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African Journal of Biotechnology

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ARTICLES

Full Length Research Paper

Genetic diversity among some productive genotypes of tomato (*Lycopersicon esculentum* Mill.)

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This research was done at Vegetable Experimental Farm, Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology, Jammu during spring and summer seasons, 2007-2009. D^2-statistics revealed that β-carotene contributed maximally towards the genetic divergence followed by ascorbic acid, total soluble solids, alcohol insoluble solids, pericarp thickness, lycopene content and polygalacturonase activity. The 60 genotypes were grouped into 20 clusters. Fourteen (14) clusters were monogenotypic and cluster I possessed highest number of genotypes numbering 25. Out of 20 clusters, cluster VII is promising for minimum polygalacturonase activity and high average fruit weight, cluster VIII had highest number of locules per fruit, fruit yield per plant and yield per hectare and cluster XVII was superior for ascorbic acid. However, cluster XX was found promising for lycopene content, β-carotene and number of fruits per plants. The highest inter cluster D^2 values were estimated between clusters XII and XX, followed by clusters XI and XX, clusters VII and XX, and clusters XV and XX, indicating that there is enough scope for the improvement of tomato crop by hybridization and selection.

Key words: Genetic variability, genetic gain, heritability, tomato.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill) is the world's major vegetable crop and known as protective food because of its special nutritive value. Tomato crop has wider adaptability, high yielding potential and multipurpose uses in fresh as well as processed food industries. An improvement in yield and quality in self pollinated crops like tomato is normally achieved by selecting the genotypes with desirable character combinations existing in nature or by hybridization (Reddy et al., 2013). It is considered as important commercial and dietary vegetable crop. Tomato is the most popular vegetable grown throughout the world with the production of 126.24 million tonnes. According to FAOSTAT (2007), the top producers of tomatoes in 2007 were China with a production of 33.64 million tonnes, followed by USA with 11.5 million tonnes, Turkey with

9.91 million tonnes, India with 8.85 million tonnes and Egypt with 7.55 million tonnes. The annual production of tomato in India during 2007-2008 (NHB, 2008) was 10261 thousand metric tons from 572000 ha of land. The leading tomato producing states are Uttar Pradesh, Karnataka, Maharashtra, Haryana, Punjab and Bihar. In Jammu, the area under tomato is 1824 ha and production is 36650 metric tons with productivity of 20.08 tonnes per hectare (Anonymous, 2008, 2009). Genetic divergence refers to the genetic distance between species or between populations within a species. A variety of parameters are used to measure the genetic distance. Smaller genetic distances indicate a close genetic relationship whereas large genetic distances indicate a more distant genetic relationship. Genetic distance can be used to compare the genetic dis-similarity between different species. Within a species, genetic distance can be used to measure the divergence between different sub-species or different varieties of a species. The importance of genetic diversity is evident in terms of survival and adaptability of a species. For instance, a species with high genetic diversity will tend to produce a wider variety of offspring, where some of them may become the fit variants. In contrast, a species that has little or no genetic diversity will produce offspring that are genetically similar and, therefore, will likely be susceptible to diseases or problems like those of their parent. Hence, little or lack of genetic diversity reduces biological fitness and increases the chances of species extinction (Gadekar et al., 1992). Genetic divergence studies have helped in designing the hybridization programmes in crop plants effectively to generate noble variants having adaptation and yielding potential far better than parental types (Sekhar et al., 2008). In vegetable crops like tomato, estimates of genetic divergence have been proposed to provide diverse parents for getting high yielding hybrids (Sharma et al., 2008). Tomato pulp and juice is digestible mild aperients, a promoter of gastric secretion and blood purifier. It is reported to have antiseptic properties against intestinal infestations. Apart from these, lycopene is valued for its anticancer property. It acts as an antioxidant (scavenger of free radicals), which is often associated with carcinogenesis. An improvement in yield and quality in self-pollinated crops like tomato is normally achieved by selecting the genotypes with desirable character combinations existing in nature or by hybridization (Reddy et al., 2013).

