sup>-1</sup> GA<sub>3</sub> proved superior in terms of shoot initiation from meristem in both mother plants collected from Holeta and Welayta.

Meristems cultured *in vitro* on shoot culture medium containing only low amounts of growth regulators produced one shoot, but they can be induced to form multiple shoots on cytokinin containing medium (Puonti-Kaerlas, 1998). In our study, most of the meristems induced multiple shoots.

The shoots induced from higher cytokinin (2.0 to 5.0 mg l<sup>-1</sup> BAP) treatments were numerous, but grew slowly, formed callus like structure at their base, and were not effectively multiplied in successive subcultures. Such shoot phenotypes were reported also by Yasmin et al. (2011) in potato; most of combinations of higher concentration of BAP and NAA explants formed shoots and calli at the base except on 0.5 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA which regenerated shoots without callus formation.

Usually, no exogenous growth regulators are required if meristems contain two or more leaf primordia (Altaf, 2009). But the report on potato proliferation by Yasmin et al. (2011) showed that growth of explants is slow in media devoid of growth regulator. This result agree with the present study where the least shoot induction percentage was observed in growth regulators free medium (about 10% from Holeta and 13% from Welayta); growth was slow and shoots were stunted.

All combinations of growth regulators tested led to shoot induction from meristem during the early stage. Each treatment showed different patterns and qualities of growth during later stages. All these results showed that 1.0 mg l<sup>-1</sup> BAP combined with 1.0 mg l<sup>-1</sup> GA<sub>3</sub> and 0.1 mg l<sup>-1</sup> NAA in MS solid medium is the optimum concentration to induce shoots from meristem of *P. edulis*.

### Shoot multiplication

Application of BAP and NAA in shoot multiplication MS medium decreased shoot formation and rooting of single nodes of 'Agrida' and 'Marfona' cultivars of potato. The best medium for shoot formation and rooting was modified solid MS medium without NAA and BAP (Armin et al., 2011). Contrary to this report, in our study, both BAP and Kinetin performed well in multiple shoot induction. However, Kinetin exhibited higher shoot number explant<sup>-1</sup> than BAP, alone or in combination with NAA. This was in agreement with the result of Salehi (2006) who obtained the best shoot proliferation on MS medium containing 3.0 mg l<sup>-1</sup> kinetin and 0.5 mg l<sup>-1</sup> NAA or 1.0 mg l<sup>-1</sup> BA and 1.0 mg l<sup>-1</sup> NAA for cultivars of *Dianthus caryophyllus* species. On the other hand, using

BAP (0.5 mg l<sup>-1</sup>) alone was found to be sufficient for high numbers of shoot formation (Nagib et al., 2003).

On the other hand, Badoni and Chauhan (2009) reported that combinations of higher concentration of Kinetin (1.0 mg l<sup>-1</sup>) and low concentration of NAA resulted in the least mean shoot height and number of nodes in potato cultivar 'Kufri Himalini'. In the present study, the highest mean number of multiple shoots was observed in 1.0 mg l<sup>-1</sup> Kinetin in combination with 0.1 mg l<sup>-1</sup> NAA for Holeta and 3.0 mg l<sup>-1</sup> Kinetin in combination with 0.05 mg l<sup>-1</sup> NAA for Welayta. These shoots originated from different sites and showed variation in the number of shoots at different concentrations of NAA and Kinetin. This is possibly due to the presence of genotype difference between plants collected from these two sites as different genotypes of the same plant species respond differently in tissue culture (Dagnino et al., 1991; Feyissa et al., 2005; Jamshieed et al., 2010). *P. edulis* treated with PGR free medium exhibited strong apical dominance with little tendency to branch. Due to this, most of the controls had only a single shoot and the mean length of the shoots was maximum, a result of apical dominance. This agree with the findings of Ezeibekwe et al. (2009) who reported in white yam that shoots cultured on PGRs free medium resulted in the longest shoot. The result of this study also showed that the number of shoots increased and the mean length of shoots decreased as the concentration of BAP increased. This is due to the fact that BAP decreases apical dominance.

A comparatively lower response was recorded when BAP or Kinetin was added alone in the medium of the water yam (Behera et al., 2009). In our study, when both types of cytokinins were used alone, they produced multiple shoots and Kinetin was slightly more effective. When BAP or Kinetin was combined with NAA in multiplication medium, the frequency of multiple shoot development was significantly improved as indicated by significant difference in the number of shoots between explants treated with 1.0 mg l<sup>-1</sup> BAP alone and its combination with 0.05 mg l<sup>-1</sup> NAA. This was in agreement with the report of Adeniyi et al. (2008) as there was significant BAP x NAA interaction indicating that the effectiveness of each of the phytohormones in inducing shoots was influenced by the presence or absence of the other. The work of Hoque (2010) also supports this finding as kinetin induces multiple shoots but the rate of shoot multiplication was slightly higher in combination with IAA.

### Rooting and acclimatization

In this study, among the *in vitro* rooting cultures, the maximum number of roots shoot<sup>-1</sup> was observed in half strength MS medium containing 1.0 mg l<sup>-1</sup> IBA. Statistically significant differences were seen in the percentage of rooted shoots between full and half strength MS medium of the

same IBA concentration ( $p \geq 0.05$ ). Ahmad et al. (2003) reported the highest root number per explants of peach rootstock on half strength MS medium supplemented with  $3.0 \text{ mg l}^{-1}$  IBA. The auxins, NAA and IBA were used by Behera et al. (2009) to induce rooting from *in vitro* raised shoots of white yam. In their study half strength MS basal medium containing NAA ( $2.0 \text{ mg l}^{-1}$ ) exhibited better rooting response than that of IBA ( $2.0 \text{ mg l}^{-1}$ ).

The overall best result in rooting was observed in *ex vitro* rooting condition. Although *ex vitro* rooting showed lower percent of survival (90 and 76.7% from Holeta and Welayta respectively) compared to acclimatization of *in vitro* rooted shoots (100 and 96.7% from Holeta and Welayta respectively), it showed the highest result in percentage of root induction, number of roots explant<sup>-1</sup> and length of root. This is promising in terms of cost reduction by avoiding the *in vitro* rooting. This *ex vitro* rooting reduced the cost of *in vitro* rooting by 99.2%. Reducing the cost of *in vitro* production is a key issue for increasing the application of the method. Rooting of microcuttings *in vitro* is expensive and can even double the price of the cutting. Acclimatization of *in vitro* rooted plantlets was highly successful in that most plants survived. All explants of Holeta origin and 96% of Welayta origin survived and established as healthy plant. Similar results were observed by Wondimu et al. (2012) in sweet potato who reported 100% survival. The present study is the first of its kind in tissue culture of *P. edulis*. In addition to the direct application in disease-free production of this crop, the present protocol contributes to further biotechnological research of this crop.

### Conflict of Interests

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

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## Short Communication

## Technique for en-masse cryo-fixation and processing of second-stage juveniles of *Meloidogyne incognita* for scanning electron microscopy

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Large number of second-stage juveniles of root-knot nematode, *Meloidogyne incognita* was prepared for scanning electron microscopy over a millipore filter (pore size 0.45  $\mu\text{m}$ ). The J2 juveniles were quickly immobilized and killed before fixation using the cryo-fixation technique followed by the primary fixation in 2.5% glutaraldehyde pre-cooled at 4-8°C and then secondary vapor fixation in 1% osmium tetroxide and drying with the series of ethanol without subsequent critical point drying. The specimens were vacuum dried in a desiccator for a period of 24-48 h. Cryo-fixation emphasizes the need to quickly stop cellular activity and thus prevent any surface artifacts that occur due to conventional fixation techniques.

**Key words:** Scanning electron microscopy, cryo-fixation, root-knot nematode, *Meloidogyne incognita*, surface morphology.

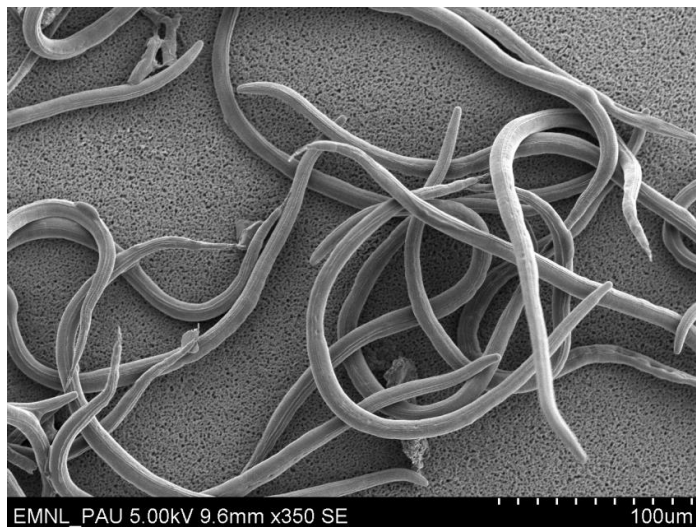
### INTRODUCTION

*Meloidogyne incognita* is a widely distributed plant-parasitic nematode considered as one of the most damaging crop pests affecting crop production (Trudgill and Blok, 2001). The second-stage juveniles of *M. incognita* (J2) after hatching from egg masses crawl in their soil environment to invade the root system of the host plant in which they undergo three moults and develop into adult females. Because of their significance in carrying out the life cycle of *M. incognita*, J2 are considered to be the key in managing the nematode population and therefore have been the subject of extensive studies (Mukhtar et al., 2013; Vanholme et al., 2004; GravatoNobre and Evans, 1998).

The scanning electron microscope (SEM) provides a

means to define the surface topographical features of species. Morphological and taxonomic studies of nematodes have been greatly enhanced by the use of SEM, and species descriptions increasingly include scanning electron micrographs. Unfortunately, morphological details are often obscured as a result of poor preservation and by artifacts produced by preparative techniques. Specimen preparation remains a limiting factor in the quality of information provided by SEM. The distortions occurring in the initial stages of preparation become magnified in the subsequent steps. Earlier conventional and SEM investigations of plant-parasitic nematodes compared several different methods of specimen preparation (Green et al.,

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**Figure 1.** Scanning Electron Micrograph of second-stage juveniles of *Meloidogyne incognita* obtained on an Axiva Millipore filter paper.

1975; Wergin, 1981, Wergin and Stone 1981; Wergin, 1982; Eisenback 1986; Carta et al., 2003, Viaene et al., 2007; Kumar et al., 2012). Cryofixing the live organisms has the advantage of processing them to a more life-like state as it takes a few milliseconds and the specimens remain fully hydrated. It also emphasizes the need to quickly stop cellular activity and thus prevent destruction of tissues through autolytic activities.

The major constraint in the studies on J2 include: (1) their small size (300-500  $\mu\text{m}$ ) which impedes their handling and processing. In previous electron microscopic studies J2 were generally picked up manually and processed which limits their number for studies; (2) the impervious nature of the cuticle as a result of which proper fixation is affected; (3) response of the nematode to the fixing fluid resulting in the coiling and distortion of the surface features. In order to overcome these difficulties we developed a cryo-fixation technique involving quick immobilization and killing before fixing with the conventional glutaraldehyde-osmium tetroxide fixing solution. The same is described in the present paper.

## MATERIALS AND METHODS

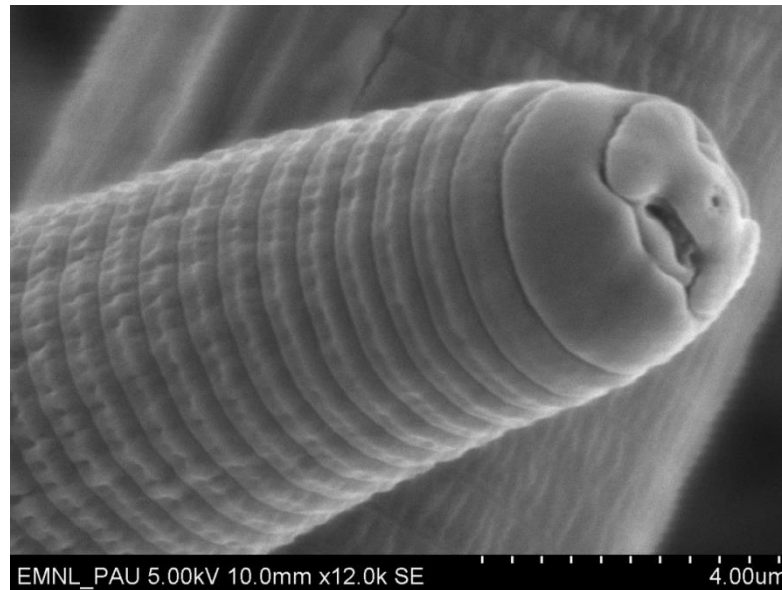
*M. incognita* cultured on tomato (*Solanum lycopersicon* cv. Castlerock) was maintained in the laboratory environment at 22-28°C. Freshly hand-picked egg masses from the roots of tomato plants were hatched in distilled water to collect J2 at room temperature (23 $\pm$ 2°C). After 24 h, the hatched juveniles were collected in a test tube, washed with triple distilled water (TDW) and re-suspended in 0.5 ml of TDW. This suspension was filtered through 0.45  $\mu\text{m}$  millipore syringe filter assembly. The filter containing nematodes was placed in a cryo chamber containing liquid nitrogen for 5 min to immobilize live J2. Once cryo-fixed, the filter paper was immediately placed in 2.5% glutaraldehyde buffered with 0.2 M sodium cacodylate adjusted to a pH of 7.2 in a cavity block for 48 h at 4°C. This was followed by washing the fixed

specimens with 0.1 M sodium cacodylate washing buffer thrice for 5 min each. Secondary vapor fixation was carried out in a dessicator using 2% osmium tetroxide ( $\text{OsO}_4$ ) (buffered in 0.1 M sodium cacodylate, pH 7.2) for 12 h at 4°C followed by a second washing with 0.1 M washing buffer thrice to remove any unreacted  $\text{OsO}_4$  at 4°C. The specimen was dehydrated in a six-step ethanol series for a period of 3 h and later vacuum dried at 650 Pa at room temperature for 24 h. In the present study, the nematodes were not critical point dried because most of the J2 due to their small size were lost during drying in CPD even after using BEEM capsule cartridge (Eisenback, 1985) with Millipore filters. Large number of dried J2 on a millipore filter paper were mounted on aluminum stubs using double sided carbon tape sputter coated with gold to prevent the build-up of high voltage changes on the specimen surface in an ion sputter coater (Hitachi E-1010) at a current of 18 - 20 mA for 20 s. The specimen was viewed and imaged with a Scanning Electron Microscope (Hitachi S-3400N) using SE imaging mode at 3-15 kV acceleration voltage.

## RESULTS AND DISCUSSION

Processing technique was evaluated on the basis of the final image obtained with the SEM. The quality of preservation of the J2 surface using the cryo-fixation technique and obtaining them in large number using the millipore filter paper technique were the most important observations seen with the SEM image.

In earlier studies poor preservation of the tissue usually resulted in wrinkles caused by shrinkage of the specimen. Most of the techniques are associated with surface precipitates, wrinkled nematode surface, and collecting large number of J2 for observation under SEM which obscure morphological details. Filtering the clean suspension with large number of J2 through Millipore filters (Figure 1) helped in obtaining them in large number for viewing under SEM and better comparison between the specimens of the same species as compared to the



**Figure 2.** Scanning Electron Micrograph of second-stage juveniles of *Meloidogyne incognita* showing very clear features of its anterior end.

conventional method of picking single J2 with bamboo pick. The cryo-immobilization and killing of J2 before the conventional fixation procedure helped in obtaining J2 free of any preparation artifacts and shrinkage of the nematode surface (Figure 2) due to their direct immersion in glutaraldehyde fixative at 4°C. Eisenback (1985) compared various methods of preparing nematodes for scanning electron microscopy. The initial comparison of preparative techniques used formaldehyde-based fixatives (Green et al., 1975) which have been replaced by glutaraldehyde (Eisenback, 1985). The immobilization, fixation and drying methods are extremely important in preparing nematodes for SEM. During the present study much better results were obtained by first cryo-immobilizing the J2 followed by glutaraldehyde fixation and without critical point drying of the J2.

### Conflict of Interests

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

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Full Length Research Paper

## ***In vitro* evaluation of roots, seeds and leaves of *Sesamum indicum* L. for their potential antibacterial and antioxidant properties**

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It is important to exemplify different types of medicinal plants for their potential antioxidant and antimicrobial properties as it has been evident that diverse nutrient and non-nutrient molecules which are produced from aromatic and medicinal plants display antioxidant and antimicrobial properties, and can protect human body against cellular oxidation reaction and pathogens. Methanol and aqueous extracts of different parts (seeds, roots and leaves) of *Sesamum indicum* L. were screened to detect *in vitro* antioxidant [1,1-diphenyl-2-picryl hydrazyl (DPPH) and thiobarbituric acid (TBA)] and antimicrobial (disc diffusion and deep well diffusion) activity. On the basis of the results, different parts of *S. indicum* L. showed promising antimicrobial and antioxidant activity in methanol extract instead of aqueous extracts. It has also been observed that *S. indicum* L. has a powerful antioxidant and antimicrobial activity and can be used as accessible source of natural antioxidants and antimicrobial agent in pharmaceutical industry and as a possible food supplement.

**Key words:** *Sesamum indicum* L., 1, 1-diphenyl-2-picryl hydrazyl (DPPH), thiobarbituric acid (TBA), antioxidant, antimicrobial.

### INTRODUCTION

Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent and produces free

radicals. These radicals are highly reactive, unstable and by-product compounds of metabolic functions in the

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human body. Most free radicals come from the oxygen atoms and are called reactive oxygen species (ROS). These are introduced in the body from both exogenous and endogenous sources such as pollutants, drugs, disease etc. (Kohen and Nyska, 2002). These radicals play deleterious effects in the body resulting in a condition known as oxidative stress. Human bodies are protected from oxidative damage of free radicals through complex defense systems which are known as antioxidants (Aris et al., 2009). Due to the fact that most radicals are short-lived species and react quickly with other molecules, thus the direct detection of ROS is difficult. Secondary products (such as derivatives of amino acids, nucleic acids and lipid peroxidation) are the marker of oxidative damage as it is analyzed by the measurement of these products (Kohen and Nyska, 2002).

In developing countries, bacterial infectious diseases directly affect the large population and these infections have been partially controlled by antibiotic therapies (Alanis, 2005) but Cordell (1995) reported that antibiotics cause hypersensitivity in host. Therefore, there is a need to develop an alternative for the treatment of various infectious diseases that is an antimicrobial drug.

Natural products of higher plants may be a new source of antimicrobial agents possibly with novel mechanism of action (Barbour et al., 2004). Plants possess a variety of beneficial factors that can be used to control human diseases. For example, polyphenols derived from plants have potential health benefits including anticancerous, antimicrobial and antioxidant activities (Yamamoto and Gaynor, 2001; Cushnie and Lamb, 2005).

Sesame (*Sesamum indicum* L.) is an ancient oil containing kernel that has been commonly used as a source of food and medicines (Hu et al., 2004; Kobayashi et al., 2004; Hsiao et al., 2006). Including sesame, all living organisms are able to develop self defense mechanisms against pathogen attack by producing important compounds such as phenols, secondary compounds and antimicrobial peptides (AMPs) (Pelegri and Franco, 2005). Sesame belongs to the family Pedaliaceae and found in tropical, subtropical and southern temperate areas of the world, particularly in India, China, South America and Africa. It plays a crucial role in Indian Ayurvedic medicines. The oil of this plant is used as an antibacterial agent and also used to cure *Verruca vulgaris* (Common Wart) and *Verruca plana* (clusters of warts, which are usually found on the soles of the foot and around the toes). Das et al. (2012) reported that Sesame peptide (molecular mass less than 1 kDa) involves in inhibition against growth of *Pseudomonas aeruginosa* as compared to *Bacillus subtilis* and this is the new discovery to make this crop more important at medicinal level. The present study was undertaken to evaluate the antioxidant and antibacterial activity of different parts of an important oil-yielding crop *Sesamum*

*indicum* L.

## MATERIALS AND METHODS

### Plant material

Seeds of *S. indicum* L. were procured from Krishi Vigyan Kendra (KVK), Banasthali University. Leaves and roots were collected from 15 days old plants, grown under controlled conditions in green house viz. temperature 30±2°C and 77% humidity.

### Bacterial strains

*Escherichia coli* (MTCC 82), *P. aeruginosa* (MTCC 741), *Staphylococcus aureus* (MTCC 737), *B. cereus* (MTCC 1272) and *Xanthomonas campestris* (MTCC-6843) were purchased from Institute of Microbial Technology, Chandigarh, India.

### Preparation of plant extract

#### Aqueous extract

10 g air-dried powder of leaves, roots and seeds were taken and these samples were dissolved in pre-sterilized distilled water. Extracts were filtered using a Buckner funnel and Whatman No.1 filter paper. Each filtrate was concentrated to dryness under reduced pressure at 40°C using a rotary evaporator.

#### Methanol extract

10 g air-dried powders of leaves, roots and seeds were mixed separately with appropriate quantity of methanol in a conical flask and then kept on a rotary shaker at 25°C for 24 h. Extracts were filtered using a Buckner funnel and Whatman No.1 filter paper. Each filtrate was concentrated to dryness under reduced pressure at 40°C using a rotary evaporator.

### Determination of antioxidant activities

The antioxidant activity in different parts was analyzed by using two methods:

#### DPPH method

The anti-oxidant potentials of the both extracts were determined on the basis of their scavenging activity of the stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical (Uddin et al., 2008). DPPH method is most widely used and easiest method to determine antioxidant activity. Absorbance at 517 nm was determined after 30 min and percent inhibition was calculated by using the formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

#### Thiobarbituric acid (TBA) test

The TBA test was conducted according to the combined method of Kikuzaki and Nakatani (1993) and Ottolenghi (1959) for determining



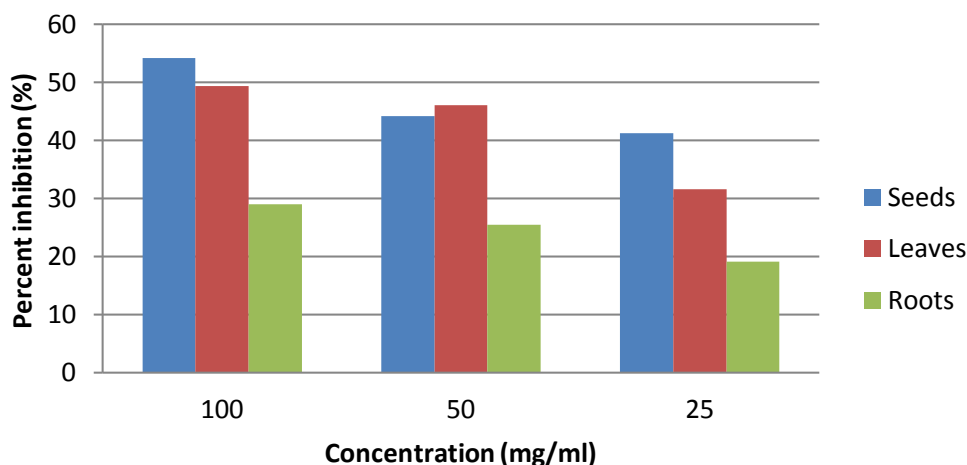


Figure 1. Free radical scavenging activity (DPPH) in methanol extracts.

the antioxidant potential of different parts of *S. indicum* L. The samples were prepared to 2.0 ml of the sample solution, 1.0 ml of 20% aqueous trichloroacetic acid (TCA) and 2.0 ml of aqueous TBA solution were added. The final sample concentration was 0.02% w/v. The mixture was placed in a boiling water bath for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min. Absorbance of the supernatant was measured at 532 nm. This TBA method described antioxidant activity by percent inhibition:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

#### Determination of antibacterial activity

The bacterial strains used as inoculums were grown at 37°C to get OD 0.6 at 600 nm and used for susceptibility testing. The antimicrobial assay was performed by two methods viz: agar disc diffusion method and agar well diffusion method (Parekh et al., 2005). The results were obtained by measuring the zone of inhibitions. For each bacterial strain, controls (c) were maintained where pure solvents were used instead of the extract. 20 µl of the test compound with concentration of 25 and 100 µg/ml was introduced into the well and on the disc. The result was obtained by measuring the zone diameter.