Therefore, the present investigation was aimed at ascertaining the nature and magnitude of genetic diversity among 60 of tomato genotypes for quality and yield attributing traits, to help the breeders in selecting promising and genetically diverse parents for desired improvement.

MATERIALS AND METHODS

The materials used for the present investigation comprised of 60

genotypes of tomato, which were collected from IIVR, Varanasi and nearby Jammu area during 2007-2009. The local germplasm was collected by approaching the farmers of different areas and making sure that the seeds collected for the investigation were the ones which the farmers were growing on their own for several years, in order to maintain the native flavour and taste. The experimental area is located in the sub-tropical zone of Jammu and Kashmir at 32° 40′ N latitude and 74° 58′ E longitude at an elevation of 332 m above mean sea level. The experiment was laid out in randomized block design with three replications with painting distance of 60 x 45 cm, total number of plots of 180 and total number of plants in experimental field of 4860. All the recommended cultural practices were followed during the growth and development period of the crop in order to raise a healthy crop. Observations were recorded on 16 physical and chemical qualities and yield related traits from ten randomly selected plants from each genotype in each plot and replications and their means were worked out for statistical analysis as per formulae given by Panse and Sukhatme (1989). Ten competitive representative plants were selected at random from each experimental plot in each replication and tagged for recording the observations.

Statistical analysis

The diversity of the competitive representative plants was estimated by using D^2 - statistics (Mahalanobis, 1936) between genotypes. The analysis of variance and covariance of 60 lines was carried out for all the characters. Using the common error dispersion matrix, the D^2 between all possible combinations were computed. The lines were grouped into different clusters. Intra and inter-cluster distances were calculated as per the method envisaged by Rao (1952). After recording data analysis of the genetic diversity and Mahalonobis, D^2 analysis was done by using Torcher's method (Figure 1) as suggested by Rao (1952).

RESULTS AND DISCUSSION

Genetic divergence

The pooled results pertaining to the contribution of each character towards the genetic divergence are presented in Table 1. Each character was ranked on the basis of their contribution (percentage) to divergence of that character. The pool depicted in Table 1 shows that the β-carotene contributed maximally (49.49%), followed by ascorbic acid (16.44%), total soluble solids (7.57%), alcohol insoluble solids (7.12%), pericarp thickness (5.82%), lycopene content (4.80%) and polygalacturonase activity (3.73%), whereas, average fruit weight (2.15%), fruit pH (1.64%), number of fruits per plant (0.85%) and fruit yield per plant (0.40%) contributed minimally towards total divergence. However, the number of locules per fruit and yield in quintals per hectare had insignificant contribution towards the total genetic divergence in tomato genotype.

The D^2 values of intra and inter cluster distance for 60 genotypes of tomato (*L. esculentum* Mill.) are presented in Table 2. The D^2 technique measures the forces of differentiation at two levels, namely intracluster and intercluster level and thus helps in the selection of genetically divergent parents for exploitation in
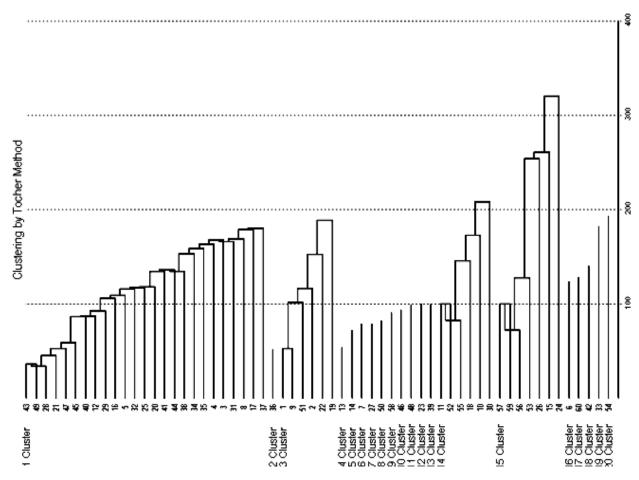
Figure 1. Clustering of 60 genotypes of tomato by Torcher's method.

Table 1. Contribution of characters towards genetic diversion.

Source	Times ranked 1st	Contribution (%)
Lycopene	85	4.80
β-Carotene	876	49.49
Polygalacturonase activity	66	3.73
Ascorbic acid	291	16.44
Fruit pH	29	1.64
Total soluble solids	134	7.57
Alcohol insoluble solids	126	7.12
Pericarp thickness	103	5.82
Number of locules	0	0.00
Number of fruits per plant	15	0.85
Fruit yield per plant	7	0.40
Average fruit weight	38	2.15
Yield in quintals per hectare	0	0.00

hybridization programmes. The intracluster distance shows divergence among the genotypes within a cluster, whereas the intercluster distance expresses relative divergence among the clusters. The genotypes were grouped into 20 clusters. 14 clusters were monogenotypic and cluster I possessed highest number of genotypes,

Table 2. Intra and inter cluster distance of 60 genotypes of tomato.

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
I	**151.44**	205.32	559.97	209.82	276.24	447.68	259.12	229.65	267.5	216.5	257.29	256.71	335.09	901.35	340.61	436.89	559.25	802.95	393.08	1694.03
II		0	290.13	65.48	97.38	78.9	296.96	82.74	136.32	198.13	341.32	490.02	100.04	396.15	386.14	280.34	172.98	454.01	181.97	874.99
III			**181.86**	403.12	502.96	266.54	836.33	248.81	440.51	312.32	845.82	1015.22	488.39	348.96	1014.44	353.88	651.48	382.1	439.28	776.5
IV				0	84.92	154.98	280.66	129.05	237.07	315.82	288.81	492.45	235.86	571.42	354.18	504.44	198.58	634.76	240.1	1179.16
V					0	203.65	171.27	143.45	333.14	443.79	301.42	568.23	200.49	671.95	409.28	606.29	185.56	680.86	288.97	1160.57
VI						0	531.97	169.51	269.93	402.82	597.55	849.38	191.95	273.7	638.67	419.88	128.45	494.34	293.5	589.91
VII							0	325	571.65	552.03	217.05	380.35	381.47	1189.91	399.95	867.55	482.67	1039.08	495.18	1966.96
VIII								0	246.9	215.41	377.22	531.79	248.71	492.56	508.9	334.68	343.92	477.8	240.91	1029.55
IX									0	200.98	437.4	353.25	186.4	555.37	343.92	156.75	375.27	691.98	372.81	1027.62
X										0	554.51	469.82	306.48	609.16	557.28	123.7	709.39	529.93	383.53	1272.25
XI											0	197.51	603.1	1202.27	403.83	849.68	579.5	974.01	447.06	2088.75
XII												0	589.23	1424.73	311.41	656.96	858.08	1257.8	659.98	2403.86
XIII													0	502.87	405.76	274.66	219.64	658.89	346.41	875.41
XIV														**203.29**	1212.03	470.57	523.57	311.59	413.54	356.78
XV															**281.79**	703.87	519.78	1280.35	607.19	1926.49
XVI																0	720.96	536.96	483.02	899.86
XVII																	0	776.8	347.67	804.79
XVIII																		0	196.85	763.5
XIX																			0	1008.49
XX																				0

numbering 25. Clusters III, XIV and XV had six, six and seven genotypes, respectively. The highest inter cluster D^2 value was observed between clusters XII and XX (2403.86), followed by XI and XX (2088.75), VII and XX (1966.96), XV and XX (1926.49), I and cluster XX (1694), X and XX (1272.25), XIV and XV (1212.03), IV and XX (1179.16), V and XX (1160.57), VIII and XX (1029.55), IX and XX (1027.62), III and XII (1015.22), and XIX and XX (1008.49); indicating that genetic material is diverse and there is enough scope for the improvement of tomato crop by hybridization and selection. The lowest inter cluster distance was observed between clusters II

and IV (65.48), II and VI (78.9), II and VIII (82.74), and II and V (97.38). However, the intra cluster distance was found to be maximum within cluster XV (281.79) followed by cluster XIV (203.29), whereas clusters III and I showed lower value (181.86) and 151.44, respectively).