#### Statistical analysis

Experimental results are expressed as means ± SD. All the same three samples for one treatment were measured three times.

## RESULTS AND DISCUSSION

It is clear from the data that there are differences in the antioxidant and antimicrobial effects of different part of the sesame plant using methanol and water extracts. It could be presumed that antioxidant and antimicrobial constituents may not be in sufficient concentration, so as to be effective. Stainer et al. (1986) supported this

statement. They revealed during their experimental work on different plant species that it could be possible that the active antimicrobial chemical constituents were not soluble in methanol or water.

In today's environment, stress, drugs and diet generates excessive free radicals in human body and causes the imbalance in homeostatic phenomenon between oxidants and antioxidants. Plants and plant products are known to possess excellent antioxidant properties and play a significant role in preventing the complications caused by excessive free radicals (Jain et al., 2010). The correlation between total phenol contents and antioxidant activity has been widely studied in different foodstuffs such as fruit and vegetables (Kiselova et al., 2006; Klimczak et al., 2007; Kedage et al., 2007; Jayaprakasha et al., 2008).

Phenolic compounds are involved in resistance to pathogens due to their antimicrobial activity, exhibits extensive range of physiological properties, including anti-allergenic, anti-inflammatory, antimicrobial, cardio-protective and vasodilatory effects (Balasundram et al., 2006). Polyphenols protect cell constituents against destructive oxidative damage. Antioxidant activity of polyphenols play an important part in absorbing and neutralizing free radicals, quenching oxygen and decomposing peroxidase, which is mainly due to their redox properties (Villano et al., 2005).

In this experiment, the antioxidant activity of methanol and aqueous extracts of different parts of *S. indicum* L. were measured by different method like DPPH scavenging activity and thiobarbituric acid method. Different concentrations of methanol and aqueous extracts (ranging from 25 to 100 mg/ml) were tested for their antioxidant activity. The antioxidant activity increased with increasing concentration of extract. In DPPH method methanol extract presented more activity than aqueous extract (Figures 1 and 2). The percent of inhibition in

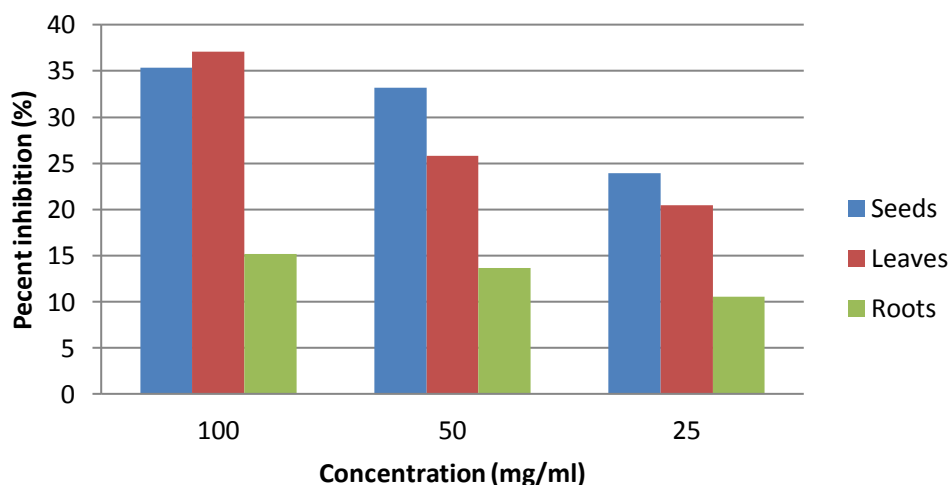


Figure 2. Free radical scavenging activity (DPPH) in aqueous extracts.

Table 1. Antioxidant activity of different parts of *Sesamum indicum* by DPPH and TBA method.

Plant part	Concentration (mg/ml)	DPPH (mg/ml)		TBA (mg/ml)	
		Methanol	Aqueous	Methanol	Aqueous
Leaves	25	31.60±0.01	20.48±0.03	67.66±0.03	43.53±0.07
	50	46.05±0.00	25.84±0.02	68.61±0.02	48.89±0.09
	100	49.42±0.01	37.09±0.06	70.84±0.02	51.57±0.06
Roots	25	19.10±0.04	10.53±0.03	39.13±0.14	28.86±0.15
	50	25.46±0.01	13.64±0.03	43.95±0.13	30.84±0.15
	100	28.97±0.01	15.16±0.01	47.08±0.13	34.18±0.16
Seeds	25	41.62±0.02	23.95±0.03	66.87±0.03	41.76±0.09
	50	44.25±0.01	33.18±0.04	69.53±0.02	43.77±0.09
	100	54.24±0.00	35.39±0.07	72.89±0.02	48.01±0.07

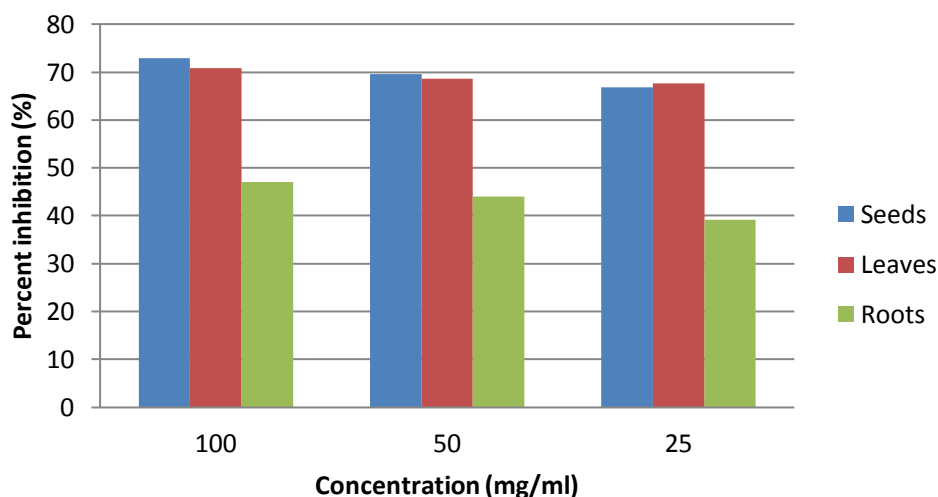
methanol extract ranged from 41.62 to 54.24% in leaves, 31.60 to 49.42% in seeds and 19.10 to 28.97% in roots while in aqueous extract, the percent of inhibition ranged from 23.95 to 35.39% in leaves, 20.48 to 37.09% in seeds and 10.53 to 15.16% in roots (Table 1).

Methanol extracts in TBA method also presented more activity than aqueous extract and the percent of inhibition in methanol extract ranged from 67.66 to 70.84% in leaves, 66.87 to 72.89% in seeds and 39.13 - 47.08% in roots while in aqueous extract the percent of inhibition ranged from 43.53 to 51.57% in leaves, 41.76 to 48.01% in seeds and 28.86 to 34.18% in roots.

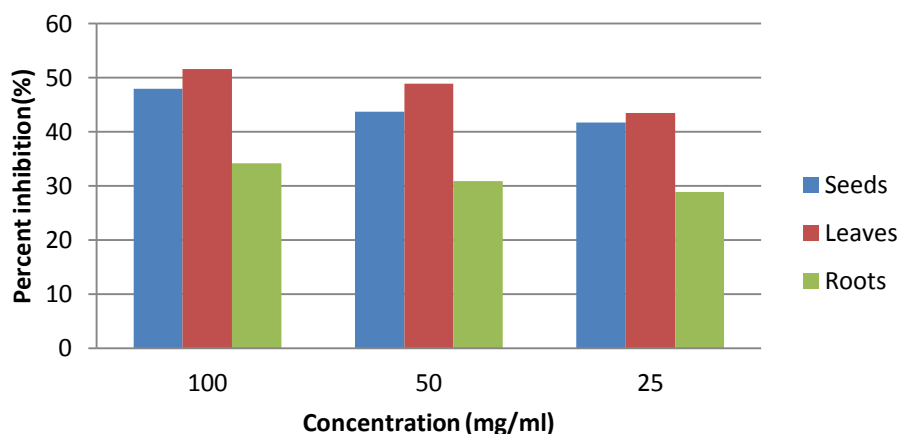
The methanol extract activity was more than that of aqueous extracts (Figures 3 and 4). This can be explained by the methanol and aqueous extracts containing a high proportion of antioxidants with the

higher activity in the TBA assay than in the DPPH method. Halvorsen et al. (2002) reported the data of antioxidant capacity of methanol extracts in dietary plants.

In developing countries, an uncontrolled increase of pathogens with antibiotic resistance including several Gram-negative and Gram-positive bacteria such as *E. coli*, *Pseudomonas* sp., *Staphylococcus* sp., *Xanthomonas* sp. and *Bacillus* sp. is the cause of serious pathological conditions which have led to search for new strategies to control these infectious agents (Paterson, 2006). All living organisms including sesame are able to develop self-defense mechanisms against pathogen attack by producing secondary compounds such as polyphenols (Pelegriani and Franco, 2005). These compounds contribute to plant innate host defense and represent an important



**Figure 3.** Antioxidant activity of methanol extracts of different parts of *Sesamum indicum* (TBA method).



**Figure 4.** Antioxidant activity of aqueous extracts of different parts of *Sesamum indicum* (TBA method).

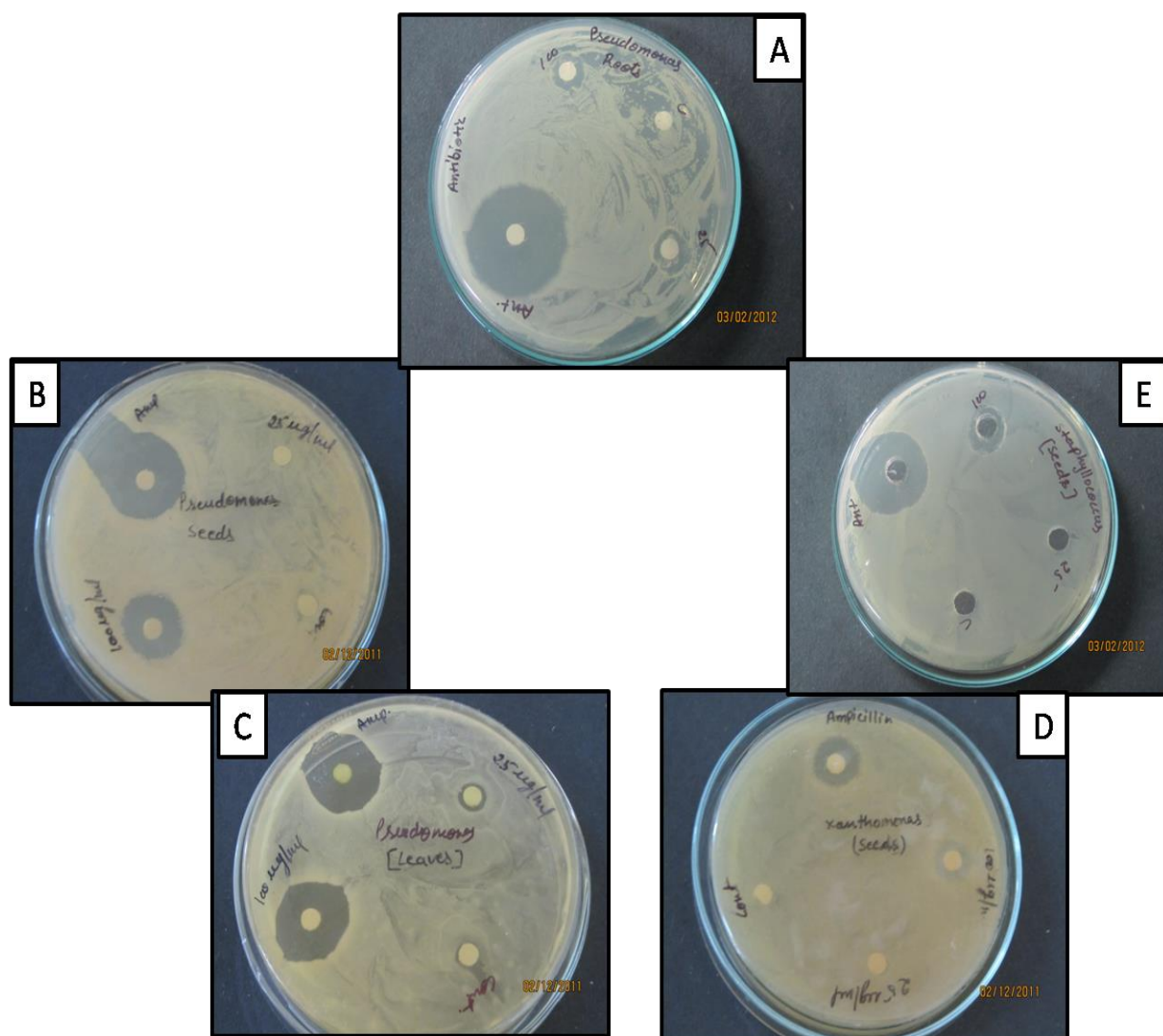
source of antibiotics (Costa et al., 2007).

In this study, *in vitro* antibacterial activity of extracts of different parts (roots, seeds and leaves) of sesame plant was assayed by agar disc diffusion (Figure 5) and agar well diffusion method (Figure 6) against five bacterial species. Table 2 summarizes the inhibition zone of ampicillin as positive control and Tables 3 and 4 summarizes the microbial growth inhibition of both methanol and aqueous extracts by disc diffusion and agar well diffusion method.

The methanol extracts of different parts of sesame plant have antibacterial effect against few tested micro-organisms except the growth of *E. coli* and *B. cereus*. Rios and Recio (2005) reported the significance of the preservation of some of the active ingredients - sesame

lignans such as Sesaminol and its glucosides which are water soluble in nature and were extracted effectively during extraction processes of the Sesame leaves. The essential oil from the leaves of *S. radiatum* inhibit the growth of *E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa*, *S. marcescens*, *S. albus* and *S. aureus* (Osibote et al., 2010).

The seed oil has been found to contain natural antibacterial agents that are effective against common skin pathogens such as *Staphylococcus* and *Streptococcus* bacteria as well as common skin fungi including the athlete's foot fungus (Annussek, 2001). Costa et al. (2007) showed in their report that antimicrobial peptides from white and black cultivars of sesame were capable of reducing the growth of *Proteus*

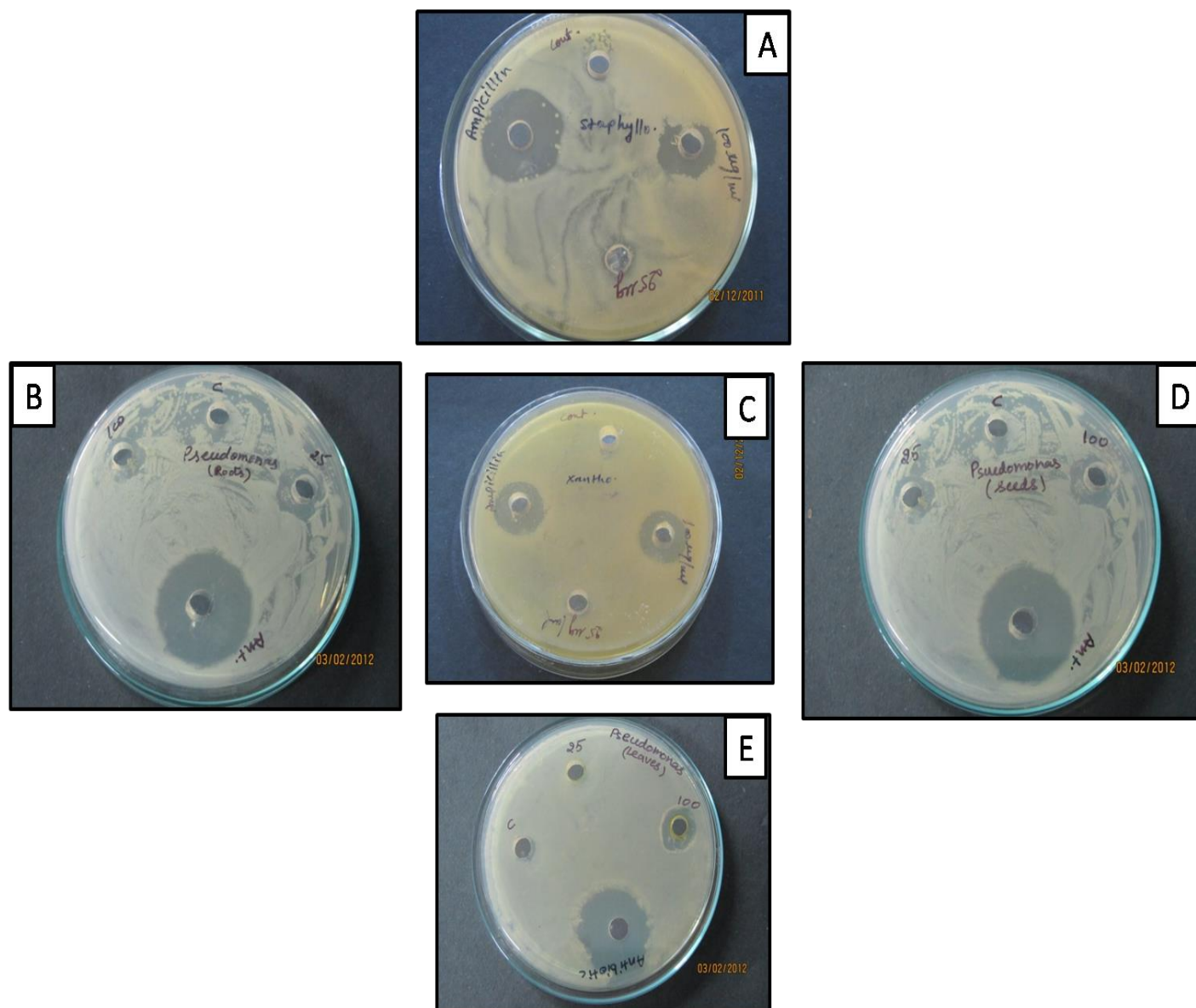


**Figure 5.** Antibacterial activity in different parts of *Sesamum indicum* L. by disc diffusion method. **A.** Activity against *Pseudomonas* for root extract (methanol extract) of *Sesamum*. **B.** Activity against *Pseudomonas* for seed extract (methanol extract) of *Sesamum*. **C.** Activity against *Pseudomonas* for leaves extract (methanol extract) of *Sesamum*. **D.** Activity against *Xanthomonas* for seed extract (methanol extract) of *Sesamum*. **E.** Activity against *Staphylococcus* for seed extract (methanol extract) of *Sesamum*.

sp. and *Klebsiella* sp. The methanol extract exhibited a mild antimicrobial activity against *Staphylococcus aureus* (Shittu et al., 2007). This confirms the use of Sesame leaves extracts as antimicrobial agent in folk medicine.

Aqueous extracts had no inhibitory effects on all the five tested microorganisms (Figure 7) but Shittu et al. (2007) explained in their earlier report that the aqueous extract of *Sesamum radiatum* leaves mildly inhibited the growth of *Candida albicans*. Aqueous extracts of *Curcuma amada* showed the inhibitory effect against *S. aureus*, and *Urgenia indica* against *Klebsiella aeruginosa* (Swarnkar and Katewa, 2009).

Based on these results, it is possible to conclude that methanol extracts of different parts of *S. indicum* L. had different level of antioxidant and antimicrobial activity. The obtained results might be considered sufficient for further studies for the isolation and identification of the active principles and to evaluate the possible synergism among extract components for their antioxidant and antimicrobial activity. These results open the possibility of finding new clinically effective antioxidative and antibacterial compounds. Our study emphasizes the accuracy of traditional remedies and also illustrates the strong dependence of certain people on traditional medicine and



**Figure 6.** Antibacterial activity in different parts of *Sesamum indicum* L. by Deep Well Diffusion Method. A. Activity against *Staphylococcus* for seed extract (Methanol extract) of *Sesamum*. B. Activity against *Pseudomonas* for root extract (methanol extract) of *Sesamum*. C. Activity against *Xanthomonas* for seed extract (methanol extract) of *Sesamum*. D. Activity against *Pseudomonas* for seed extract (methanol extract) of *Sesamum*. E. Activity against *Pseudomonas* for leaves extract (methanol extract) of *Sesamum*.