Cluster means for yield and quality contributing characters

Sixty (60) genotypes were placed in 20 clusters. The means of the clusters for yield and quality characters are shown in Table 3. For lycopene,

top ranking clusters are XX (4.52), III (3.98 mg) and VI (3.9 mg).

Clusters XX, XIV and XVIII are rich in β-carotene with the value of 2.55, 2.36 and 2.33 mg; whereas, the cluster number VII (38.66), III (38.99) and VIII (41.05) exhibited minimum polygalacturonase activity. However, clusters number XVII (37.8 mg), VII (32.72 mg) and VI (33.41 mg) had highest values for ascorbic acid content while the clusters with greater acidity are clusters number XVIII (3.41), XI (3.42) and XIX (3.56). For total soluble solids, the highest values were observed in clusters number V (5.03), VII (4.92) and IV (4.82). Clusters XIII, XVI and XX

Table 3. Cluster means for yield and quality contributing characters of 60 genotypes of tomato.

Cluster	lycopene	β-Carotene	Polygalacturonase activity	Ascorbic acid	Fruit pH	Total soluble solids (%)	AIS	Pericarp thickness (mm)	Number of locules	Number of fruits/plant	Fruit yield/plant (g)	Average fruit weight (g)	Yield (q/ha)
I	2.87	1.41	45.63	25.01	4.1	4.47	31.38	4.37	2.73	14.41	898.68	62.53	323.44
II	3.33	1.68	52.66	29.37	3.96	4.53	32.49	3.85	2.67	14.28	812.77	56.64	292.48
III	3.98	2.09	38.99	26.16	4.22	4.42	30.34	4.61	2.67	16.97	821.36	52.62	296.06
IV	3.22	1.6	52.52	28.92	4.04	4.82	25.32	3.85	2	14.39	677.47	47.33	243.77
V	2.98	1.5	49.61	32.05	4.06	5.03	32.18	4.17	2.5	20.72	822.29	39.58	295.97
VI	3.9	1.89	54.42	33.41	4.1	4.5	29.12	3.8	3.17	13.33	771.99	57.52	277.84
VII	2.44	1.2	38.66	32.72	4.23	4.92	35.35	4.82	2.83	12.78	968.72	75.55	348.66
VIII	3.32	1.72	41.05	28.78	4.11	4.6	30.17	4.4	3.33	24.78	1459.83	59.1	556.75
IX	3.3	1.63	58.86	26.44	4.05	3.85	31.73	3.38	2.67	18.16	593.87	32.48	213.66
X	3.43	1.68	44.27	20.04	4.03	4.32	35.38	4.07	3.33	12.97	866	65.79	311.66
XI	2.35	1.22	43.16	29.98	3.42	4.25	27.57	4.92	2.5	11.9	520.52	43.84	187.3
XII	2.05	1.16	45.09	26.01	4.23	3.67	30.49	4.42	2.33	12.05	623.72	48.62	224.48
XIII	3.15	1.66	60.18	30.42	4.59	4.48	39.13	3.33	3	12.82	725.12	56.54	260.97
XIV	3.73	2.36	59.31	29.16	3.98	4.32	31.77	3.54	2.47	14.5	771.54	54.35	279.94
XV	2.48	1.24	59.09	28.74	4.2	4.31	29.4	3.32	2.67	17.93	895.23	54.82	322.17
XVI	3.55	1.9	55.82	21.53	4.33	3.85	38.92	3.68	2.83	19.55	1120.29	57.09	403.26
XVII	3.22	1.7	63.7	37.8	3.97	4.6	28.95	3.07	2.5	14.44	671.88	50.25	260.79
XVIII	2.51	2.33	46.64	24.62	3.41	4.45	35.41	4.37	2.83	11.51	589.92	51.3	212.27
XIX	2.07	1.94	52.39	27.86	3.56	4.5	30.05	3.88	3.17	13.91	789.29	56.64	284.08
XX	4.52	2.55	64.55	34.52	3.79	4.35	38.05	2.58	2.5	24.92	875.29	35.39	315.05

had the maximum value for alcohol insoluble solids with the values of 39.13, 38.92 and 38.05 mg/100 g, respectively. The pericarp thickness was found to be highest in clusters V (4.92), XI (4.82) and VII (4.61). However, clusters VIII and X had same number of locules (3.33) followed by cluster XIX (3.17). Number of fruits per plant was observed to be maximum in clusters XX (24.92), VIII (24.78) and V (20.72). It was observed that clusters I, VII, VIII and XVI had highest values of 898.68, 968.72, 1459.83 and 1120.29 g, respectively, for fruit yield per plant. Average fruit weight was observed to be maximum in cluster

number VII (75.55 g) followed by cluster number X (65.79 g) and I (62.53 g). The top ranking clusters for yield in quintals per hectare were clusters VIII (556.75q/ha), XVI (403.26), VII (348.66 q/ha) and I (323.44 q/ha). The clusters II, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII, XVI, XVII, XIX and XX had single genotype each, namely, EC-29914, EC 27995, EC-521041, JTP- 02-05, EC- 5888, Improved Shalimar and CGNT-12 CO-2.

Composition of cluster based on D^2 values

Composition of cluster based on D^2 values (Figure

1) indicated a lot of genetic diversity among the sixty (60) genotypes of tomato. Genetic diversity among the sixty (60) genotypes of tomato was worked as per the procedure given by Mahalanobis (1936) and presented in Table 4 and Figure 1. The results indicate that all the sixty (60) genotypes were grouped into 20 clusters. Cluster I had 25 genotypes namely, EC-381213, EC-2517, PAU-2371, CO-3, EC-52077, KS-229, VR-415, EC-521044, PAU-2372, EC-3526, EC-521056, EC-2798, EC-521079, Pant T-8, Pant T-10, KS-227, VTG-85, EC-528374, NDT-9, Pant T-7, EC-538151, EC-529081, EC-521086, EC-9046 and Local-2707, followed by cluster XV with seven genotypes,

Table 4. Composition of cluster based D^2 values of 60 genotypes of tomato.

Cluster	Number of genotypes	Genotypes
I	25	EC-381213, EC-2517, PAU-2371, CO-3, EC-52077, KS-229, VR-415, EC-521044, PAU-2372, EC-3526, EC-521056, EC-2798, EC-521079, Pant T-8, Pant T-10, KS-227, VTG-85, EC-528374, NDT-9, Pant T-7, EC-538151, EC-529081, EC-521086, EC-9046, Local-2707.
II	1	EC-29914.
III	6	EC-164660, EC-521067, CGNT-1, EC-363942, EC-521045, Punjab Chhuhara.
IV	1	EC-27995.
V	1	EC-521041.
VI	1	JTP-02-05.
VII	1	EC-5888.
VIII	1	Improved Shalimar
IX	1	CGNT-12
X	1	CO-2
XI	1	EC-135580
XII	1	CTS-02
XIII	1	VTG-86
XIV	6	EC-521059, CGNT-2, CGNT-6, DT-2, EC-251581, EC-3668,
XV	7	CGNT-11, CGNT-13, CGNT-10, CGNT-3, EC-35293, EC-538151/3, CTS-06-19
XVI	1	EC-520059.
XVII	1	CGNT-14.
XVIII	1	EC-521054.
XIX	1	PAU-1374
XX	1	CGNT-5