**Table 2.** Zone of inhibition (in cm) of antibiotic (ampicillin, 1 mg/ml) against five bacterial strains.

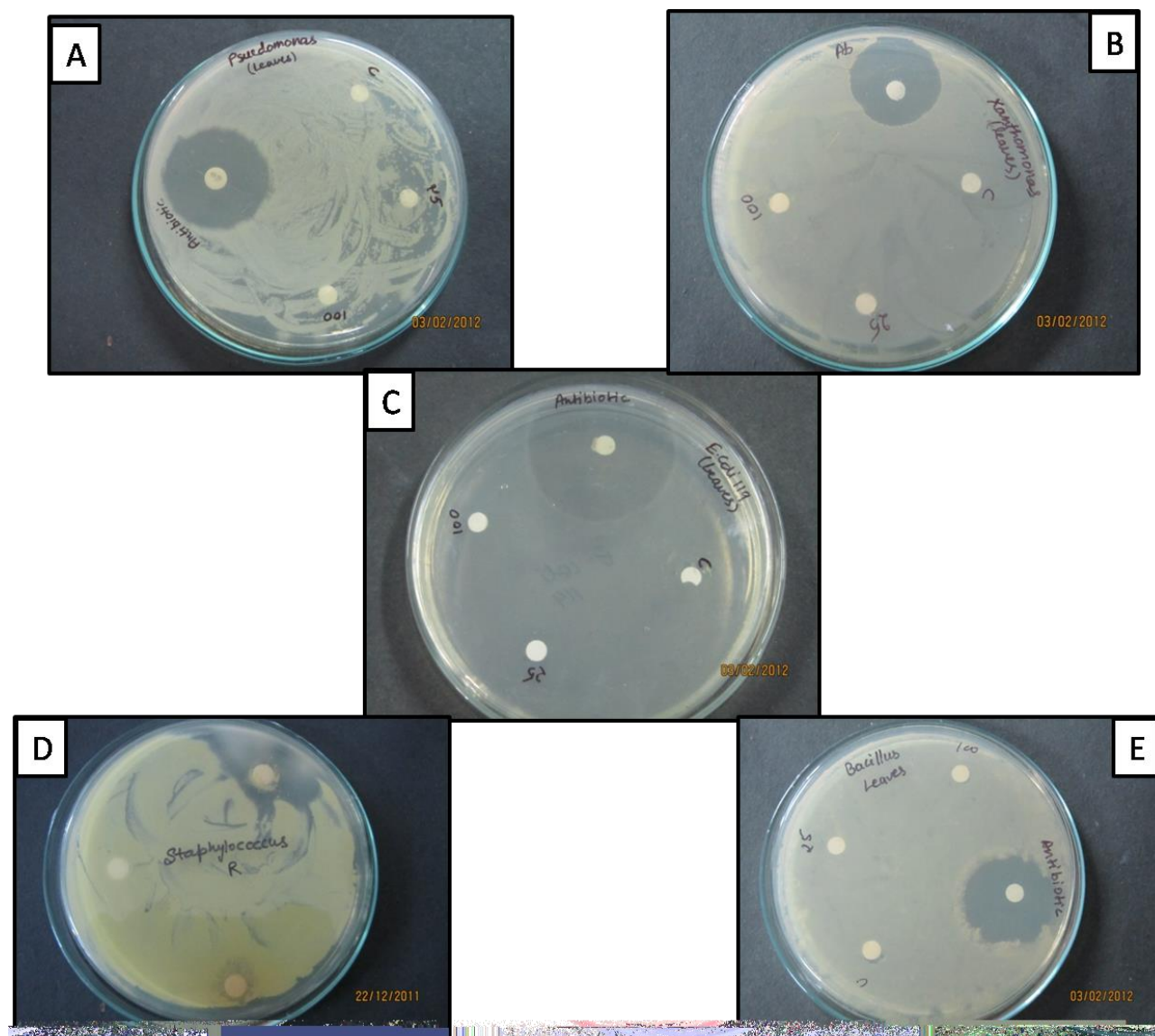
Bacterial strain	Zone of inhibition (cm)
<i>E. coli</i> 119	2.4
<i>Bacillus cereus</i>	2.6
<i>Pseudomonas aeruginosa</i>	3.1
<i>Xanthomonas compestris</i>	3.0
<i>Staphylococcus aureus</i>	4.0

**Table 3.** Zone of inhibition (cm) of methanol and aqueous extracts of different parts of *Sesamum indicum* L. against five bacterial strains (Disc method).

Plant part	Solvent	Concentration (µg/ml)	<i>E. coli</i> 119	<i>Bacillus cereus</i>	<i>Pseudomonas aeruginosa</i>	<i>Xanthomonas compestris</i>	<i>Staphylococcus aureus</i>
Roots	Aqueous	Control	-	-	-	-	-
		25	-	-	-	-	-
		100	-	-	-	-	-
	Methanol	Control	-	-	-	-	-
		25	-	-	0.6	-	-
		100	-	-	1.2	-	-
Seeds	Aqueous	Control	-	-	-	-	-
		25	-	-	-	-	-
		100	-	-	-	-	-
	Methanol	Control	-	-	-	-	-
		25	-	-	-	-	-
		100	-	-	2.4	2.0	1.4
Leaves	Aqueous	Control	-	-	-	-	-
		25	-	-	-	-	-
		100	-	-	-	-	-
	Methanol	Control	-	-	-	-	-
		25	-	-	0.6	-	-
		100	-	-	2.8	-	-

**Table 4.** Zone of inhibition (cm) of methanol and aqueous extracts of different parts of *Sesamum indicum* L. against five bacterial strains (Bore well method).

Plant part	Solvent	Concentration (µg/ml)	<i>E. coli</i> 119	<i>Bacillus cereus</i>	<i>Pseudomonas aeruginosa</i>	<i>Xanthomonas compestris</i>	<i>Staphylococcus aureus</i>
Roots	Aqueous	Control	-	-	-	-	-
		25	-	-	-	-	-
		100	-	-	-	-	-
	Methanol	Control	-	-	-	-	-
		25	-	-	0.8	-	-
		100	-	-	1.4	-	-
Seeds	Aqueous	Control	-	-	-	-	-
		25	-	-	-	-	-
		100	-	-	-	-	-
	Methanol	Control	-	-	-	-	-
		25	-	-	1.2	-	-
		100	-	-	1.8	2.6	3.2
Leaves	Aqueous	Control	-	-	-	-	-
		25	-	-	-	-	-
		100	-	-	-	-	-
	Methanol	Control	-	-	-	-	-
		25	-	-	-	-	-
		100	-	-	2.2	-	-



**Figure 7.** Aqueous extracts of different parts of *Sesamum indicum* L. showing no inhibitory zones for the tested microorganisms.

the creativeness in which plants and their secondary metabolites can be utilized.

### Conflict of Interests

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

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Full Length Research Paper

## Statistical optimization of rapid production of cellulases from *Aspergillus niger* MA1 and its application in bioethanol production from rice hulls

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The aim of this study was the utilization of rice hulls (RH) as a clean, cost effective, renewable and abundant agro-waste in bioethanol production by simple and applicable methods; for this purpose *Aspergillus niger* MA1 was isolated from RH and selected from many other isolates. This isolate produced 15.0, 3.38 and 49.1 U/g RH for CMCase, FPase and  $\beta$ -glucosidase, respectively, by solid state fermentation of RH after 36 h. The cellulases of the fungus showed good thermal and pH stability with maximum activity at 50°C and pH 5. An increase in cellulases productivity by statistical optimization and multi-response of SSF medium was revealed. Addition of 0.25% brej 35 to tap water or saline is very effective in elution of *A. niger* MA1. Cellulase which was successfully used in saccharification of steam explosion pretreated RH, revealed 16.36 g/L reducing sugars and subsequently fermentation by *Saccharomyces cerevisiae* which produced 9.42 g/L ethanol after 24 h.

**Key words:** Rice hulls, bioethanol, cellulases, solid state fermentation, *Aspergillus niger* MA1, statistical optimization, multi-response.

### INTRODUCTION

Trade-off between food and energy in most African countries make it difficult to use agricultural crops to produce bioethanol. The alternative option is the use of lignocellulosic agro-wastes. One of these abundant agro-wastes in Egypt is rice hulls (RH) which represents 20% (mass fraction) of the harvested rice. Its composition comprises around 20 to 25% lignin, 35 to 40% cellulose and 15 to 20% hemicelluloses (Saha and Cotta, 2008).

The essential key in this bioconversion is cellulases which are multi-enzyme system, composed of several enzymes with numerous isozymes which act synergistically and catalyze the hydrolysis of cellulose and cello-oligosaccharide derivatives (Chinedu et al., 2008), but the contribution of the commercial enzyme cost to bioethanol production cost is quite significant (Klein-Marcuschamer et al., 2012). For economic

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production of cellulases from wastes, solid state fermentation (SSF) is highly an effective method, in which cellulose substrate acts as the carbon source in sufficient moisture without free water. Optimal design of the culture medium by application of statistical experimental design techniques or response surface methodology (RSM) in fermentation process can result in improved product yields, reduced process variability, closer confirmation of the output response to nominal target requirements and reduced development time and overall costs.

In this work RH is the substrate from which *A. niger* MA1 was isolated and upon which rapid production of cellulases was statistically optimized which was subsequently used in the saccharification of pretreated RH and used successfully in bioethanol production.

## MATERIALS AND METHODS

### Solid state fermentation

In 100 ml glass flask, one gram of dried RH moisten with 1 ml of basal salt solution contains (g/L)  $\text{KH}_2\text{PO}_4$ , 15;  $(\text{NH}_4)_2\text{SO}_4$ , 5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.6;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.14;  $\text{MnSO}_4 \cdot 6\text{H}_2\text{O}$ , 0.16;  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.37; and peptone, 2, pH 4.8; autoclaving was done at 120°C for 20 min. Inoculated was done with 0.5 ml of fungal spore suspension ( $10^6$  spore/ml) of the tested species and incubated at  $28 \pm 2^\circ\text{C}$  for 10 days.

### Elution of cellulases enzymes with different eluents

After fermentation, elution of cellulase enzymes were carried out by different eluents such as tap water, saline solution and buffer solution alone (1:10 w/v) or mixed with different surfactant Triton, Berj 35 and Tween 80 (0.25%). After shaking at 200 rpm for 30 min, the supernatant was separated by filtration and used for cellulases assay.

### Carboxy methyl cellulose (1.4 - $\beta$ - D - glucan glucanohydrolase EC 3.2.1.4)

A mixture of 0.5 ml of substrate (0.5 w/v of carboxymethyl cellulose, sigma) in 0.2 M acetate buffer, pH 4.8 plus 0.5 ml of enzyme was incubated for 30 min at 50°C (Magnelli and Forchiassin, 1999). One unit of endoglucanase activity is the amount of enzyme required to release reducing sugars equivalent to 1  $\mu\text{mole}$  glucose per min under the above experimental conditions.

### Filter paper activity (FPase)

According to Ghose (1987), 1 ml of supernatant of culture is incubated with 50 mg filter paper Whatman No. 1 (1.0  $\times$  6.0 cm) in 1 ml of 0.2 mol acetate buffer (pH 4.8) at 50°C for 60 min. One unit of FPase activity corresponds to 1  $\mu\text{mole}$  of glucose equivalent released per minute under the experimental assay conditions.

### $\beta$ -Glucosidase (cellbiase or $\beta$ D-glucoside glucohydrolase E C 3.2.1.21)

$\beta$ -glucosidase activity against cellobiose was determined by 0.1 ml of culture supernatant to 0.5 ml of cellobiose in 0.2 mol acetate

buffer (pH 4.8). The reaction mixture was incubated at 50°C for 30 min. One  $\beta$ -glucosidase activity unit is equivalent to 1  $\mu\text{mole}$  of glucose per minute under the above experimental conditions.

The released reducing sugars of three enzymes were measured by glucose oxidase Kit using glucose as standard (Spinreact Company, Spain).

## Characterization of CMCase and FPase and $\beta$ -glucosidase produced by *A. niger* MA1

### Determination of optimum incubation temperature

The influence of temperature on cellulase activities was determined by assaying the cellulase activities in the standard reaction mixture at various degrees (20, 30, 40, 50 and 60°C).

### Thermal stability

Thermostability were determined by incubating the enzyme for different times (60, 120, 180 and 240 min) at various temperatures (30, 40, 50, 60, 70, 80 and 90°C); the remaining cellulase activities were then assayed in its optimum conditions.

### Determination of optimum pH

The pH of moistening agent was adjusted using 0.05 M acetate buffers to different pH levels (4.6, 4.8, 5, 5.4, 5.7 and 6). Then the flasks were incubated for 42 h at 30°C. The flasks were harvested for extraction and determination of cellulases activity.

### pH stability

Mixture of the enzyme solution and 0.5 acetate buffers were adjusted to cover the pH range from 3.6 to 5.6 and were incubated at 4°C for 6, 12, and 18 and 24 h, the remaining cellulase activities were then assayed in its optimum conditions.

### Pretreatment of RH

**Alkali pretreatment:** NaOH at the concentrations of 10% (w/v), was mixed with RH in 1:7 weight of RH to volume of NaOH, autoclaved at 121°C for 20 min, washed with distilled water thoroughly and then dried at 50°C.

**Steam explosion:** Ten grams of RH was placed in 100 ml glass flask, treated with 2 par of saturated steam in autoclave. After 5 min, the pressure is suddenly reduced to atmospheric pressure.

**Acidic pretreatment:** Ten grams of RH was treated with diluted  $\text{H}_2\text{SO}_4$  (2% )then autoclaved at 121°C for 20 min, washed with distilled water thoroughly and then dried at 50°C.

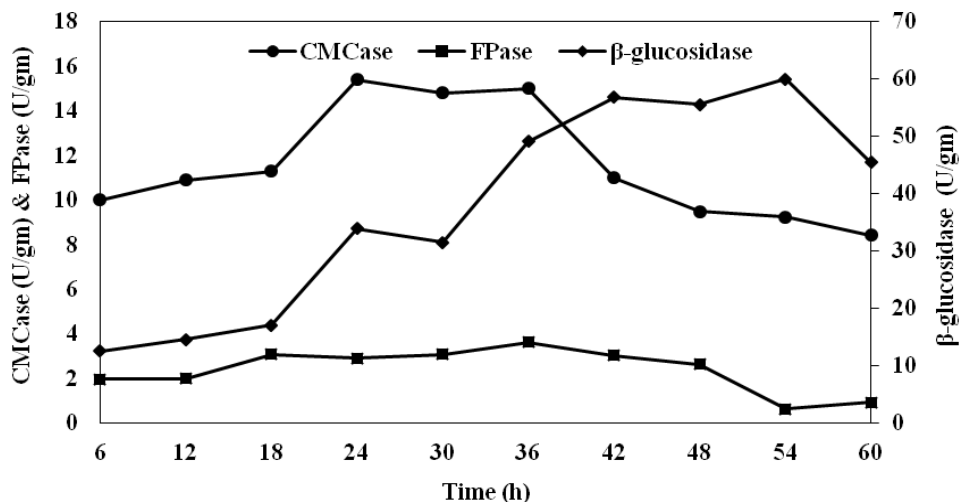
## Statistical optimization of cellulases enzyme production in solid-state fermentation

### Plackett-Burman design

For each variable, a high (+1) and low (-1) level was tested. All trials were performed in triplicate, and each experiment was repeated twice, with the mean considered for the response. Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for the determination of variable significance.

**Table 1.** The levels of variables chosen for the Box-Behnken optimization experiment.

Variable	Code	(-1)	0	(+1)
Yeast extract (mg/gm)	X <sub>1</sub>	2	4	6
Peptone (mg/gm)	X <sub>2</sub>	2	4	6
Corn steep liquor (μl/gm)	X <sub>3</sub>	20	40	60

**Figure 1.** Time course of CMCase, FPase and β-glucosidase production by *A. niger* MA1 onSSF of RH.

### Box-Behnken design

Once critical factors were identified via screening, a Box-Behnken design for three independent variables (Table 1), each at three levels with three replicates at the center points was done (Box and Behnken, 1960).

### Fermentation for bioethanol production

At the beginning of the experiment the fermentor was sterilized. The tested sugary material was estimated for total fermentable sugars by the Anthrone method (Hedge and Hofreiter, 1962) and supplemented with nitrogen and phosphorus as follow; KH<sub>2</sub>PO<sub>4</sub> 0.1%, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.5%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05%, and Yeast extract 0.1%. The pH of the medium was adjusted to 5.0. Then, the prepared sugary syrup was loaded in the fermentor followed by the yeast, *S. cerevisiae* inoculums and incubated at 30°C. The appropriate air flow was provided through port that entered through the top of the fermentor (for about 2 h) to allow the yeast to begin growth and reproduction. Samples were extracted from the fermentor every 1 h therefore to measure the changes in temperature, pH and density of the solution. When two similar results were obtained the fermentation process was stopped. The fermentation broth was obtained in order to measure the ethanol (Caputi et al., 1968) and remaining sugar concentrations.

### Fermentation efficiency

Fermentation efficiency = (Actual ethanol recovery / Theoretical recovery) × 100

Theoretical recovery = Total sugars × 0.64

Actual ethanol recovery = Actual ethanol obtained

## RESULTS

### Fungal isolate

One of the best source of fungal strains capable of hydrolyzing RH is RH itself and we achieved that by plating RH samples from different localities in Egypt over sterilized and moisten filter paper. *A. niger* MA1 was selected from many mesophilic fungal isolates which were tested for their cellulolytic activity.

### Time course of cellulases productivity of *A. niger* MA1 in SSF of RH

The highest production of CMCase (Unit/gm RH) was observed in the time range from 24 to 36 h of fermentation (around 15 U/g), then decreased gradually to 8.43 U/g after 60 h of fermentation period. The maximum production of FPase is released after 36 h (3.63 U/g), and decreased gradually to 0.63 and 0.93 U/g after 54 and 60 h. The activity of β-glucosidase take relatively more time, it gave its maximum activity after 54 h of fermentation (60 U/g) then decreased to 45.4 U/g after 60 h (Figure 1).

**Table 2.** Box-Behnken factorial experimental design, representing the response of cellulases activity as influenced by yeast extract, peptone and corn steep liquor by *A. niger* MA1 on RH after 42h of incubation.

Trial	Yeast extract	Peptone	Corn steep	CMCase (U/g)		FPase (U/g)		β-glucosidase (U/g)	
				Response	Predicted	Response	Predicted	Response	Predicted
1	-1	-1	0	16.81	16.71	3.70	3.76	64.60	63.87
2	1	-1	0	13.04	12.86	3.44	3.32	60.80	61.44
3	-1	1	0	13.33	13.43	4.20	4.31	58.01	57.36
4	1	1	0	20.07	20.08	4.10	4.03	48.90	49.62
5	-1	0	-1	13.04	13.16	3.10	2.97	47.70	49.25
6	1	0	-1	18.01	18.21	3.60	3.66	48.20	48.38
7	-1	0	1	17.30	17.08	4.80	4.74	66.00	65.81
8	1	0	1	15.02	14.83	3.21	3.33	58.07	56.51
9	0	-1	-1	10.21	10.12	1.88	1.93	60.09	59.25
10	0	1	-1	17.52	17.20	2.31	2.31	35.06	34.15
11	0	-1	1	15.23	15.50	2.42	2.41	54.76	55.66
12	0	1	1	12.30	12.32	3.33	3.27	61.60	62.43
13	0	0	0	18.01	17.30	3.90	3.97	86.10	85.98
14	0	0	0	17.28	17.30	4.05	3.97	87.05	85.98
15	0	0	0	16.72	17.30	3.98	3.97	84.80	85.98

### Statistical optimization of medium using response surface methodology for cellulase production by *A. niger* MA1 under SSF of RH

#### Plackett Burman experimental design

The analysis of randomized Plackett-Burman experimental of 15 independent variables (data not shown) namely; weight, moisture content, pH, inoculum size, sand particles, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, NH<sub>4</sub>NO<sub>3</sub>, NaNO<sub>3</sub>, peptone, yeast extract, corn steep liquor, wheat bran, MgSO<sub>4</sub>, CaCl<sub>2</sub>, and Tween 80, showed that, yeast extract, corn steep liquor and peptone had significantly influence on the productivity of CMCase, FPase and β-glucosidase of *A. niger* MA1.

#### Box-Behnken design (BBD)

The result of 15 runs in BBD of yeast extract (X<sub>1</sub>), peptone (X<sub>2</sub>), and corn steep liquor (X<sub>3</sub>) chosen for optimization of *A. niger* MA1 cellulases are shown in Table 2. All the three activities varied distinctly with the conditions tested. CMCase showed divergence from 10.21 to 20.07 U/g, FPase activity between 1.88 and 4.80 U/g while β-glucosidase varied from 35.06 to 87.05 U/g.

The experimental results suggest that these variables strongly affected the fermentation process. The analysis of variance (ANOVA) of the model for CMCase, FPase and β-glucosidase (Table 3) showed validity of predictions in terms of p-value (<0.05) and the coefficients of determination (R<sup>2</sup>) of their models were 98.78, 98.97 and 99.60%, where the R<sup>2</sup>(adj) were 96.57, 97.13 and 98.89% respectively. The R<sup>2</sup> and adj R<sup>2</sup> value

provides a measure of variability in the observed response values that can be explained by the experimental factors and their interactions. The closer the R<sup>2</sup> and adj R<sup>2</sup> value to 1, along with non-significant lack of fit, the stronger the model is, the better it predicts the response.

#### CMCase productivity optimization

The interactive effects of the studied variables on cellulases activity were studied by plotting 3D surface curves against any two independent variables, while keeping other variables at its central (0) level. The response surface of CMCase is shown in Figure 2a to c. It shows the increase in CMCase at high level of any of the corn steep liquor and yeast extract, interaction between high levels of peptone and corn steep liquor and low levels of peptone and yeast extract; it was also enhanced by high levels of yeast extract which interacted with moderate level of corn steep liquor. The final response function to predict CMCase activity was

$$Y_{CMCase} = 17.30 + 0.70X_1 + 0.98X_2 + 0.13X_3 + 2.62 X_1 X_2 - 1.82X_1 X_3 - 2.55 X_2 X_3 + 0.25 X_1^2 - 1.77 X_2^2 - 1.72X_3^2$$

#### FPase productivity optimization

As shown in Figure 3a-c, the interaction of moderate levels of yeast extract, corn steep liquor and peptone supported high FPase activity. For predicting the optimal point; within experimental constrains, the following second-order polynomial function was fitted to the experimental results of FPase activity:

**Table 3.** ANOVA of CMCase, FPase and  $\beta$ -glucosidase production of *A. niger* MA1 under SSF of RH.

Enzyme	Source	DF	Adj SS	Adj MS	F	P
<sup>1</sup> CMCase	Regression	9	100.71	11.19	44.85	0
	Linear	3	11.873	3.9575	15.86	0.005
	Square	3	21.938	7.3128	29.31	0.001
	Interaction	3	66.895	22.298	89.37	0
	Residual error	5	1.248	0.2495		
	Lack-of-fit	3	0.388	0.1292	0.3	0.827
	Pure error	2	0.86	0.43		
<sup>2</sup> FPase	Regression	9	8.6613	0.9624	53.62	0
	Linear	3	2.0737	0.6912	38.51	0.001
	Square	3	5.4316	1.8105	100.87	0
	Interaction	3	1.156	0.3853	21.47	0.003
	Residual Error	5	0.0897	0.018		
	Lack-of-Fit	3	0.0785	0.0262	4.64	0.182
	Pure Error	2	0.0113	0.0056		
<sup>3</sup> $\beta$ -Glucosidase	Regression	9	3115.3	346.15	139.89	0
	Linear	3	524.69	174.9	70.68	0
	Square	3	2311.9	770.63	311.44	0
	Interaction	3	278.74	92.91	37.55	0.001
	Residual error	5	12.37	2.47		
	Lack-of-fit	3	9.82	3.27	2.57	0.293
	Pure error	2	2.55	1.28		

<sup>1</sup>S = 0.4995, PRESS = 8.135, R<sup>2</sup> = 98.78%, R<sup>2</sup> (pred) = 92.02%, R<sup>2</sup> (adj) = 96.57%; <sup>2</sup>S = 0.1339, PRESS = 1.280, R<sup>2</sup> = 98.97%, R<sup>2</sup> (pred) = 85.36%, R<sup>2</sup> (adj) = 97.13%; <sup>3</sup>S = 1.5730, PRESS = 162.86, R<sup>2</sup> = 99.60%, R<sup>2</sup> (pred) = 94.79%, R<sup>2</sup> (adj) = 98.89%; DF: degree of freedom; SS: sum of squares; MS: mean of squares; F:F-value; P: significance level of P-value (at significance level<0.05).

$$Y_{FPase} = 3.97 - 0.18X_1 + 0.31X_2 + 0.35X_3 + 0.04 X_1 X_2 - 0.52 X_1 X_3 + 0.012 X_2 X_3 + 0.53 X_1^2 - 0.65 X_2^2 - 0.83 X_3^2$$

### $\beta$ -Glucosidase productivity optimization

For  $\beta$ -glucosidase (Figure 4a to c), the interaction between the moderate concentration of the three variable give maximum production of the enzyme. Elliptical contour plots obtained from the data of the present study clearly show significance of the mutual interactions between the variables. The following second-order polynomial function was fitted to the experimental results of  $\beta$ -glucosidase activity:

$$Y_{\beta\text{-glucosidase}} = 85.98 - 2.54X_1 - 4.58X_2 + 6.17X_3 - 1.32 X_1 X_2 - 2.10 X_1 X_3 + 7.96X_2 X_3 - 12.89 X_1^2 - 15.01 X_2^2 - 18.09X_3^2$$

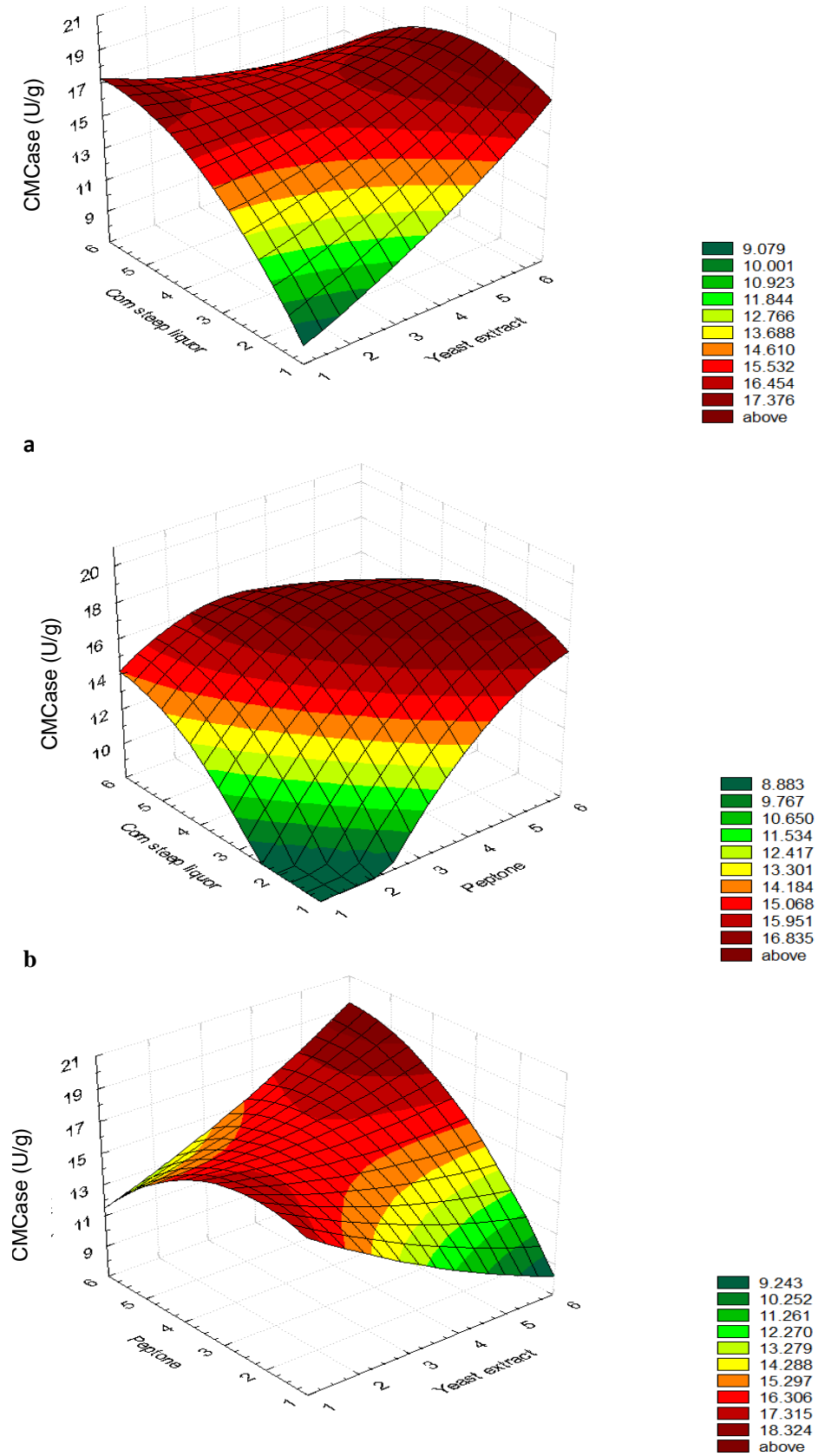
### Multi-response optimization

Based on actual values of the multi-response optimiza-

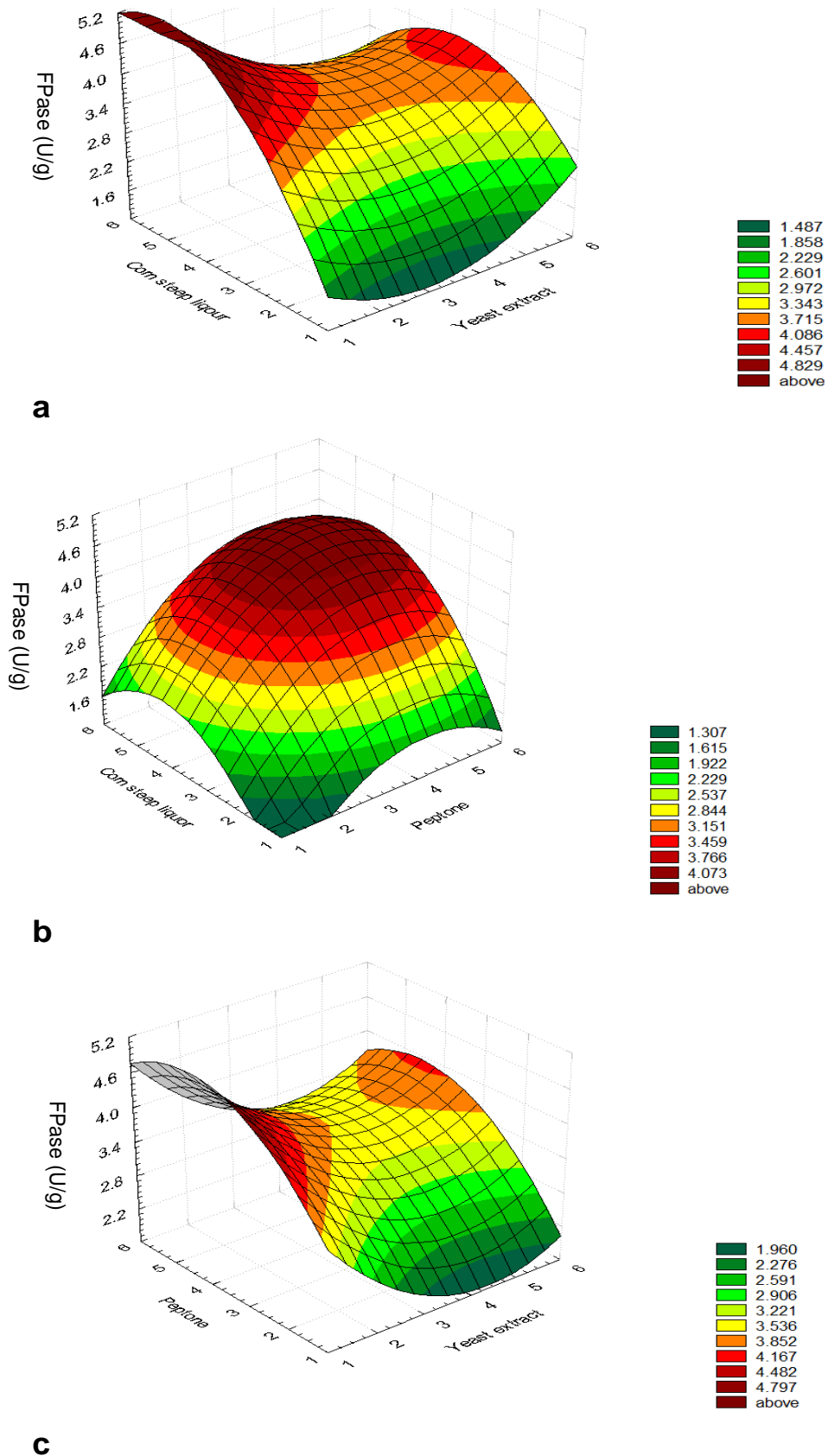
tion of the three enzymes, the data in Table 4 shows that the amounts of the tested nutrient could be added to RH for maximizing the cellulases production simultaneously, in this respect, the desirability function recorded 0.846; this value is somehow accepted.

### Scaling up via solid-state tray fermentation

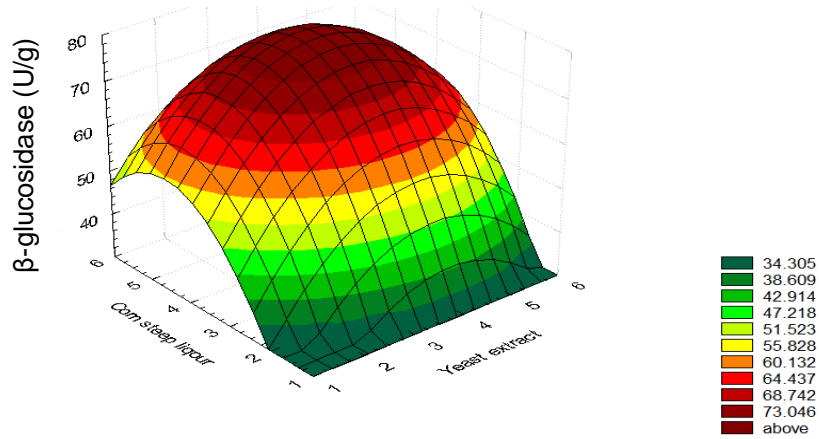
Taking into consideration the multi-response optimization, *A. niger* MA1 cellulases production process was scaled-up in the laboratory using simple and cheap solid-state tray fermentation. The total productivity of the three enzymes increased with increasing in the size of fermentation container in contrast to productivity per gram of RH. As shown in Table 5 the maximum productivity of CMCase, FPase and  $\beta$ -glucosidase are noticed when *A. niger* MA1 was cultivated in Petri dishes (20 g in 20 cm diameter Petri dish) giving 16.7, 3.02 and 55.9 U/g and in glass flask (10 g in 120 ml) giving 10.02, 3.17 and 52.32 U/g respectively. In trays the three enzymes productivity decreased gradually with increasing amount of RH parallel to tray size.



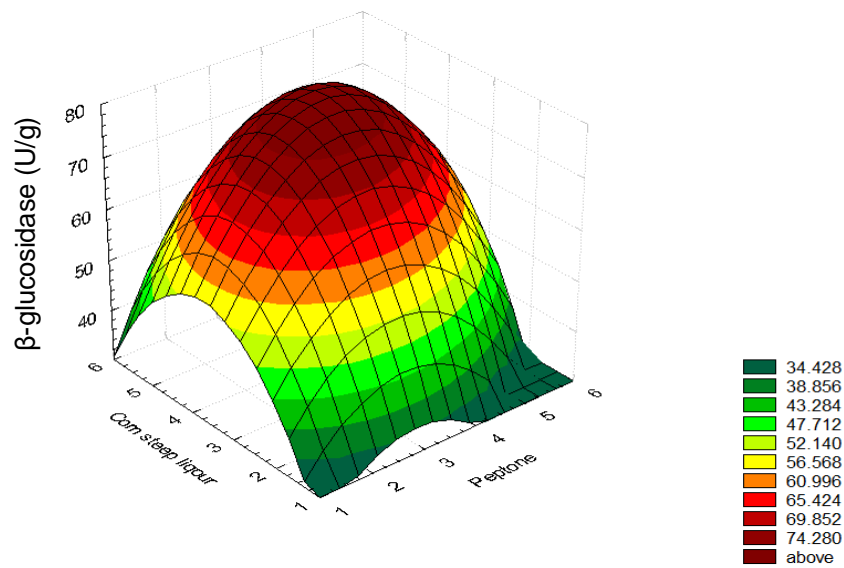
**Figure 2.** a. Response surface graphs illustrating the effect of yeast extract and corn steep liquor. b. Peptone and corn steep liquor. c. Yeast extract and peptone on CMCCase production.



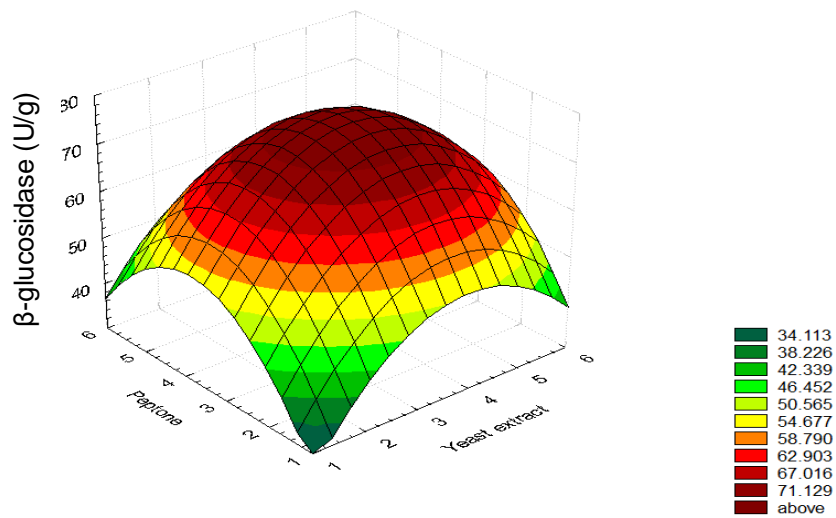
**Figure 3a.** Response surface graphs illustrating the effect of yeast extract and corn steep liquor; **3b.** Peptone and corn steep liquor; **3c** Yeast extract and peptone on Fpase production.



a



b



c

**Figure 4a.** Response surface graphs illustrating the effect of yeast extract and corn steep liquor; **4b.** Peptone and corn steep liquor; **4c.** Yeast extract and peptone on  $\beta$ -glucosidase production.



**Table 4.** Multi-response optimization of CMCase, FPase and  $\beta$ -glucosidase production of *A. niger* MA1 under SSF of RH after 42 h of fermentation

Yeast extract (mg/gm)	Peptone (mg/g)	Corn steep ( $\mu$ l/gm)	CMCase (U/g)		FPase (U/g)		$\beta$ -glucosidase (U/g)		*Desirability
			Response	Predicted	Response	Predicted	Response	Predicted	
2.06	3.68	47.87	16.41	17.73	4.28	4.80	78.60	76.39	0.846

\*Desirability function is used to test the possibility of optimizing more than one response at the same time, the closer to 1, the more accuracy of desirability function.

**Table 5.** Scaling up of cellulases production by *A. niger* MA1 after 42 h of SSF.

RH (g)	Container	CMCase		FPase		$\beta$ -glucosidase	
		u/g	Total	u/g	Total	u/g	Total
10	Glass flask (120 ml)	10.02	100	3.17	32	52.32	523
20	Petri dishes (20 cm)	16.70	334	3.02	60	55.90	1118
<b>Tray</b>							
100	(2494 cm <sup>3</sup> )	8.20	820	2.70	270	47.60	4760
150	(3438 cm <sup>3</sup> )	6.60	990	2.04	306	45.02	6753
200	(4582 cm <sup>3</sup> )	6.20	1240	1.80	360	42.13	8426

### Elution of *A. niger* MA1 cellulases by different eluents

After scaling up, elution process is very critical for this purpose. Many eluents were tested and as shown in Figure 5, maximum activity of CMCase were released with Tap water + 0.25% Brej 35 which gave 16.87 U/g with 149.37% efficiency as well as FPase; 4.65 U/g with 184.40% efficiency, for  $\beta$ -glucosidase Brej 35 was also more efficient but this time with saline solution.

### Characterization of CMCase, FPase and $\beta$ -glucosidase of *A. niger* MA1

#### Temperature of incubation

In different incubation temperature from 20 to 60°C, cellulases activity was increased with

increase in temperature with maximum activity of CMCase, FPase and  $\beta$ -glucosidase at 50°C which recorded 16.01, 4.90 and 62.94 U/g respectively, while further increase in temperature showed decrease in cellulases activity.

#### Thermal stability

CMCase enzyme showed good thermal stability in temperatures below 70°C as 81.69, 74.65 and 73.71% of the activity remained after 12, 18 and 24 h respectively at 50°C and 68.54 and 58.22% of the activity remained after 12 and 18 h respectively at 60°C with nearly half life time reaching to 24 h at 60°C; similar results were obtained for FPase activity where  $\beta$ -glucosidase is more resistance to thermal denaturation as its activity was 93.3% after 12 h of exposure to 50°C and retain 60% of its activity after 24 h of

exposure to 70°C and reached 32.31% of its activity after 6 h at 80°C, where it denatured and lost all of its activity after 24 h of exposure to 90°C.

#### Effect of pH

CMCase have high activity in a pH range from 4.6 to 6; the highest CMCase produced by *A. niger* MA1 was recorded at pH 5, where FPase and  $\beta$ -glucosidase showed high activity at pH range from 4.6 to 5.6 with optimum at 4.8.

#### pH stability

CMCase have a high pH stability, it retained 99.00, 96.25, 95.25, 99.00, 97.25, 98.25 and 95.75% of (3.6 to 5.6) respectively and showed 95.75 and

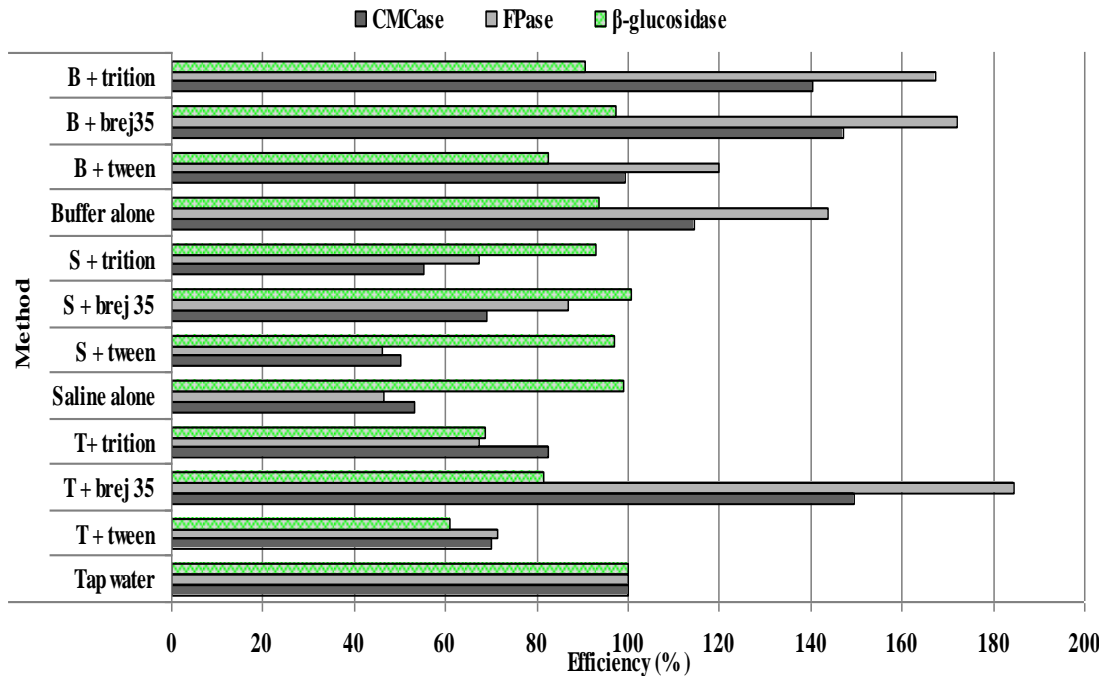


Figure 5. Effect of different eluent solutions in *A. niger* MA1 cellulases recovery from SSF of RH.

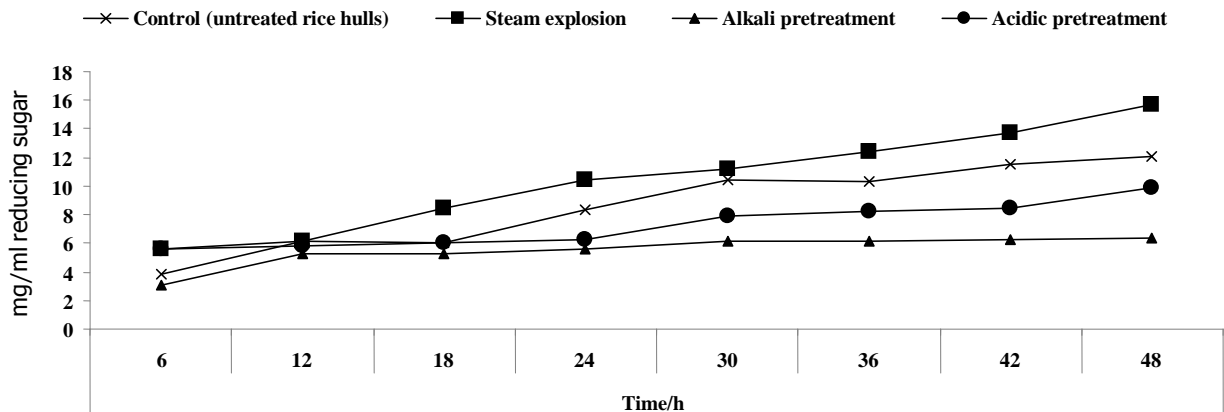


Figure 6. Effect of different treatment of RH in its saccharification by *A. niger* MA1 cellulases.

95.00% activity after 24 h at 3.6 and 4 pH values respectively. FPase was stable in pH range 3.6-5.6 with high stability at pH 4.8 and the enzyme lost only 7% of its activity after 24 h at pH 5.  $\beta$ -glucosidase was more stable, as it lost only 0.62% in activity after 24 h in pH value of 4.8. In more acidic pH of 3.6, 4 and 5.6 the enzyme lost only 3.4, 2.8 and 3.1% of its activity respectively after 24 h of incubation.

### Hydrolysis of RH by *A. niger* MA1 cellulases

In enzymatic hydrolysis process cellulase enzymes of *A.*

*niger* MA1 containing 2, 0.42 and 8.6 U/ml of CMCase, FPase and  $\beta$ -glucosidase respectively were used to convert cellulose of RH into sugars which then fermented into ethanol.