CGNT-11, CGNT-13, CGNT-10, CGNT-3, EC-35293, EC-538151/3 and CTS-06-19; clusters III and XIV with six genotypes in each cluster: EC-164660, EC-521067, CGNT-1, EC-363942, EC-521045, Punjab Chhuhara and EC-521059, CGNT-2, CGNT-6, DT-2, EC-251581, EC-3668. However, the clusters II, IV, V, VI, VII, VIII IX, X, XI, XII, XIII, XVI, XVII, XIX and XX had single genotype each namely, EC-29914, EC 27995, EC-521041, JTP- 02-05, EC- 5888, Improved Shalimar, CGNT-12, CO-2, EC- 135580, CTS-02, VTG-86, EC-520059, CGNT-14, EC-521054, PAU-1374 and CGNT-5.

In the present investigation, the results pertaining to the contribution of each character towards the genetic divergence revealed that β-carotene contributed maximally, followed by ascorbic acid, total soluble solids, alcohol insoluble solids, pericarp thickness, lycopene content and polygalacturonase activity, whereas average fruit weight, fruit pH, number of fruits per plant and fruit yield per plant contributed minimally towards total divergence.

The D^2 values of intra and inter cluster distance for 60 genotypes of tomato are reported. The highest inter cluster D^2 values were estimated to be between clusters XII and XX, followed by XI and XX, VII and XX, XV and XX, I and XX, X and XX, XIV and XV, IV and XX, V and XX, VIII and XX, IX and XX, III XII, and IX and I, indicating that there is enough scope for the improvement of tomato crop by hybridization and selection. However,

the lowest inter cluster distance was observed between cluster II and IV followed by clusters II and VI, II and VIII, and II and V. However, the intra cluster distance was found to be maximum within cluster XV, followed by cluster XIV, whereas clusters III and XX reported lower values, respectively. It can be concluded that if we are interested in improving lycopene content, hybridization between clusters VIII and XX is a better option. To reduce polygalacturonase activity, cluster VII and VIII is ideal. It indicates that genotype in cluster VII is better suited for long transportation. To improve ascorbic acid content, cluster XVII and VII is an ideal combination. Improving maximum yield hybridization between clusters VII and VIII is better as it also possessed important genotypes having special features which could be better exploited by double cross or their derivatives for future selection. These findings are in close conformity with those of Kumar and Tewari (1999), Parthasarathy and Aswath (2002) and Sekhar et al. (2008).

Means of yield and quality contributing characters of different clusters reveal considerable variation for important characters such as yield, fruit weight, fruit colour and lycopene content. In the present investigation, cluster means were worked out from the pooled data on the basis of mean performance of genotypes for different traits studied. The pooled data depicted in Table 3 revealed that cluster 5 was observed to be promising for total soluble solids. Whereas, cluster VII is promising for

minimum polygalacturonase activity and high average fruit weight, cluster VIII had highest number of locules, cluster VIII was also found promising for fruit yield per plant and yield quintals per hectare, cluster XI was found superior for pericarp thickness, cluster XIII is promising for fruit pH and alcohol insoluble solids, cluster XVII was superior for ascorbic acid. However, cluster XX was found promising for lycopene content, β-carotene and number of fruits per plants. Similarly, Parthasarathy and Aswath (2002) also recorded same trend for fruit weight and yield.