As shown in Figure 6, untreated, steam explosion, alkaline and acid treated RH were used in hydrolysis trials. Generally the liberated reducing sugar is produced with different values according to type of pretreatment of RH and increases gradually till its maximum activity after 48 h. It is clear that, steam explosion pretreatment is the most efficient method as it recorded 16.36 mg/ml of reducing sugars after 48 h of enzymatic hydrolysis, followed by untreated RH which release 12.10 mg/ml.

**Table 6.** Bioconversion of enzymatically hydrolyzed RH sugars to bio-ethanol by *Saccharomyces cerevisiae*.

Analyses (%)	Steam explosion
Total reducing sugars (g/L)	16.36 ( $\pm 0.03$ )
Ethanol content (g/L)	9.42 ( $\pm 0.08$ )
Fermentation efficiency (%)	89.93 ( $\pm 4.9$ )
Remaining reducing sugars (g/L)	0.40 ( $\pm 0.03$ )

Contrarily, amount of reducing sugar are lower in alkali and acidic pretreatment of RH which recorded 6.33 and 9.90 mg/ml.

### Bioconversion of enzymatically hydrolyzed RH sugars into bioethanol

As shown in Table 6, the resultant yield of total sugars after enzymatic saccharification of steam exploited RH using cellulases enzymes of *A. niger* MA1 to final concentration of 16.36 mg/ml; this amount of sugar was fermented by *S. cerevisiae* for 24 h to 9.42 g/L ethanol.

## DISCUSSION

In fungal bioconversion, the selection of active isolate is essential. In this work the RH is the target of hydrolysis and in the same time the source of fungal isolates; by this strategy the selected *A. niger* MA1 is more adapted to produce cellulases upon RH and showed maximum activity after only 36 h of incubation. This relatively short incubation time is shorter than 3 to 8 days reported as optimum incubation period of cellulolytic enzymes during SSF of lignocellulosic residues (Abo-State et al., 2010) and shorter than 80 h recorded by *A. niger* in production of cellulases from potato peel (Santos et al., 2012) also shorter than 96 h recorded by *A. niger* in saw dust (Acharya et al., 2008) and in rice straw (Pericin et al., 2008) whereas the results obtained by Panagiotou et al. (2003) and Narasmish et al. (2006) are in agreement with our results. Generally the optimum incubation time of cellulase production depends on the substrate and the tested fungus (Alam et al., 2005).

Solid state fermentation (SSF) is an attractive and economic process to produce cellulases due to lower capital investment, lower operating expenses (Yang et al., 2004), ease of use, superior productivity, use of simpler fermentation media, reduced production of waste water and easier control of bacterial contamination (Pandey et al., 1994). Among the advantages of SSF, it is often cited that enzyme titer is higher in SSF than in submerged fermentation (SMF) (Gonzalez et al., 2002). In this study using RH as a substrate for cellulases production is based on reduction in the production cost

which is a critical target in cellulase research. *A. niger* MA1 cellulases productivity using RH as a sole carbon source is more than that produced by *Trichoderma* sp. FETL on RH or on mixture of sugar cane baggase and palm kernel cake via SSF (Pang and Ibrahim, 2006). More recently, it was also better than that produced by *A. fumigatus* in SSF of alkali treated rice straw (Sherief et al., 2010). Our result confirms the high  $\beta$ -glucosidase productivity of *A. niger* similar to records of Wen et al. (2005) and Kang et al. (2004).

In screening the factors affecting production of cellulases enzymes, it's very important to test as many factors as possible to identify the significances of each of them. Plackett-Burman designs offers good and fast screening procedure and mathematically computes the significance of large number of factors in one experiment, which is time saving and maintain convincing information on each component. Among fifteen independent variables, yeast extract, peptone and corn steep liquor have significant effect on production of *A. niger* MA1 cellulases which further optimized in BBD and revealed considerable increases in the three enzymes. In this connection Gao et al. (2008) and Ng et al. (2010) reported that the organic nitrogen sources favored cellulases production by *A. terreus* M11 and  $\beta$ -glucosidase production by *P. citrinum* YS40-5. On the contrary, Sasi et al. (2012) found that *A. flavus* showed the highest production of cellulase enzyme utilizing ammonium sulfate as nitrogen source than yeast extract. In scaling up trials, the cellulases enzyme production per gram decreases with increase in quantity of RH and size of tray, due to low aeration and heat transfer with increase in the depth of substrate (Gowthaman et al., 2001).

The effectiveness of elution is necessary to recovery of enzyme from the fermented biomass, to best of our Knowledge; Brij 35 is not used before in extraction of cellulases, the addition of this surfactant gives maximum elution of CMCase and FPase activity with tap water. Tap water is commonly available, save and low cost extraction and used by other workers (Ahmed, 2008). The eluted CMCase and FPase enzymes of *A. niger* MA1 in the present investigation, showed good thermal stability with half-life time reached to 24 hours at 60°C, similar to (CMCase and Avicelase) of *A. fumigatus* (Parry et al., 1983), *T. reesei* (Busto et al., 1996) and more than that of *A. oryzae* KBN616 (Kitamoto et al., 1996).  $\beta$ -glucosidase of *A. niger* MA1 is more resistant to thermal denaturation with activity 60% after 24 h of exposure to 70°C which is more than that reported by *A. niger* 322 which lost most of its activity at temperature higher than 50°C (Peshin and Mathur, 1999). These data encourage the application of *A. niger* MA1 cellulases in saccharification trials of RH. For enhancing this process, pretreatment of RH make the process more efficient. In the present study, amount of reducing sugar produced from cellulases treatment of steam exploited RH are

**Table 7.** Comparing the results of the present investigation of reducing sugar and ethanol content with the previous work on RH.

Type of pretreatment	Enzyme used	Amount of reducing sugars	Amount of ethanol (g/L)	Strain of fermentation	Fermentation time (h)	Reference
Steam explosion	Cellulases enzyme (mix of CMCase, FBase and $\beta$ -glucosidase) of <i>A. niger</i> MA1	16.36 g/l 163 mg/g hulls	9.42	<i>Saccharomyces cerevisiae</i>	24	This study
Diluted H <sub>2</sub> SO <sub>4</sub> 1% pretreatment	Commercial enzyme (cellulase, xylanase, esterase, beta-glucosidase and Tween20)	42.0±0.7 g/l (acid, enzyme, overliming)	9.1±0.7	(1) <i>E. coli</i> strain FBR5 simultaneous saccharification and fermentation with recombinant <i>E. coli</i> FBR5	39	Saha and Cotta, 2005
Alkaline H <sub>2</sub> O <sub>2</sub> pretreatment	Commercial enzyme (cellulase, beta-glucosidase and xylanase)	428±12 mg/g	8.2±0.2 (1) 8.0±0.2 (2)	(1) <i>E. coli</i> strain FBR5 (2) recombinant <i>E. coli</i> FBR5	(1) 24 (2) 48	Saha and Cotta, 2007
Lime pretreatment	Commercial enzyme preparations (cellulose, beta-glucosidase and hemicellulase)	154±1 mg/g 19.8±0.6 g/l	9.8±0.5 11.0±0.1	1) <i>E. coli</i> strain FBR5 simultaneous saccharification and fermentation with recombinant <i>E. coli</i> FBR5 2)	(1) 19 (2) 53	Saha and Cotta, 2008
Fungal pretreatment with <i>A. awamori</i> and <i>Pleurotus</i>	FPU	34 mg/g	8.5	<i>Saccharomyces cerevisiae</i>	168	Patel et al., 2007

16.36 g/L (163 mg/g RH). This result (Table 7) is much more than 34 mg/g RH recorded by Patel et al., (2007) with fungal pretreatment by *A. awamori* and *Pleurotus sajorcaju* and application of FPase enzyme and closer to 154 mg/g RH which recovered after lime pretreatment and hydrolysis by commercial enzyme preparations (cellulose,  $\beta$ -glucosidase and hemicellulase) (Saha and Cotta., 2008). On the other hand it lower than 428±12 mg/g RH that obtained after pretreatment with alkaline H<sub>2</sub>O<sub>2</sub> and application of commercial enzyme (cellulase,  $\beta$ -glucosidase and xylanase) (Saha and Cotta, 2007). With respect to variation in amount of reducing sugar produced pretreatment with steam explosion is preferred because it save method, have no chemical wastes that can be produced from acid and alkalia treatment.

In continual improvements in the yield of ethanol the amount of ethanol produced from steam explosion pretreated RH are 9.42 g/L higher and faster than that exhibited by Patel et al (2007) who produce (8.5 g/L) from fungal pretreatment of RH with *A. awamori* and *P. sajorcaju* and fermentation with *Saccharomyces cerevisiae* after 168 h. other researchers who used commercial cellulases in scarification of RH like Saha et al., (2005) who obtained similar yield (9.1 g/L) by simultaneous saccharification and fermentation of H<sub>2</sub>SO<sub>4</sub> treated RH using recombinant *E. coli* FBR5 after 39 h. as well as Saha and Cotta (2008) when used *E. coli* strain FBR5 in fermentation of lime pretreatment RH with simultaneous saccharification they obtained 9.8 and 11.0 g/L ethanol after 19 and 53 h, respectively.

### Conclusion

Simple optimization of RH as medium for *A. niger* MA1 can be used in rapid production of considerable amount and mixture of cellulases which used in scarification of RH and subsequently fermented to bioethanol.

### Conflict of Interests

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

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## Full Length Research Paper

# Bioethanol production from *Curcubita pepo* and *Opilia amentacea* fruits using four strains of *Saccharomyces cerevisiae*

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Ethanol is commercially produced by fermenting agricultural products that contain high sugar or starch contents. In this study, the juices of *Opilia amentacea* and *Curcubita pepo* were used to produce bioethanol. A mixture of Urea ( $\text{CON}_2\text{H}_4$ ), monosodium glutamate ( $\text{C}_5\text{H}_8\text{NNaO}_4$ ), potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ), and magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) is used to promote batch ethanol production with four strains of *Saccharomyces cerevisiae* as the fermenting organisms. The results obtained have shown that the nutrients mixture significantly affected kinetic parameters and enhanced bioethanol production. Subsequently, the highest outputs of  $60.72 \pm 0.68$  and  $50.93 \pm 1.61$  g ethanol/kg were obtained respectively with *O. amentacea* and *C. pepo*. In the same time,  $460.97 \pm 8.66$  g ethanol/kg were got as maximum output from sucrose (NG).

**Key words:** Fruit juices, enrichment, *Saccharomyces cerevisiae*, fermentation, bioethanol.

## INTRODUCTION

Growing environmental concerns and increasing petroleum products prices have recently stimulated interest in optimizing fermentation processes for large-scale production of alternative fuels such as bioethanol (Wyman, 2001). The yeast "*Saccharomyces cerevisiae*" is the major industrial ethanol producer, because it is generally acknowledged as a safe microorganism that can produce ethanol by fermentation up to 20% (v/v) from carbon sources (Cot et al., 2007). Typically, ethanol fermentation is carried out under Normal Gravity (NG) technology, which is defined

as the preparation and fermentation of mashes containing 20-24% of dissolved solids (Thomas et al., 1996).

Ethanol can be produced from many different raw materials such as corn grain (in USA), sugarcane (in Brazil), tapioca and molasses (in Thailand) (Khongsay et al., 2010). In Togo, ethanol is commonly produced in artisanal way by using palm wine, corn, sorghum or millet as raw materials for local beer brewing. Ethanol is also produced in industrial scale with sugarcane molasses and cassava chips at Anié (Togo) by a Chinese company

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called "Sinto". The Actual challenge is to use less agricultural value products/residues or wastes which are not competing with common food production.

Therefore more interest are devoted to ethanol production by developing and implementing advanced process technologies (Mielenz, 2001). Thus, wild fruits from different plants with potentially higher level of sugars can be used as an alternative low-cost feedstock (Mazmanci, 2011).

In this study, bioethanol production from two types of local fruits (*Opilia amentacea* and *Curcubita pepo*) is compared to sucrose fermentation using four strains of *S. cerevisiae*. The cells growth and effects on main fermentation kinetic parameters were investigated.

## MATERIALS AND METHODS

### Microorganism and inoculum preparation

Four strains of *S. cerevisiae* were used as fermentative yeasts. The first strain of *S. cerevisiae* called "Safelevure" (noted S1) is generally used in bakery and in the artisanal production of local beers in Togo. The three other ones (noted S2, S3 and S4) obtained from "Ryan Wu" Company, are the first new biotechnology products in China which has been widely used in the production of drinking alcohol, dehydrated alcohol and fuel ethanol. 1.00 g of each strain of active dry yeast was introduced into 250 mL of a preculture medium which had the following composition (in g/L): Yeast extract, 10.00; Tryptone, 20.00; Glucose (Prolabo®), 20.00 and NaCl (Prolabo®), 9.00. After homogenization, the liquid mixture is incubated at the room temperature (30-32°C) with continuous stirring using a magnetic stirrer. After 24 h of incubation, the active cells were then harvested and used as inoculum for ethanol production (EP) (Novidzro et al., 2013).

### Raw materials

*O. amentacea* (Opiliaceae) and *C. pepo* (Cucurbitaceae) fruits were harvested in June 2010 in Agou-Akplolo forests, located on the foot of Mount Agou in Togo. The juices were extracted with distilled water and autoclaved at 121°C during 15 min to prevent contamination as well as to enhance their sugar concentration (Mazmanci, 2011). Then the samples were centrifuged and filtered. Filtrates were kept at -23°C against bacterial contaminations until their use for reducing sugars analysis and alcoholic fermentation.

### Ethanol production media

*O. amentacea* and *C. pepo* fruit juices containing total soluble solids of 20°Bx supplemented with/without a mixture of four nutrients were used as EP media. Referring to the literature, four nutrients were mixed in following proportions (Al-Obaidi et al., 1987; Acourene and Tama, 2001; Novidzro et al., 2013) and used as additional supplements to study their effects on EP with the mashes. Their contents in the fermentation broth were (g/L): Urea (CON<sub>2</sub>H<sub>4</sub>), 2.00; Monosodium Glutamate (C<sub>5</sub>H<sub>8</sub>NNaO<sub>4</sub>), 1.00; Potassium Dihydrogen Phosphate (KH<sub>2</sub>PO<sub>4</sub>), 0.25 and Magnesium sulfate heptahydrate (MgSO<sub>4</sub>.7H<sub>2</sub>O), 0.25. A comparison is made with sucrose syrup in the same conditions.

The pHs of the mashes were adjusted then in 4.5 ± 0.2. The EP media were transferred into a capped plastic flask of 1000 mL with a final working volume of 250 mL and then autoclaved at 121°C for

15 min to prevent the final mixtures against contamination. The sterilized mashes were used as fermentation media after cooling down (Novidzro et al., 2013).

### Fermentation conditions

Each inoculum, prepared previously, was introduced into 10 % (v/v) of each EP medium so that the concentration of viable cells at the beginning of the fermentation must be equal to 2.8 × 10<sup>6</sup> viable cells/mL 10<sup>6</sup> viable cells/mL. After a step of pre fermentation during 12 h, the 90% of the staying mashes were added to each pre fermented EP medium. The fermentation was operated in batch mode and performed in a shaker at 100 rpm (Khongsay et al., 2010) in the laboratory at room temperature of 30 ± 2°C for 10-15 days. Data were expressed as the mean of four experiments. Samples were taken at appropriate time intervals for tests.

### Analytical methods

The fruits juices have been characterized to determine chemical groups present in the mesocarps of the fruits such as polyphenols, via the reaction with ferric chloride (Rafael et al., 2005), and flavonoids via the reaction "with cyanidine" (Brock et al., 2006; Konkon et al., 2008).

The cell numbers in the fermentation broth were determined by direct counting method with methylene blue staining on Motic/B1 Series/System Microscopes instrument using Malassez hemacytometer, while the total soluble solids (TSS) were determined by *Fabre Mesurelec* hand-held refractometer ((RAM 0-80% 106), respectively as described by Khongsay et al. (2010). The pH was measured by pH-meter (CRISON/PH 25).

The fermentation broth was centrifuged at 13,000 rpm for 10 min (Laopaiboon et al., 2008). The supernatant was then determined for the total residual sugars by phenol sulfuric acid method (Mecozzi, 2005) and the reading absorbance was 490 nm, using a calibration curve produced with glucose in DMS 300 UV VISIBLE Spectrophotometer. The reducing sugars were determined by Bertrand Method and sucrose in fruit juices by the following formula (Acourene and Tama, 2001):

$$[\text{Sucrose}] = ([\text{Total Sugars}] - [\text{Reducing Sugars}]) \times 0.95 \quad (1)$$

Percentage of total sugars consumption rate (SCR: %) was calculated as the ratio of the consumed mass of total sugars to the initial mass of total sugars (Laopaiboon et al., 2008). Ethanol concentration was analyzed by high-performing liquid chromatography (using *Chrompack pump; Chromapack/RI Detector; Merck/D-2500/Chromato-Integrator* and C18 (25 cm x 4.6 mm) column. The volumetric ethanol productivity (Q<sub>p</sub>: g/L/h) and the percentage of conversion or yield efficiency (E<sub>y</sub>:%) were calculated by the following equations (Khongsay et al., 2010):

$$Q_p = \frac{P}{t} \quad \text{and} \quad E_y = \frac{Y_{p/s} \times 100}{0.54} \quad (2)$$

where, **P (g/L)** is the final ethanol concentration produced, **t (h)** the fermentation time giving the highest ethanol concentration during batch fermentation on EP media under NG conditions and **0.54** the maximum theoretical ethanol of sucrose consumption.

The ethanol yield (**Y<sub>p/s</sub>: g/g**) is calculated by the following formula (Swain et al., 2007):

$$Y_{p/s} = \left[ \frac{\text{Mass of product (Ethanol) formed}}{\text{Mass of substrate (Total sugars) consumed}} \right] \quad (3)$$

Statistical analysis was carried out via Turkey test by one-way and

**Table 1.** Characteristics of raw fruits juices of *Curcubita pepo* and *Opilia amentacea*.

Characteristic	<i>Curcubita pepo</i> juice	<i>Opilia amentacea</i> juice
Volume (mL)	1100.0 ± 4.0	1200.0 ± 5.0
Total soluble solid (°Bx)	20.0 ± 0.1	20.0 ± 0.1
Total sugars (g/L)	101.1 ± 2.4	122.7 ± 2.3
Reducing sugars (g/L)	56.9 ± 3.0	114.1 ± 2.7
Sucrose (g/L)	42.0 ± 0.6	8.2 ± 0.4
Polyphénols test	+	++++
Flavonoïds Test	++	++++

+, slightly positive test; ++, positive test; +++++, very positive test.

two-way ANOVA using MSTATC.EXE software. All the experiments were performed in four times and the results were expressed as mean ± standard deviation (SD) of the four experiments.

## RESULTS AND DISCUSSION

### The fruits juices biochemical composition

The characteristics of raw juices extracted from 1000 g of each fruit are shown on Table 1. To extract juices of identical initial concentration of 20.0 ± 0.1 Bx, the volume of water used varied from one fruit to another. *O. amentacea* juice appeared on Table 1, as the richest substratum in total sugars, with a concentration of 122.7 ± 2.3 g/L corresponding to a quantity of total sugars of 147.2 ± 2.8 g/kg of fruit while the juice of *C. pepo* could be distinguished like the weakest in total sugar, with its concentration of 101.1 ± 2.4 g/L in total sugars, equivalent to a quantity of 111.2 ± 2.6 g/kg of fruit. The quantities of total sugars in the fruit juices were low by comparison to sucrose syrup (230 g/L) with the same initial concentration of 20.0 ± 0.1°Bx.

With a concentration of 114.1 ± 2.7 g/L, or a quantity of 136.9 ± 3.2 g/kg of fruit, *O. amentacea* was also the fruit which had the most elevated content in reducing sugars. The one of *C. pepo* was only of 56.9 ± 3.0 g/L, or a quantity of 62.6 ± 3.3 g/kg of fruit. But, about the sucrose content, *C. pepo* was the best, with a concentration of 42.0 ± 0.6 g/L, or a quantity of 46.2 ± 0.7 g/kg of fruit, against a concentration of 8.2 ± 0.4 g/L and quantity of 8.2 ± 0.4 g/kg of fruit, for *O. amentacea*. Probably, with these contents in sugars presented in this study, *O. amentacea* would be the most favorable substratum to the alcohol production. Therefore, the same total soluble solids does not mean the same reducing sugar contents of which different juices should be stated.

The pHs of the fruit juices were 5.35 ± 0.08 and 3.25 ± 0.06, respectively for *C. pepo* and *O. amentacea*. These values could contribute to a better conservation of juices, then for good alcohol production. Indeed, it had been proven that, in fermentation broth, an acidic pH allowed to avoiding contaminations or the bad reactions, due to the presence of other micro-organisms, such as the bacteria (Ban et al., 1988).

The two phytochemical tests revealed that *O. amentacea* juice contained more phenolic compounds (Polyphenols and flavonoïds) in relation to the one of *C. pepo*. However, phenolic compounds were considered as poisons to yeasts growth, so they could have negative impacts for alcohol production (Almeida et al., 2007).

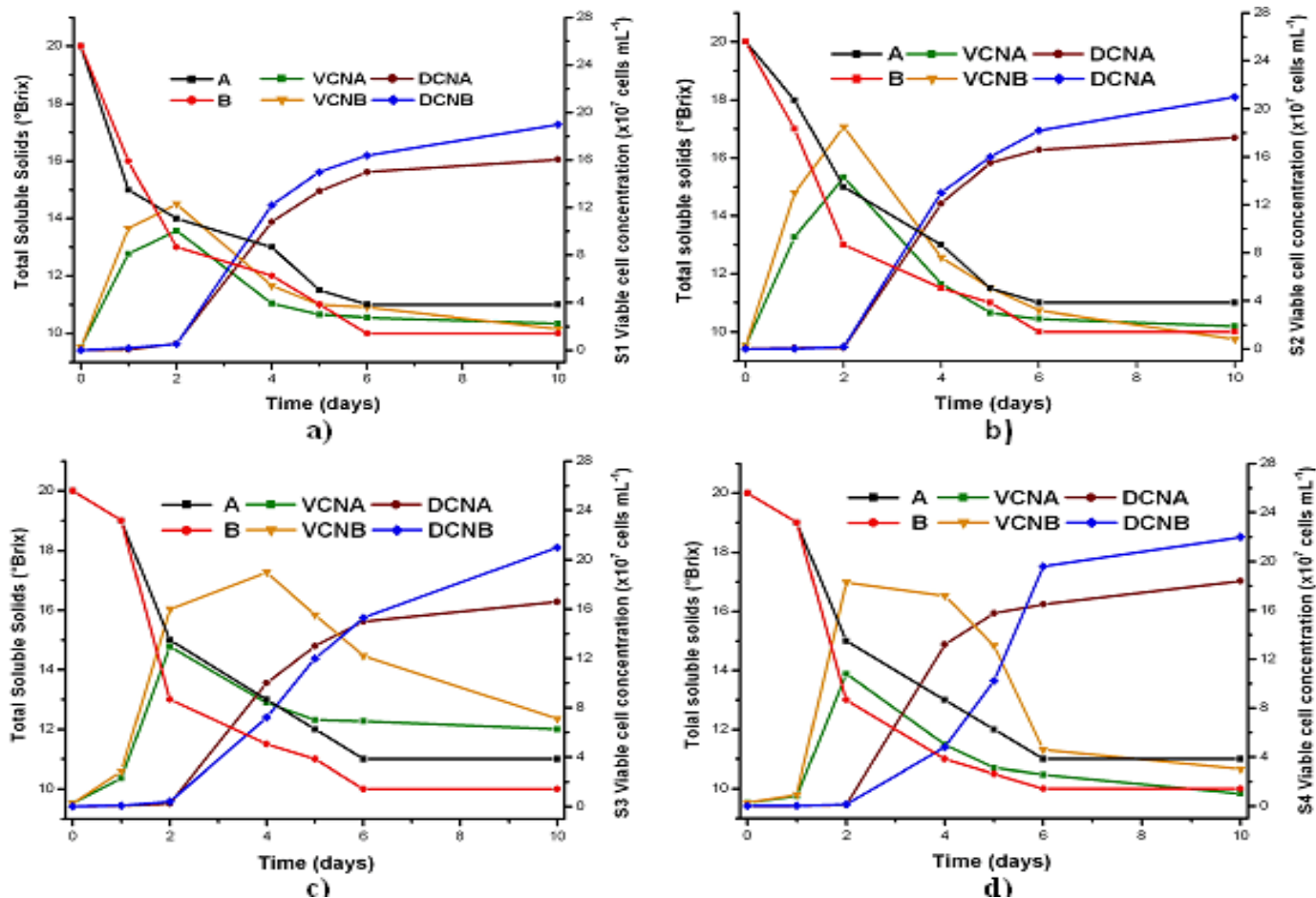
### Evolutions of brix and yeasts population during fermentation period

The curves of Figures 1 and 2 showed the profiles of Brix and the number of the living and dead yeast cells variations, during the fruit juices fermentation. After the inoculation, there is a continuous dropping of the Brix for each strain. However, the dropping of the Brix show three phases: a lag phase at the beginning, followed by an acceleration phase, and then finishing by the deceleration phase. It is known that the Brix reduction is favorable to ethanol production under anaerobic conditions.

Simultaneously, a slow rhythm increase of the living cells number was observed as the fermentation begun. This was followed respectively by an acceleration phase, an exponential growth phase and finally, by a decline phase, characterized by a rapid decrease in viable cell numbers. These developments are in accordance with works achieved by Aiba et al. (1973). On the other hand, the presence of dead cells happened before a very quick intensified phase. Microbial growth in juices reveals four phases namely lag, exponential, stationary, and death phases. During lag phase, cells got progressively accustomed to the new environment and were growing (there is no increase in cell numbers). Exponential phase was the period when cell numbers increases rapidly. When the growth and death rates of cells were approximately equal, the cells entered the stationary phase. Finally, when the living environment of cells comes to accumulate toxic wastes and to face the lack of nutrients, these eventually results in death of the cells (Fugelsang and Edwards, 2007).

With enrichment of the media by nutrients mixture, the Brix reduction became important, and the cellular growth also rose significantly. Whatever the strain used in the non-enriched media, the reduction of Brix was slow and





**Figure 1.** The effects of the mixture of nutrients on the profiles of Brix and cell growth during batch ethanol fermentation of *Curcubita pepo* fruit juice: a, with S1; b, with S2; c, with S3 and d, with S4. A= pure juice; B = enriched juice; VCNA = Viable Cell Number in A; VCNB = Viable Cell Number in B; DCNA = Dead Cell Number in A and DCNB = Dead Cell Number in B.

weak, and the cellular growth always remained lower when compared to enrich experiment case. At the end of fruit juice fermentation, it is noteworthy that number of the dead cells in *C. pepo* experiment was higher than viable cells. The inverse phenomenon was observed for *O. amentacea*.

### The maximum viable cell numbers during the fermentation

The yeasts proliferation varied from one medium to another (Table 2). The values of the maximal viable cells number of every strain were significantly different ( $P \leq 5.0\%$ ) in the enriched media as well as in the non-enriched media. Among the three different media investigated, *C. pepo* juice showed the best yeasts cells growth. So, the maximal viable cells number is obtained in the non-enriched medium with S2 ( $14.30 \pm 0.05 \times 10^7$  to  $0.02 \times 10^7$ /mL cells). But *O. amentacea* juice is the medium in which yeasts cell growth was the poorest.

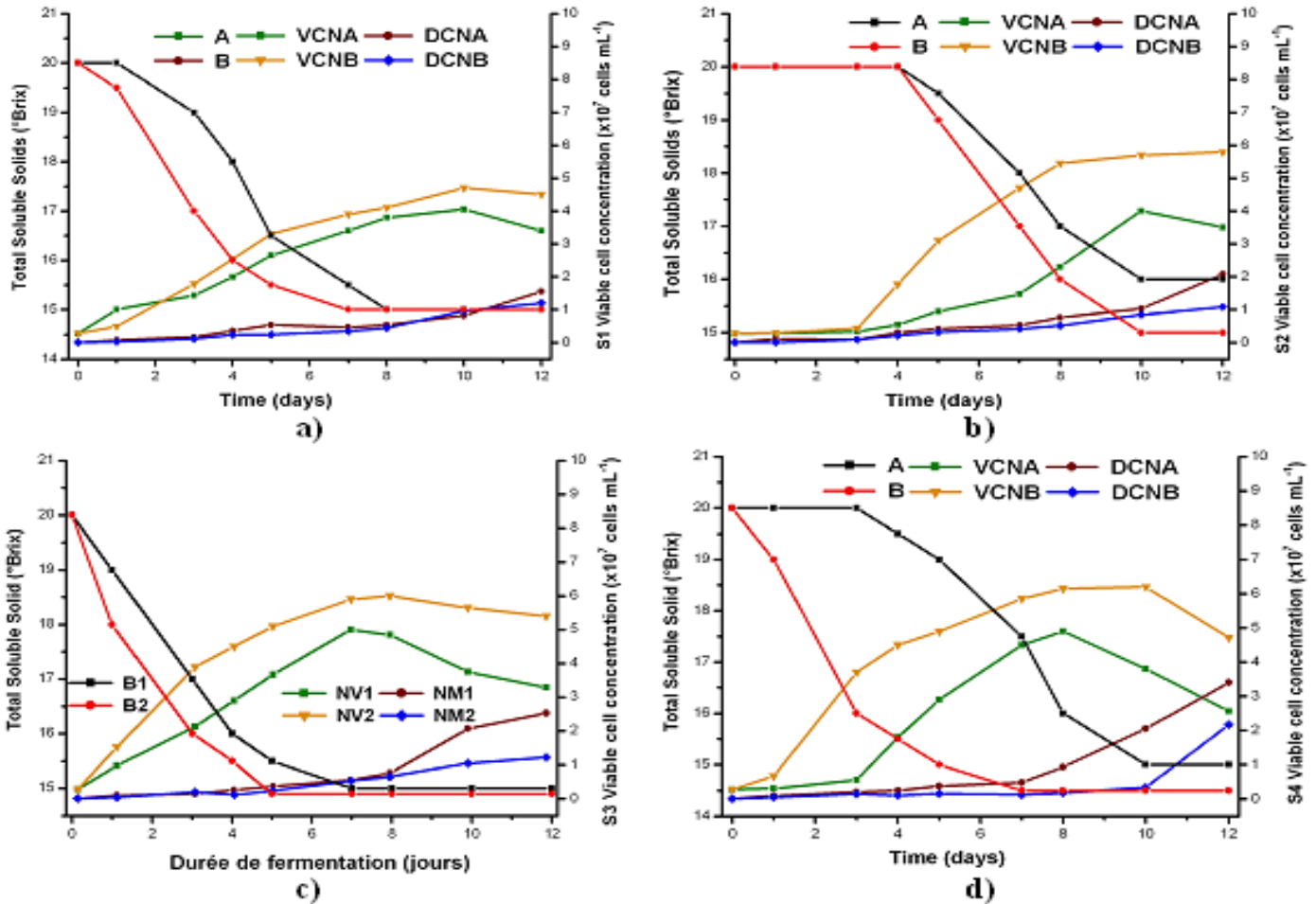
The possible existence of poisonous substances

inhibiting the development of the yeasts in this medium might likely explain the phenomenon, according to the phytochemical tests down.

On the whole, the growths of S2, S3 and S4 were better than those of S1. One can suggest that S2, S3, and S4 would be more effective than S1 for ethanol production. The weak alcoholic tolerance of S1 or other inhibiting factors could explain the weak performance of S1, although it has been proven that the presence of some by-products such as  $\text{CO}_2$ , organic acids and phenolic compounds (Almeida et al., 2007) not only disturb the development of the yeasts, but also inhibit their production capacity (Fugelsang and Edwards, 2007).

### The main kinetic parameters during ethanol production fermentation

The main kinetic parameters of fermenting sucrose, *O. amentacea* and *C. pepo* fruits mashes were shown on Tables 3, 4 and 5, showing very interesting effect of nutrients on the fermentation.



**Figure 2.** The effects of the mixture of nutrients on the profiles of Brix and cell growth during batch ethanol fermentation of *Opilia amentacea* fruit juice: (a, with S1; b, with S2; c, with S3 and d, with S4. A= pure juice; B = enriched juice; VCNA = Viable Cell Number in A; VCNB = Viable Cell Number in B; DCNA = Dead Cell Number in A and DCNB = Dead Cell Number in B.

**Table 2.** The maximum viable cells numbers obtained in the fermentation broths.

EP medium	Maximum viable cells numbers (x107 cells.mL-1)			
	S1	S2	S3	S4
Non-enriched sucrose syrup	2.61 ± 0.01 <sup>c</sup>	8.05 ± 0.03 <sup>b</sup>	5.80 ± 0.07 <sup>b</sup>	7.61 ± 0.01 <sup>b</sup>
Enriched sucrose syrup	8.80 ± 0.06 <sup>b</sup>	18.70 ± 0.04 <sup>a</sup>	17.07 ± 0.08 <sup>b</sup>	14.60 ± 0.04 <sup>b</sup>
Non-enriched <i>Curcubita pepo</i> juice	10.05 ± 0.02 <sup>a</sup>	14.30 ± 0.05 <sup>a</sup>	12.95 ± 0.02 <sup>a</sup>	10.80 ± 0.04 <sup>a</sup>
Enriched <i>Curcubita pepo</i> juice	12.30 ± 0.02 <sup>a</sup>	18.50 ± 0.11 <sup>b</sup>	19.00 ± 0.02 <sup>a</sup>	18.30 ± 0.09 <sup>a</sup>
Non-enriched <i>Opilia amentacea</i> juice	4.05 ± 0.08 <sup>b</sup>	4.10 ± 0.04 <sup>c</sup>	5.00 ± 0.05 <sup>c</sup>	4.90 ± 0.02 <sup>c</sup>
Enriched <i>Opilia amentacea</i> juice	4.70 ± 0.03 <sup>c</sup>	5.79 ± 0.07 <sup>c</sup>	6.00 ± 0.04 <sup>c</sup>	6.40 ± 0.07 <sup>b</sup>

S1, *S. cerevisiae* "Safelevure" strain generally used in bakery and in the artisanal production of local beers in Togo; S2, S3 and S4 were obtained from "Ryan Wu" Company in China and has been widely used in the production of drinking alcohol, dehydrated alcohol and fuel ethanol. Values are in the mean ± standard deviation of 4 experiments. Mean followed by the same letter (a, b and c/a', b' and c') next to the values within a same column are not significantly different using Turkey's multiple range test at the level of 5%.

**Sugars consumption**

The sugar consumption rate (SCR: %) were respectively

79-89 and 76-87% (Table 2) in the enriched and non-enriched sucrose medium, depending on yeast strains. However, total sugars utilization in the fruit juices was

**Table 3.** The main fermentation kinetic parameters of ethanol production with sucrose mash ( $C_0=20^\circ\text{Brix}$ ).

Kinetic parameter	S1		S2		S3		S4	
	A	B	A	B	A	B	A	B
SCR (%)	75.98 ± 2.02	79.10 ± 1.48	86.72 ± 1.32	87.72 ± 1.79	80.01 ± 2.33	82.77 ± 1.55	87.39 ± 1.45	88.56 ± 2.51
t (h)	120	48	120	48	120	48	144	72
$P_{th}$ (g/L)	94.02 ± 2.50	97.88 ± 1.83	107.31 ± 1.63	108.54 ± 2.21	99.00 ± 2.88	102.42 ± 1.92	108.14 ± 1.79	109.58 ± 3.11
$P_{exp}$ (g/L)	74.82 ± 1.32	92.91 ± 2.48	90.66 ± 2.18	105.97 ± 1.99	85.94 ± 1.16	98.53 ± 2.04	93.73 ± 1.22	104.83 ± 3.44
$Q_p$ (g/L/h)	0.624 ± 0.011	1.936 ± 0.052	0.756 ± 0.018	2.208 ± 0.041	0.716 ± 0.010	2.053 ± 0.043	0.651 ± 0.008	1.456 ± 0.048
$Y_{p/s}$ (g/g)	0.428 ± 0.008	0.511 ± 0.014	0.455 ± 0.011	0.525 ± 0.010	0.467 ± 0.006	0.518 ± 0.011	0.466 ± 0.006	0.515 ± 0.017
$E_y$ (%)	79.29 ± 1.40	94.57 ± 2.52	84.17 ± 2.02	97.27 ± 1.83	86.48 ± 1.12	95.85 ± 1.98	86.36 ± 1.12	95.31 ± 3.13

**Table 4.** The main fermentation kinetic parameters of ethanol production with *Curcubita pepo* fruit juice ( $C_0 = 20^\circ\text{Brix}$ ).

Kinetic parameter	S1		S2		S3		S4	
	A	B	A	B	A	B	A	B
SCR(%)	48.50 ± 2.44	72.82 ± 2.07	54.89 ± 1.78	85.28 ± 2.52	59.17 ± 1.49	88.80 ± 1.91	55.29 ± 2.56	83.75 ± 2.33
t (h)	120	72	120	72	120	72	144	96
$P_{th/c}$ (g/L)	26.38 ± 1.33	39.61 ± 1.13	29.86 ± 0.97	46.38 ± 1.37	32.18 ± 0.71	48.30 ± 1.04	30.07 ± 1.39	45.55 ± 1.26
$P_{exp}$ (g/L)	23.03 ± 1.00	37.66 ± 1.35	27.02 ± 1.12	45.19 ± 0.54	25.92 ± 1.37	46.3 ± 1.46	26.98 ± 1.12	44.27 ± 0.79
$Q_p$ (g/L/h)	0.192 ± 0.008	0.523 ± 0.019	0.225 ± 0.009	0.628 ± 0.008	0.216 ± 0.011	0.643 ± 0.020	0.187 ± 0.008	0.461 ± 0.008
$Y_{p/s}$ (g/g)	0.470 ± 0.020	0.512 ± 0.018	0.487 ± 0.020	0.524 ± 0.006	0.433 ± 0.023	0.516 ± 0.016	0.483 ± 0.020	0.523 ± 0.009
$E_y$ (%)	87.06 ± 3.78	94.83 ± 3.40	90.26 ± 3.74	97.15 ± 1.16	80.31 ± 4.24	95.61 ± 3.01	89.45 ± 3.71	96.91 ± 1.73

S1, *S. cerevisiae* "Safelevure" strain generally used in bakery and in the artisanal production of local beers in Togo; S2, S3 and S4 were obtained from "Ryan Wu" Company in China and has been widely used in the production of drinking alcohol, dehydrated alcohol and fuel ethanol.

relatively low. Only 49-59 and 53-63% of total sugars are used in *C. pepo* and *O. amentacea* non enriched juices, respectively.

When the juices were enriched, these values were improved up 73 to 89% and 63 to 78%, respectively (Tables 3 and 4). All the obtained results showed that the enrichment of the medium provokes an increase of the sugar consumption rates.

Approximately 11-37% of initial total sugars content still remained at the end of enriched juices fermentation. Complete sugar utilization may be

achieved by optimizing aeration rate, agitation, and other nutrients supplementation, as already evoked by Laopaiboon et al. (2008).

#### Fermentation time

Fermentation times were very low in enriched media, unlike those of non-enriched media. S1, S2, and S3 gave the same fermentation times which were always lower than those of S4. Moreover, the fermentation times were identical in

sucrose and *C. pepo* media. Comparatively, *O. amentacea* juices media showed high fermentation times. Possibly, the intensity of inhibitory substances in *O. amentacea* juice might slow down significantly the fermentation process. The cells growth (Figures 1 and 2) could support this possibility.

#### Final ethanol concentration ( $P_{exp}$ ) produced

The final  $P_{exp}$  values from sucrose media are two

**Table 5.** The main fermentation kinetic parameters of ethanol production with *Opilia amentacea* fruit juice  $C_0 = 20^\circ$ Brix).

Kinetic parameter	S1		S2		S3		S4	
	A	B	A	B	A	B	A	B
SCR. (%)	53.04 ± 1.52	62.74 ± 2.01	61.34 ± 1.77	76.89 ± 1.55	63.04 ± 1.28	74.89 ± 2.25	59.80 ± 1.12	78.19 ± 2.11
t (h)	192	120	192	120	192	120	240	144
$P_{th/c}$ (g/L)	35.01 ± 1.00	41.42 ± 1.33	40.49 ± 1.17	50.76 ± 1.02	41.61 ± 0.84	49.44 ± 1.49	39.48 ± 0.74	51.62 ± 1.39
$P_{exp}$ (g/L)	32.85 ± 1.44	35.31 ± 1.30	37.12 ± 1.27	48.98 ± 1.08	38.97 ± 1.75	47.55 ± 1.07	36.95 ± 1.47	50.60 ± 0.57
$Q_p$ (g/L/h)	0.171 ± 0.008	0.294 ± 0.011	0.193 ± 0.007	0.408 ± 0.009	0.203 ± 0.009	0.396 ± 0.009	0.154 ± 0.006	0.351 ± 0.004
$Y_{p/s}$ (g/g)	0.505 ± 0.022	0.459 ± 0.016	0.493 ± 0.017	0.519 ± 0.011	0.504 ± 0.023	0.517 ± 0.012	0.504 ± 0.020	0.527 ± 0.006
$E_y$ (%)	93.47 ± 4.10	84.94 ± 3.13	91.33 ± 3.12	96.15 ± 2.12	93.30 ± 4.19	95.82 ± 2.16	93.27 ± 3.71	97.67 ± 1.10

SCR (%) = Sugar Consumption Rate ; t = fermentation time;  $P_{th/c}$  = theoretical final ethanol concentration which must be produced;  $P_{exp}$  (g/L) = experimental final ethanol concentration produced;  $Q_p$  = volumetric ethanol productivity;  $Y_{p/s}$  = ethanol yield;  $E_y$  = yield efficiency; A = non enriched medium; B = enriched medium with urea ( $CON_2H_4$ : 2.00 g/L), monosodium glutamate ( $C_5H_8NNaO_4$ : 1.00 g/L), potassium dihydrogen phosphate ( $KH_2PO_4$ : 0.25 g/L) and magnesium sulfate heptahydrate ( $MgSO_4 \cdot 7H_2O$ : 0.25 g/L). S1, *S. cerevisiae* "Safelevure" strain generally used in bakery and in the artisanal production of local beers in Togo; S2, S3 and S4 were obtained from "Ryan Wu" Company in China and has been widely used in the production of drinking alcohol, dehydrated alcohol and fuel ethanol.

or three times higher than those of fruit juices. The maximum  $P_{exp}$  values with enriching approach are  $50.60 \pm 0.57$  g/L (equivalent to  $60.72 \pm 0.68$  g ethanol/kg of fruit) for *O. amentacea* fruits using S4 (Table 5) and  $46.3 \pm 1.46$  g/L (equivalent to  $50.93 \pm 1.61$  ethanol/kg) for *C. pepo* fruits using S3 (Table 4). The maximum  $P_{exp}$  value with sucrose medium enriched were  $105.97 \pm 1.99$  g/L (equivalent to  $460.97 \pm 8.66$  g ethanol/kg sucrose NG), reached with S2. From Tables 3, 4 and 5, it can be deduced that ethanol production with *O. amentacea* juice was slightly better than that with *C. pepo*. In addition, ethanol produced using S2, S3 and S4 gave better  $P_{exp}$  values relatively to those obtained with S1. It is known that high sugar consumption can lead to a good ethanol production (Khongsay et al., 2012 and Khongsay et al., 2010). So, the weak sugars quantity and the presence of poisonous substances such as phenolic compounds in fruit juices (Almeida et al., 2007), might likely have impacts on the outputs of ethanol production. Therefore, a preliminary detoxification process could contribute to the

improvement of ethanol production from these kinds of raw materials (Mazmanci, 2011). Unfermentable carbon source's existence in the juices might also explain why the ethanol yields were low in the juices.

#### Ethanol productivity ( $Q_p$ : g/L/h)

With sucrose, the best productivity ( $Q_p$ ) obtained was  $2.208 \pm 0.041$  g/L/h with S2, and the lowest one was  $1.456 \pm 0.048$  g/L/h with S4. Without nutrients, the best  $Q_p$  reached with S2 in sucrose mashes was  $0.756 \pm 0.018$  g/L/h, while the lowest one was  $0.624 \pm 0.011$  g/L/h obtained with S1 (Table 3). For *C. pepo* enriched juice, the best and the lowest  $Q_p$  obtained by S3 and S4 were  $0.643 \pm 0.020$  g/L/h and  $0.461 \pm 0.008$  g/L/h, respectively. But in the absence of enriching nutrients, *C. pepo* juice best productivity was  $0.225 \pm 0.009$  g/L/h with S2 and the lowest is  $0.187 \pm 0.008$  g/L/h by S4 (Table 4). In the case of *O. amentacea* enriched juices, it was S2 which

gave the best productivity of  $0.408 \pm 0.009$  g/L/h and the lowest one of  $0.294 \pm 0.011$  g/L/h was given by S1. Without the enrichment of *O. amentacea* juices, the best productivity of  $0.203 \pm 0.009$  g/L/h was given by S3, and the lowest one of  $0.154 \pm 0.006$  g/L/h by S4 (Table 5). So, to get high ethanol productivity, the fermentation time must be kept in a short time (Khongsay et al., 2010).

#### Ethanol yield ( $Y_{p/s}$ : g/g) and yield efficiency ( $E_y$ : %)

The experimental  $Y_{p/s}$  values ( $0.428 \pm 0.008$  g/g to  $0.527 \pm 0.006$  g/g) and the  $E_y$  values ( $79.29 \pm 1.40$  % to  $97.67 \pm 1.10$  %) (Tables 3 and 4), in relation to the final sugars consumptions, were slightly lower than what were hypothetically the theoretical predicted. The ethanol concentrations produced were also slightly lower than the calculated theoretical ethanol concentrations. Many factors explained this decrease in the expected

results. First, the production of by-products such as organic acids, other alcohols, ketones, etc. was harmful for the good productivity. Secondly, the use of parts of sugars by the yeasts to get energy, useful and compulsorily to their growth was also seen as a source of output loss (Ballerini and Alazard-Toux, 2006). The previous reported Yp/s of cane molasses (Reed, 2002), dried sweet potato chips/flour (Woolfe, 1992), and cassava chips/flour (Balagopalan et al., 1987) were 265 to 272 g ethanol/kg, 280 to 320 g ethanol /kg, and (420 to 450 g ethanol /kg), respectively.

In the present study, the maximum ethanol yields calculated were 41.296 g ethanol /kg for *O. amentacea* fruits and 36.134 ethanol g/kg for *C. pepo* fruits. Ameyapoh et al. (2006) produced bioethanol from *Manguifera indica* L. (Anacardiaceae) mash of 6°Bx with four strains of *S. cerevisiae* (AYS01, AYS02 AYS001 and L2056). They got outputs (g/L ethanol) of 7.83; 6.52; 5.21 and 8.65, respectively. On the other hand, beer production achieved by Dahouenon-Ahoussi et al. (2012) from *Sorghum bicolor* and *Musa acuminata* mashes, gave the output of 4.86%Vol, equivalent to approximately 37 g ethanol /L. Our results were better than those obtained by Ameyapoh et al., (2006) and Dahouenon-Ahoussi et al. (2012). However, they were lower than those obtained with *Washintonia robusta* fruit juice containing 105 g/L of reducing sugars, and which succeeded to a maximal production of ethanol of 25 g/L, equivalent to  $71.42 \pm 1.4$  g of ethanol/kg (Mazmanci, 2011).

The improvement of ethanol production yields by using other different microorganisms such as *Zymomonas mobilis* (Claassen et al., 1999), *Saccharomyces bayanus* (Castellar et al., 1998), *Saccharomyces pastorianus* (Fujii et al., 2001), *Kluyveromyces fragilis* (Szambelan et al., 2004) or genetically modified microorganisms (Ostergaard et al., 2000), will be investigated in the future.

## Conclusion

The outputs of bioethanol production obtained in this study were  $50.60 \pm 0.57$  g ethanol/L (equivalent to  $60.72 \pm 0.68$  g ethanol/kg of fruit) and  $46.3 \pm 1.46$  g/L (equivalent to  $50.93 \pm 1.61$  g ethanol/kg of fruit), respectively for *O. amentacea* and *C. pepo*. Moreover, all the other fermentation kinetic parameters measured in this study showed clearly that the media supplementation with the mixture of nutrients (urea, monosodium glutamate, potassium dihydrogen phosphate, and magnesium sulfate heptahydrate) improved significantly the batch ethanol production performances. However, the maximal concentration of ethanol produced with sucrose ( $105.97 \pm 1.99$  g/L) was comparatively more elevated. All the results we obtained suggested that S4 is the most suitable strain for ethanol production under normal gravity(NG). The use of these conditions can be very attractive in fuel ethanol production domains.

The use of *C. pepo* and *O. amentacea* fruits as alternative carbon sources for ethanol production was proposed. Ethanol production yields for the two fruits were very low. However, as these fruits were abundant and inedible, so their eventual exploitation for bioethanol production could be a source of added value for the local population.

This would decrease the use of edible products for fuel production. But, fruit juices detoxification and supplementation with little quantity of sucrose or other nitrogen sources and/or other nutrients as well as other fermentation processes should be further investigated to improve and complete sugar consumption under normal gravity and very high gravity conditions.

## Conflict of Interests

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

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Full Length Research Paper

## Effect of various concentrations of *Aloe vera* coating on postharvest quality and shelf life of mango (*Mangifera indica* L.) fruits Var. 'Ngowe'

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**Mango (*Mangifera indica* L.) is a popular and economically important tropical fruit throughout the world due to its excellent nutritional composition, eating and visual qualities. However, the fruit is highly perishable and as a result high post-harvest losses continue to be reported especially in Africa. In order to address this problem, four concentrations of *Aloe vera* (AG) (0, 25, 50 and 75%) and chitosan (1%) were tested at two temperature levels (room temperature and 13°C) to determine their effect on the postharvest life of mango (var.'ngowe'). The experimental design was a 5 by 2 factorial experiment embedded in a complete randomized design with three replications. Data were recorded on weight loss, pH and total soluble solids (TSS) among others. The results show that at both temperatures 50 and 75% aloe concentrations significantly increased the shelf life evidenced by reduced percentage weight loss. Fruit firmness and total soluble solids concentration and pH were also maintained for longer periods in these treatments. Findings of this study demonstrate the potential of using *A. vera* gel at 50% as a coating for improved postharvest shelf life and maintaining quality of mango fruits hence reduced postharvest losses.**

**Key words:** *Aloe vera* gel, postharvest shelf life, mango fruit quality.

### INTRODUCTION

Mango (*Mangifera indica* L.) is the most economically important fruit in the Anacardiaceae family (Tharanathan et al., 2006). World trade in mangoes has been increasing over the years, and both exports from Kenya and local consumption is growing. The world market continues to become more price-competitive in spite of postharvest challenges for example, losses caused by diseases (HCDA, 2011). Mango is one of the most popular fruits all over the world as it has an attractive color, delicious taste and excellent nutritional properties. However, mango fruits

are climacteric and ripen rapidly after harvest, this limits their storage, handling and transport potential (Lalel et al., 2003). Mango has an easy access to post-harvest disease infection and production and consumption imbalances after harvesting lead to considerable losses (Zeng et al., 2006). Therefore, scientists are working towards prolonging the shelf life of the fruit by slowing down the ripening process while maintaining quality and flavor. Fruit coating after harvesting is becoming popular in this respect (Gill et al., 2005). However, possible health risks

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associated with the residue of the coating materials like fungicides are reducing the scope of coatings. Edible coatings have no residue associated risks and are possible alternative options (Ergun and Satici, 2012).

The use of *Aloe vera* gel has drawn interest in the food industry (Arowora et al., 2013). *A. vera* based edible coatings have been shown to prevent loss of moisture and firmness, control respiration rate and development and maturation, delay oxidative browning, and reduce micro-organism proliferation in fruits such as sweet cherry, table grapes and nectarines (Valverde et al., 2005; Matinez-Romero et al., 2005; Ahmed et al., 2009). In addition to the traditional role of edible coatings as a barrier to water loss and delaying fruit senescence, the new generation coatings are being designed for incorporation and/or for controlled release of antioxidants, nutraceuticals, chemical additives and natural antimicrobial agents (Vargas et al., 2008). It has also been reported that *A. vera* extracts possess antimicrobial activity against Gram positive and Gram negative bacterial pathogens (Adetunji, 2008). The use of *A. vera* gel as an edible surface coating has been reported to prolong the shelf life and to delay changes in parameters related to deterioration of quality in sweet cherry and table grapes (Martinez- Romero et al., 2006; Serrano et al., 2006), yet no studies have demonstrated the use of *A. vera* natural plant extract based on its antifungal properties on enhancement of shelf life and quality of mango fruits. Therefore, this study was conducted with the objective of evaluating the effects of the different *A. vera* concentrations on postharvest life of mango fruits.

## MATERIALS AND METHODS

### Research site

The postharvest study was carried out in a laboratory at Egerton University, Njoro, Kenya. The laboratory lies at a latitude of 0° 23' South, longitude 35° 35' East, altitude of approximately 2,238 m a.s.l in the Lower Highland 3 (LH3) agroecological zone (Jaetzold and Schmidt, 1983). The laboratory records average maximum and minimum temperatures of 19 to 22°C and 5 to 8°C, respectively (Egerton Metrological Station, 2009).

### Mango

The variety 'Ngowe' was used: it is popular, has little fibre and has excellent eating quality but it is susceptible to anthracnose. All the fruits that were used in this study were acquired from a grower in Masii in Machakos County, Kenya. The fruits were harvested at the mature green stage. The mature green fruits were without any visible blemish. The fruits were transported to the laboratory the same day.

### *Aloe vera*

Leaves of *A. vera* were harvested from Lare in Nakuru County, Kenya. Only the fully extended mature leaves were harvested. The leaves were then stored in plastic papers and transported to the

laboratory within same day.

### Chitosan

Crushed chitosan powder food grade was purchased from Kobian Chemicals Company Nairobi.

### Preparation of coating solutions

*Aloe vera* gel was obtained from fresh *aloe vera* leaves, the matrix was separated from the outer cortex of the leaves and the colourless hydroparenchyma homogenized in a blender. The resulting mixture was filtered using Watman filter paper number 100 to remove the fibres. The liquid constituted fresh *A. vera* gel. The gel matrix was pasteurized at 70°C for 45 min. For stabilizing, the gel was cooled immediately to an ambient temperature and 4.5 g of ascorbic acid was added; 4.5 g of citric acid was then added to adjust the pH to 4.

To prepare chitosan coating, 1% Chitosan (Kobian Chemical Co.) was dissolved in a 0.5% glacial acetic acid and distilled water. The pH value of the chitosan solution was then adjusted to 5.6 using 0.1 M NaOH.

### Application of treatments and experimental design

The coating solutions were: *aloe vera* gel (0%) as a negative control, *aloe vera* gel (25%), *aloe vera* gel (50%), *aloe vera* gel (75%), and chitosan (1%) as a positive control. Fresh fruits were dipped completely into the coatings solutions at room temperature for 25 min. The fruits were allowed to drain and then dried at room temperature to allow a thin film layer to be formed on the fruits. The fruits were then stored at room temperature and at 13°C. Mature, green fruits, without any visible blemish, were selected and the pedicels were removed. The fruits were then randomly divided into eight lots of 20 fruits each. The first lot constituted the positive control and was coated with chitosan. The second, third, fourth and fifth lots were coated by dipping completely in *A. vera* gel at concentrations of 0, 25, 50 and 75%, respectively and stored at room temperature and at 13°C (recommended optimum storage temperature for mangoes). The experiment was laid out as a 5 by 2 factorial experiment embedded in a completely randomized design with three replications. Various parameters were evaluated at 4 day intervals until the overall acceptability became unsatisfactory for each lot of samples (the fruit was considered as waste when it is infected by disease and/or its firmness value is less than 2).

### Weight loss

Three fruits in each replication for each treatment were marked before storage, and weighed using a digital balance (EK-600H, Japan). The same fruits were weighed at the beginning of the experiment and at the end of each storage period. The results were expressed as percentage loss of initial weight.

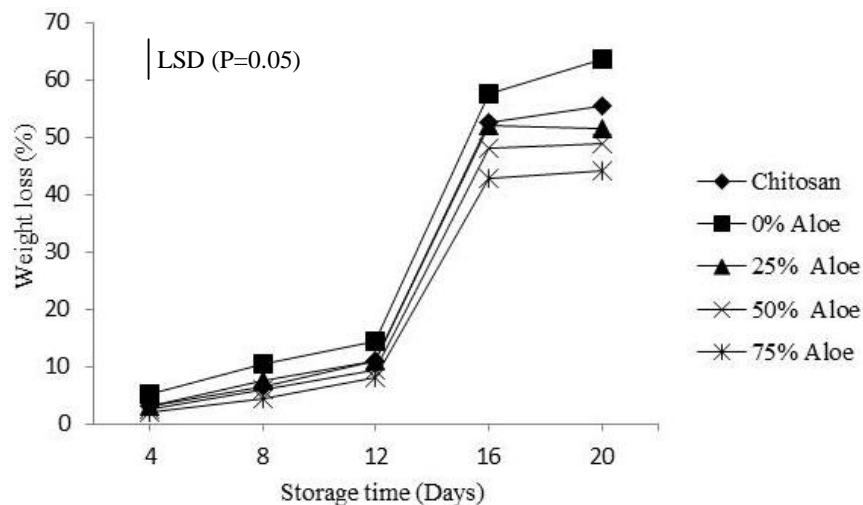
### Total soluble solid (TSS)

Total soluble solids were determined using hand held refractometer (0-30 °Brix) (RHW refractometer, Optoelectronic Technology Company Ltd. UK). Individual mango fruits from each treatment were ground in a blender to obtain soluble solids readings from the freshly prepared juice.

### Firmness

Three mango fruits from each treatment were used to determine





**Figure 1.** Percentage weight loss in Ngowe mango fruits treated with different concentrations of *Aloe vera* coatings and chitosan as a positive control.

fruit firmness using a hand held penetrometer (model 62/DR, UK) with a 8 mm diameter probe. The results were reported in Kg Force.

#### pH

This was measured with a standard calibrated pH meter (ADWA CO.). This measurement was made on juice expressed from flesh of the whole fruit filtered through filter papers.

#### Data analysis

The data collected was subjected to Analysis of Variance (ANOVA) at  $P \leq 0.05$ , using PROC GLM code of SAS (version 9, 2005) and means for significant treatments separated using the Tukey's Honestly Significant Different Test at  $P \leq 0.05$ .

## RESULTS AND DISCUSSION

### Weight loss

Fruits from all the treatments lost weight throughout the entire storage period (Figure 1). At day four, there was significant difference ( $P \leq 0.05$ ) between the negative control (0% *A. vera* gel) and the other treatments but there was no significant difference among 25, 50 and 75% *A. vera* gel concentrations and those coated with 1% chitosan (the positive control). At day eight, 75% *A. vera* gel was the most effective in reducing weight loss followed by 50% while the 0% aloe had the highest weight loss. At day twelve, there was significant difference between the control and 50 and 75% *A. vera* gel treatments. Seventy five percent *A. vera* gel had the lowest weight loss.

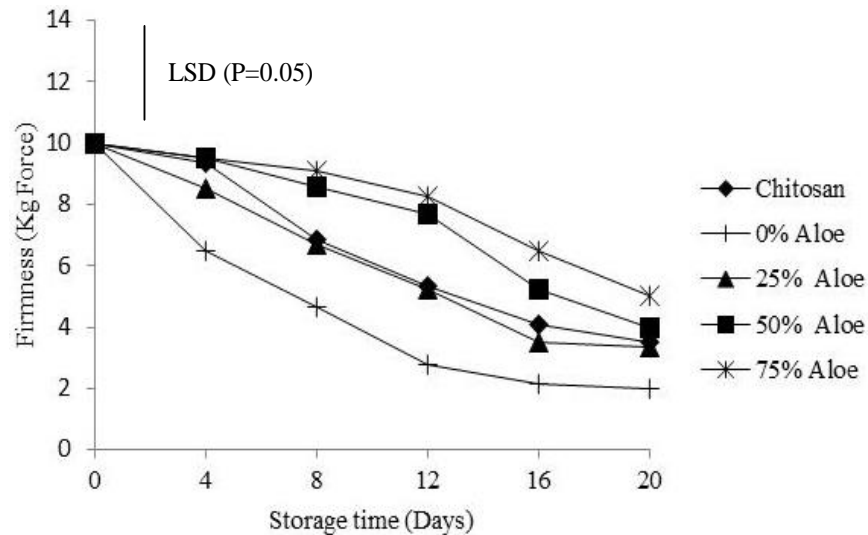
At day sixteen, there were significant effects between the control and 25, 50 and 75% *A. vera* gel treatments. The negative control had the highest weight loss though it

was not significantly different from Chitosan. At day twenty, 0% *A. vera* gel had the highest weight loss while 75% *A. vera* gel had the lowest weight loss among the other treatments. Generally weight loss was lowest in day four, eight and twelve with a sharp increase between day twelve and sixteen. After day sixteen, weight loss was highest only in the 0% *A. vera* treatments.

Fruits treated with 0% *A. vera* gel as a control recorded the highest weight loss compared to those treated with 25, 50, 75% aloe concentrations and those coated with 1% chitosan as a positive control. It was observed that the average weight loss of mango fruits significantly increased with increasing storage period. Fruit weight loss occurs as a result of dehydration and loss of water from fruit surface. *A. vera* gel must have created a semi-permeable barrier to gases and water vapor and therefore reduced water loss and hence reduced weight loss. Similar to the present results, *A. vera* gel reduced weight loss in 'Arctic Snow' nectarines (treated with 2.50%, stored at 20°C (Ahmed et al.(2009), 'StarKing' cherries (treated with 33%, stored at 1°C; Martinez-Romero et al., 2006) and 'Autumn Royal' table grapes (treated with 33%, stored at 2°C; Castillo et al., 2010).

### Fruit firmness

There was a decreasing trend in fruit firmness with storage time in both coated and uncoated mangoes during the course of storage (Figure 2). Initially there was no significant difference ( $P \leq 0.05$ ) between the treatments. At day four of the storage period, 50 and 75% *A. vera* gel coated fruits had the highest firmness value while the lowest values were observed in fruits coated with 0% *A. vera* gel. At day eight, fruits treated with 0% *A. vera* gel had the lowest value of firmness while 75% *A. vera* gel



**Figure 2.** Fruit firmness of Ngowe mango fruits treated with different concentrations of *Aloe vera* coatings and chitosan as a positive control.

had the highest value of firmness. At day twelve, there were significant differences among the treatments, 0% *A. vera* gel had the lowest fruit firmness value while 75% *A. vera* gel had the highest value. At day sixteen, similar observations as to day twelve were made and at the end of the storage period (twenty days), 0% *A. vera* gel had the lowest fruit firmness value while those coated with 75% *A. vera* gel had the highest value.

It was therefore apparent that the reduced loss in firmness in fruits coated with *A. vera* was due to the effect of the coating which delayed the softening. *A. vera* gel must have modified the internal gas composition of mangoes causing reduction of cell wall degrading-enzymes responsible for mango softening (Aguiar et al., 2011). These results demonstrated beneficial effects of the *A. vera* coating on enhancement of the mango shelf life, since it has been postulated that softening and texture changes during mango fruit ripening determine fruit storability and shelf life, as well as reduced incidences on decay and less susceptibility to mechanical damage. The present study demonstrates observations similar to those of Arowora et al. (2013) who worked on oranges coated with *A. vera* gel. It was, also, observed that fruit firmness significantly decreased with increasing storage period. Loss in fruit firmness with the progress of storage period is due mainly to decomposition, enzymatic degradation of insoluble protopectins to simpler soluble pectins, solubilization of cell and cell wall contents as a result of the increase in pectin esterase activity and subsequent development of juiciness and the loss in peel and pulp firmness.

## pH

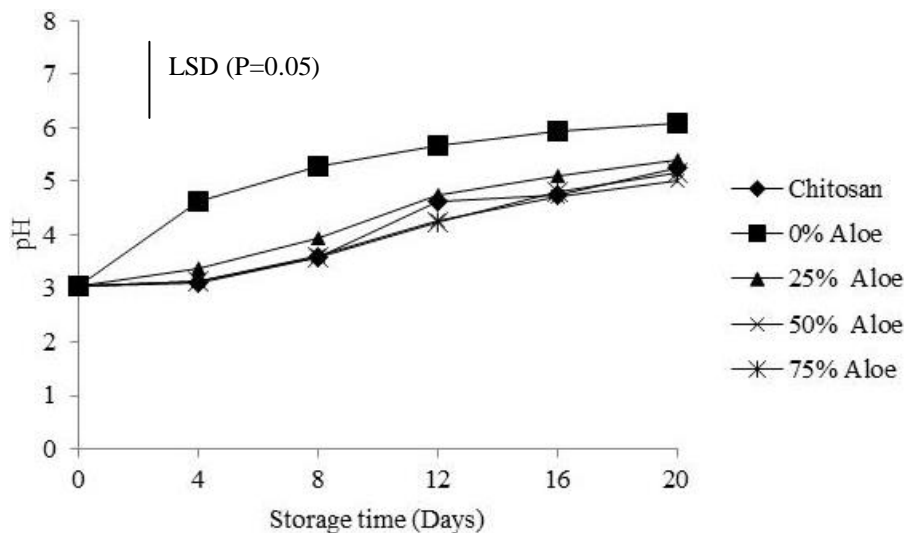
The pH of the mangoes was observed to gradually increase

during the course of storage (Figure 3). Initially there was no significant difference ( $P \leq 0.05$ ) but at day four, there was significant difference between the treatments. Fruits coated with 50 and 75% *A. vera* and chitosan had the lowest juice pH readings while 0% *A. vera* gel had the highest juice pH readings. At day eight, fruits treated with 75% *A. vera* gel had the lowest pH value and 0% *A. vera* gel had the highest pH reading. At day 12, fruits coated with 50 and 75% *A. vera* gel had the lowest pH reading while the control (0% *A. vera* gel) had the highest juice pH value. For day 16, fruits coated with 50% *A. vera* gel had the lowest pH value and 0% *A. vera* gel coated fruits had the highest pH value.

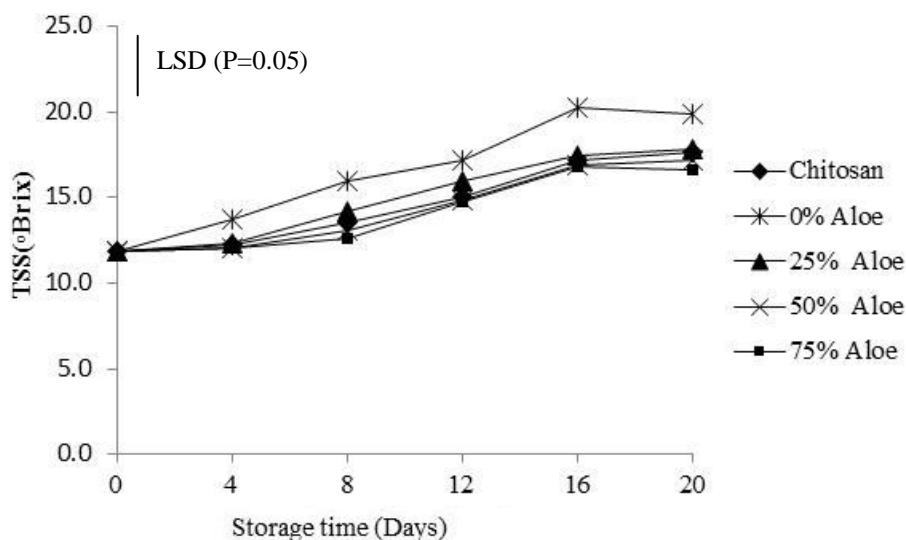
However, in day 16 there was no significant difference between chitosan coated fruits and 50% *A. vera* gel. At day twenty, it was found that 0% *A. vera* gel had the highest pH value while those coated with 50% *A. vera* gel had the lowest pH value. It was found that *A. vera* coated mangoes had lower pH values at the end of storage period with fruits treated with 75% aloe having the lowest readings; this was due to the semi-permeability created by *A. vera* coatings on the surface of the fruit, which modified the internal atmosphere that is endogenous  $O_2$  and  $CO_2$  concentrations in the fruit, thus retarding ripening. Baldwin et al. (1999) reported similar results using Nature Seal (NS) and Tropical Fruit Coating 213 (TFC) coatings on mango ripening during storage. Similar results were reported in carambola fruits (Neeta et al., 2013).

## Total soluble solid (TSS)

Fruits treated with aloe gel and those treated with chitosan had a significantly lower TSS values compared with those treated with 0% aloe concentration (Figure 4).



**Figure 3.** pH of Ngowe mango fruits treated with different concentrations of *Aloe vera* coatings and chitosan as a positive control.



**Figure 4.** TSS of Ngowe mango fruits treated with different concentrations of *Aloe vera* coatings and chitosan as a positive control.

At day zero there was no significant difference ( $P \leq 0.05$ ) between the treatments. In day four, fruits coated with 25, 50 and 75%, *A. vera* gel concentrations and those coated with chitosan had a significantly lower total soluble solids (TSS) compared with those treated with 0% *A. vera* gel. However there were no significant differences among fruits coated with 25, 50 and 75% *A. vera* treatments and chitosan. In day eight, similar observations were as those of day four.

At day 12, the highest total soluble solids were observed on fruits coated with 0% *A. vera* gel and the lowest readings

were recorded for fruits coated with 75% *A. vera*. At day sixteen of the storage period, the highest TSS was observed on fruits coated with 0% *A. vera* gel and the lowest readings were recorded for fruits coated with 75% *A. vera* gel. At day at the end of storage period (twenty days), fruits coated with 50 and 75% *A. vera* gel had the lowest TSS while the control had the highest TSS. *A. vera* gel and chitosan coatings must have modified internal atmosphere resulting in high  $\text{CO}_2$  which retards conversion of starch to sugars and less moisture loss thus reducing ripening and maintaining the TSS.

It was also observed that TSS significantly increased with storage time. This behavior of TSS was likely due to losses in water through respiration and evaporation and hydrolysis of starch during storage and hence increases in TSS (Eman et al., 2013). Ahmed et al. (2009) observed the formation of soluble pectinic acid from insoluble protopectin during senescence of fruit; and they attributed such increase in TSS to the conversion of starch into sugar. Similar results were obtained using *A. vera* gel treatment (2.5%) which suppressed the increase in TSS for 'Artic Snow' nectarines during ripening at 20°C (Ahmed et al., 2009).

## CONCLUSION AND RECOMMENDATION

Findings of this study demonstrate the potential of using *A. vera* gel as a coating for improved postharvest shelf life and maintaining quality of mango fruits hence reduced postharvest losses. The results showed that at both temperatures, 50 and 75% aloe concentrations significantly increased the shelf life evidenced by reduced percentage weight loss. Fruit firmness and total soluble solids concentration and pH were also maintained for longer periods in these treatments. Since *A. vera* is an edible plant, does not pose any environmental hazard and is easily available in Kenya and other tropical regions, *A. vera* at 50% concentration can be used as an alternative fruit coating for mangoes.

## Conflict of Interests

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

## AKNOWLEDGEMENTS

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## Full Length Research Paper

# Seed protein electrophoresis of some members of the family fabaceae

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Seed storage protein profiles of 10 members of the family Fabaceae were assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Total seed storage protein of the studied plants resolved on 10% SDS polyacrylamide gels showed variations in their banding pattern. Results of SDS-PAGE pattern also revealed a band common to all the plant species studied which suggests that this band could be tagged as generic band among members of Fabaceae. The maximum genetic affinity of 0.93 was observed between *Vigna subterranea* and *Arachis hypogaea*, while minimum genetic affinity of 0.32 was observed between *Senna siamea* and *Albizia lebeck* which further reveal wide genetic diversity among the studied plant species. This observation also suggested that *V. subterranea* (Bambara groundnut) and *A. hypogaea* (groundnut) are genetically very close and should be put together taxonomically. Nineteen (19) major bands were recorded and only *S. siamea* had two specific bands which indicate that these two bands could be used to distinguish this species from other legumes considered in this study. It could be concluded that SDS-protein electrophoresis is an important tool for genetic analysis and this protocol has revealed a considerable amount of genetic diversity among the 10 studied plant species for their discrimination.

**Key words:** Fabaceae, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), storage protein, genetic affinity, electrophoresis.

## INTRODUCTION

Legumes are among the three largest families of flowering plants. The group is the third-largest land plant family, after Orchidaceae and Asteraceae, with 730 genera and over 19,400 species comprising herbs, shrubs, trees and climbers. Members of this family include a number of important agricultural crops like *Glycine max* (soybean), *Phaseolus vulgaris* (beans), *Pisum sativum* (pea), *Cicer arietinum* (chickpeas), *Medicago sativa* (alfalfa), *Arachis*

*hypogaea* (groundnut), *Ceratonia siliqua* (carob), and *Glycyrrhiza glabra* (licorice).

Legumes are useful as human and animal food, as wood and soil-improving components of agricultural and agroforestry system. This large family is divided into three subfamilies - the Mimosoideae, Caesalpinoideae and Faboideae. Javid et al. (2004) reported that the diversity observed in protein profiles and seed storage proteins have

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potential for species classification and also serves as marker for interspecific hybridization procedure. Thus, this technique could better be used to establish interspecific diversity and phylogenetic or evolutionary relationships among various species. Suranto (2002) reported that protein electrophoresis has been regarded to be the most useful tool for resolving systematic problems in situations where morphological characters are felt inadequate to make taxonomic decisions.

Increasingly, electrophoresis has become an additional tool to unravel taxonomic and phylogenetic problems (Azeez and Morakinyo, 2004). Most applications of electrophoretic techniques in plant classifications use gel medium supports. This has resulted from the reliability of data produced by gel electrophoresis, which have been accepted widely, particularly in studies of plant population genetics (Mohammed et al., 2006; Sadia et al., 2009, Atoyebi et al. 2014). Omonhinmin and Ogunbodede (2013) opined that because of the high level of inter-specific diversity among members of the Fabaceae family, there is need to review the traditional taxonomic position of the family. Several studies have been conducted on other families like Aspidiaceae and Athyriaceae (Dhir et al., 1975), Aracaceae (Mohammed et al., 2006), Cucurbitaceae (Yadav, 2008) and Solanaceae (Bhat and Kudesia 2011) using SDS-PAGE method but such studies on Fabaceae family is still scanty.

According to Bruneau et al. (2008), sub-family Caesalpinioideae forms a basal grade from which a monophyletic Faboideae and Mimosoideae arise. In the report of Van den Bosch and Stacey (2003), most authors supported the monophyletic origin of members in the Fabaceae family while several recently published floristic accounts still refer to legumes as having a polyphyletic origin leading to their grouping into three sub-families often referred to as sub-families: Caesalpinioideae, Mimosoideae and Faboideae. Bruneau et al. (2008) opined that the family *Fabaceae* is long overdue for an extensive taxonomic re-appraisal. The present study was therefore undertaken to assess the systematic relationships among some selected species of Fabaceae using protein electrophoretic method and possibly assess the mode of evolution of the selected legumes from the three sub-families.

## MATERIALS AND METHODS

### Collection of samples

Seeds of 10 members of the family Fabaceae (Table 1) were collected in May 2013 and stored for six months to get their seed storage protein. Two of the 10 studied plant species were under the sub-family Mimosoideae, three under Caesalpinioideae and five under Faboideae. Proper identification of each plant species used was carried out at the Department of Biological Sciences of Kogi State University, Anyigba.

### SDS gel electrophoresis of seed proteins

Seeds from each sample were dried in an oven before homogenizing

with an extraction buffer containing 0.05 aM Tris-HCl (pH7.4) 4°C. Bromophenol blue was added to the sample buffer as a tracking dye to watch the movement of proteins in the gel. The homogenate was centrifuged at 10,000 r.p.m for 15 min at 4°C and the supernatant was used for electrophoresis. Twelve percent (12%) SDS-gel was used for the run following the Discontinuous Electrophoretic method of Leammli (1970). The sigma® maker used to trace the bands contains 13 proteins ranging from 6,500 to 205,000 kb.

### Data analysis

To avoid ambiguity in data, only consistent protein band between 6,500 and 205,000 kb were considered for data recording. Bands clearly visible in at least one species were scored 1 for present, 0 for absent and entered in binary matrix.

The similarity index proposed by Nei and Li (1979) was used to locate the degree of similarity ( $S_{ab}$ ), between two cultivars a and b according to the formula:

$$S_{ab} = 2N_{ab} / (N_a + N_b)$$

Where,  $N_{ab}$  = number of bands common to both species a and b;  $N_a$  = number of bands in species a;

$N_b$  = number of bands in species b; a dendrogram (hierarchical cluster) was constructed using the unweighted pair group method average (UPGMA). All computations were done using SPSS V21 window software.

## RESULTS

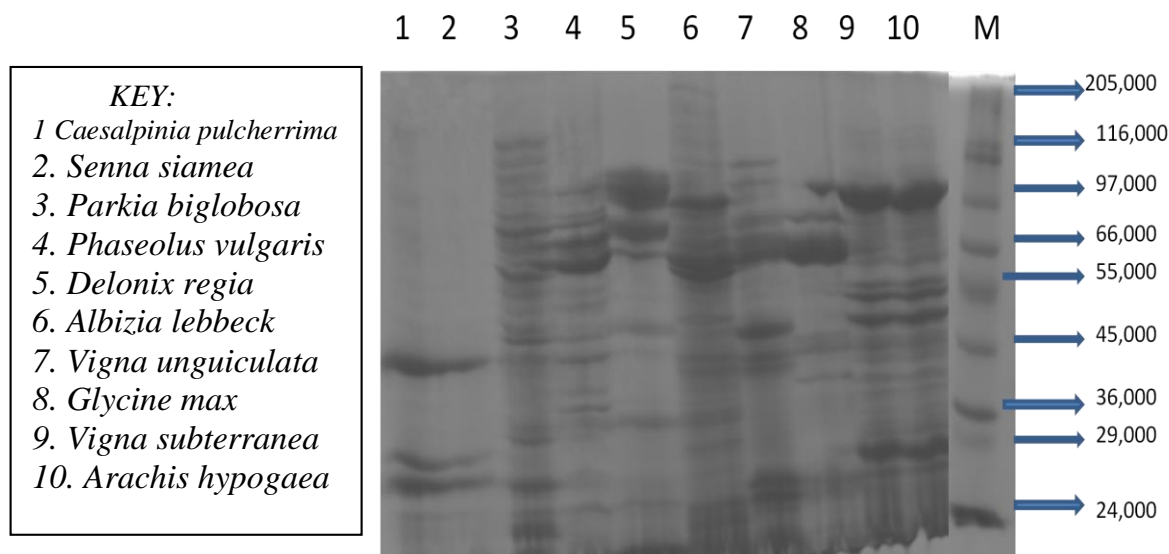
The patterns of protein in the 10 studied members of Fabaceae is shown in Plate 1. A close examination of the bands revealed that the studied plants shows differences in their banding patterns with marked difference in the numbers and intensities of the bands. A total of 19 protein bands were observed among the 10 studied legume species while band number 8 is the only band common to the studied plant species (Figure 1).

The total number of bands and number of unique bands in Table 2 shows that accession number 6 (*A. lebeck*) possessed the highest number of bands (16) while the least number of band (3) was observed in accession number 2 (*Senna siamea*). Table 3 shows that the higher similarity coefficient of 0.93 was observed between accession number 9 (*V. subterranea*) and accession number 10 (*A. hypogaea*) while the least similarity of 0.32 was observed between accession number 2 (*S. siamea*) and accession number 6 (*Albizia lebeck*).

The hierarchical cluster analysis for the 10 studied plant species (Figure 2) grouped the plants into two major clusters. The first cluster comprised of four species while six species occupied the 2<sup>nd</sup> cluster. The four species under the first cluster were accession numbers 3 (*Parkia biglobosa*), 6 (*Albizia lebeck*), 9 (*Vigna subterranean*) and 10 (*Arachis hypogaea*) while the six species under the second cluster were 1 (*C. pulcherrima*), 2 (*S. siamea*), 4 (*P. vulgaris*), 5 (*D. regia*), 7 (*V. unguiculata*) and 8 (*Glycine max*). Accessions 9 (*Vigna subterranean*) and 10 (*Arachis hypogaea*) showed the highest similarity among the studied plant species.

**Table 1.** Description of the 10 species of *Fabaceae* Studied.

Accession number	Common name	Location of collection	Habit of the plant	Scientific name	Sub-family
1	Pride of Barbados	Anyigba	Tree	<i>Caesalpinia pulcherrima</i>	Caesalpinioideae
2	Cassia tree	Anyigba	Tree	<i>Senna siamea</i>	Caesalpinioideae
3	Locust bean tree	Anyigba	Tree	<i>Parkia biglobosa</i>	Mimosoideae
4	Common beans	Anyigba market	Herb	<i>Phaseolus vulgaris</i>	Faboideae
5	Flamboyant plant	Anyigba	Tree	<i>Delonix regia</i>	Caesalpinioideae
6	Siris tree	Anyigba	Tree	<i>Albizia lebbek</i>	Mimosoideae
7	Cowpea	Anyigba market	Herb	<i>Vigna unguiculata</i>	Faboideae
8	Soybeans	Anyigba market	Herb	<i>Glycine max</i>	Faboideae
9	Bambara nut	Anyigba market	Herb	<i>Vigna subterranea</i>	Faboideae
10	Groundnut	Anyigba market	Herb	<i>Arachis hypogaea</i>	Faboideae

**Figure 1.** Electrophoregram showing protein banding patterns for the 10 legume plant species studied.

## DISCUSSION

Protein electrophoresis is a better tool for the identification of genetic diversity and tracing evolutionary processes in plants than morphological markers (Omonhinmin and Ogunbodede, 2013; Natarajan, 2014). Seed protein electrophoresis according to Berber and Yaşar (2011) is increasingly being utilized as an additional approach for species identification and as a useful tool for solving evolutionary problems in plants. Ehsapour et al. (2010) and Sinha et al. (2012) attributed this to the fact that proteins stored in the seeds are highly independent of environmental factors. Alege et al. (2013) and Yatung et al. (2014) opined that the discrepancy between morphological and protein profile is due to the impact of the environment on the former.

The 10 plant species under the Fabaceae family studied revealed that no two plants share the same

protein banding patterns which indicates that genetic diversities exist among the plant species. The presence of a common band (band number 8) among the 10 plant species suggests their close genetic affinity and common ancestry.

This band is coded for by a gene that has become fixed in different species under the Fabaceae family over evolutionary time. This is in agreement with the finding of Azeez and Morakinyo (2004) that the presence of common bands in *Lycopersicon* and *Trichosanthes* species depicts their common evolutionary origin. Also, Akinwusi and Illoh (1995) attributed the appearance of a common band in all individual in a population to the fact that the gene coding for the enzyme or protein does not vary.

*S. siamea* had two unique bands (bands number 18 and 19) which suggest that these two bands can be tagged as species specific bands for the identification of this

**Table 2.** The total number of bands and the unique bands among the 10 studied plants.

Accession number	Plant species	Total number of bands	Bands specific to each species
1	<i>Caesalpinia pulcherrima</i>	7	Nil
2	<i>Senna siamea</i>	3	Nil
3	<i>Parkia biglobosa</i>	15	Nil
4	<i>Phaseolus vulgaris</i>	11	Nil
5	<i>Delonix regia</i>	7	Nil
6	<i>Albizia lebbbeck</i>	16	2
7	<i>Vigna unguiculata</i>	9	Nil
8	<i>Glycine max</i>	6	Nil
9	<i>Vigna subterranea</i>	14	Nil
10	<i>Arachis hypogaea</i>	14	Nil

**Table 3.** Similarity index for the 10 species of Fabaceae studied.

Accession number	1	2	3	4	5	6	7	8	9	10
1	-									
2	0.60	-								
3	0.64	0.33	-							
4	0.56	0.44	0.77	-						
5	0.58	0.40	0.64	0.78	-					
6	0.60	0.32	0.84	0.67	0.52	-				
7	0.50	0.52	0.67	0.80	0.75	0.64	-			
8	0.62	0.44	0.48	0.59	0.62	0.55	0.80	-		
9	0.48	0.35	0.83	0.72	0.48	0.73	0.61	0.50	-	
10	0.57	0.35	0.83	0.72	0.48	0.73	0.61	0.50	0.93	-

1, *Caesalpinia pulcherrima*; 2, *Senna siamea*; 3, *Parkia biglobosa*; 4, *Phaseolus vulgaris*; 5, *Delonix regia*; 6, *Albizia lebbbeck*; 7, *Vigna unguiculata*; 8, *Glycine max*; 9, *Vigna subterranea*; 10, *Arachis hypogaea*.

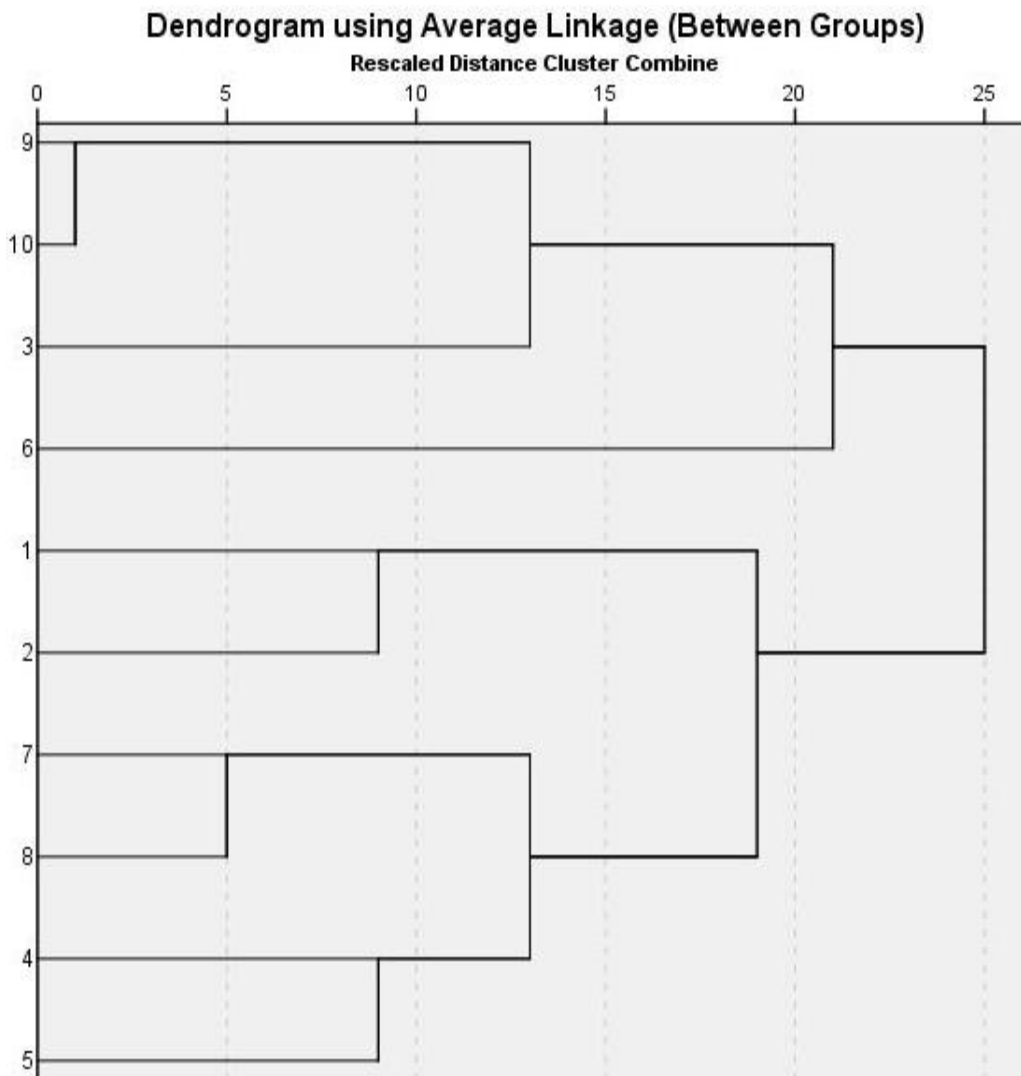
plant. This report is in line with the findings of Mohammed et al. (2006) on members of Aracaceae.

The similarity coefficient range of 0.32 to 0.93 indicates that genetic diversity exists within the family Fabaceae for their systematics. The highest similarity observed between 9 (*Vigna subterranea*) and 10 (*Arachis hypogaea*) is a clear indication that they are phylogenetically related than every other species studied. In contrary, the least similarity observed between 2 (*S. siamea*) and 6 (*A. lebbbeck*) suggests that they evolved along different evolutionary trends. This therefore justified their placement under different sub-families, Mimosoideae and Caesalpinioideae, respectively.

The hierarchical cluster analysis for the 10 studied plant species showed two major clusters. This suggests that the origin of Fabaceae may be along more than one evolutionary line. This is further supported by the fact that all the members of sub-family Mimisoideae (that is, *Parkia biglobosa* and *A. lebbbeck*) considered in this study

clustered together in the first group while the three members of the Caesalpinioideae (*C. pulcherrima*, *S. siamea* and 5 *Delonix regia*) studied remained together in the second group. This is an indication that the Mimosoideae and Caesalpinioideae lines may be the two lines of evolution in the family Fabaceae. The scattering of the members of the sub-family Faboideae between the two clusters supports origin through two evolutionary lines. This observation in a way contradicts the earlier report of Bruneau et al. (2008) that the sub-family Caesalpinioideae forms a basal grade from which a monophyletic Faboideae and Mimosoideae arise. Cluster analysis and similarity matrix revealed very close genetic similarity between the two accessions with underground pods; that is, accessions 9 (*V. subterranea*) and 10 (*A. hypogaea*), Bambara groundnut and groundnut, respectively. This strongly suggests their placement under the same genus taxonomically while the remaining eight members studied should retain their genera.





**Figure 2.** Hierarchical cluster analysis for the 10 legumes studied. 1, *Caesalpinia pulcherrima*; 2, *Senna siamea*; 3, *Parkia biglobosa*; 4, *Phaseolus vulgaris*; 5, *Delonix regia*; 6, *Albizia lebbek*; 7, *Vigna unguiculata*; 8, *Glycine max*; 9, *Vigna subterranea*; 10, *Arachis hypogaea*.

## CONCLUSION AND RECOMMENDATION

The objectives of this study which were to assess the genetic similarities among the 10 selected legumes, re-appraise their taxonomic position and unravel their possible mode of evolution have been achieved. The study suggests evolution of Mimosoideae and Caesalpinioideae along two lines from which members of the Faboideae originated. Also, it was observed that *V. subterranea* (Bambara groundnut) and *A. hypogaea* (groundnut) are genetically very close and should be put under the same genus. It is therefore recommended that other techniques especially molecular markers like random amplified polymorphic DNA (RAPD) should be employed to compliment the findings of this study. Also, a larger number of genera under the Fabaceae should be considered.

## Conflict of Interests

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

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