Composition of cluster based on D^2 values indicated a lot of genetic diversity among the sixty (60) genotypes of tomato. It was worked out as per the procedure given by Mahalanobis (1936) and presented in Table 4. The results indicate that all the sixty (60) genotypes were grouped into 20 clusters. Cluster I contains highest number of genotypes, that is, 25 genotypes, namely, EC-381213, EC-2517, PAU-2371, CO-3, EC-52077, KS-229, VR-415, EC-521044, PAU-2372, EC-3526, EC-521056, EC-2798, EC-521079, Pant T-8, Pant T-10, KS-227, VTG-85, EC-528374, NDT-9, Pant T-7, EC-538151, EC-529081, EC-521086, EC-9046 and Local-2707, followed by cluster XV with seven genotypes: CGNT-11, CGNT-13, CGNT-10, CGNT-3, EC-35293, EC-538151/3, CTS-06-19, clusters III and XIV with six genotypes in each cluster: EC-164660, EC-521067, CGNT-1, EC-363942, EC-521045, Punjab Chhuhara and EC-521059, CGNT-2, CGNT-6, DT-2, EC-251581, EC-3668. However the clusters II, IV, V, VI, VII, VIII IX, X, cluster XI, XII, XIII, XVI, XVII, XIX and XX had single genotype each namely, EC-29914, EC 27995, EC-521041, JTP- 02-05, EC- 5888, improved Shalimar, CGNT-12, CO-2, EC- 135580, CTS-02, VTG-86, EC-520059, CGNT-14, EC-521054, PAU-1374 and CGNT-5. Even though most of the varieties were developed in India, there was good diversity because of diverse parents used in the development of these varieties or some were introduction from other countries which could have contributed to diversity present in these genotypes. The grouping of genotypes into 20 clusters indicated the presence of wide range of genetic diversity among the genotypes. These findings are in close conformity with those of Parthasarathy and Aswath (2002), Arun and Kohli (2003) and Sharma et al. (2009).

For D^2 analysis, tomato genotypes were grouped into 20 clusters. Considerable inter and intra cluster distance were observed between and within the clusters. The highest inter cluster D^2 values were estimated to be between clusters XII and XX, followed by XI and XX, VII and XX, XV and XX, I and XX, X and XX, XIV and XV, IV and XX, V and XX, VIII and XX, IX and XX, III XII, and IX and I, indicating that there is enough scope for the improvement of tomato crop by hybridization and selection. Contribution of each character towards the genetic divergence was maximum for β-carotene followed by ascorbic acid, total soluble solids, alcohol insoluble solids, pericarp thickness, lycopene content and polygalacturonase activity. An improvement in yield and

quality in self pollinated crops like tomato is normally achieved by selecting the genotypes with desirable character combinations existing in nature or by hybridization. The success of hybridization programme depends upon selection of suitable parents of diverse origin. Thus, the results of the present study could have strong implications for breeding programs for development of tomato variety as a commercially important crop and would be helpful for future programs regarding tomato varieties genetic improvements, building a genetic map for the local tomato varieties. These findings were in general agreement with the earlier reports of Basavaraj et al. (2010), Evgenidis et al. (2011) and Thamir et al. (2014).

Conclusion

This study reveals that cluster V was promising for total soluble solids whereas, cluster VII was promising for minimum polygalacturonase activity and high average fruit weight, cluster VIII had highest number of locules per fruit, cluster VIII was also found promising for fruit yield per plant and yield per hectare, cluster XI was found superior for pericarp thickness, cluster XIII was promising for fruit pH and alcohol insoluble solids, cluster XVII was superior for ascorbic acid, However, cluster XX was found promising for lycopene content, β-carotene and number of fruits per plants. Highly diverse clusters were XII and XX, and XI and XX. Genotypes in these clusters are proposed for hybridization to get heterotic hybrids in F_1 generation and some promising transgressive segregants in F_2 generation.

Conflict of interests

The authors did not declare any conflict of interest

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Full Length Research Paper

Production of friable embryogenic callus and regeneration of Ugandan farmer-preferred cassava genotypes

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Generation of embryogenic callus is a key step in genetic engineering of many crop species, including cassava. Protocols for generation of friable embryogenic callus (FEC) have been lacking for Ugandan cassava genotypes, thereby delaying their genetic engineering for agronomic and other desirable traits. The objective of this study was to determine conditions suitable for production and regeneration of FEC in the Ugandan cassava genotypes; Aladu, Bukalasa and Ebwanateraka, and control cultivar 60444. Immature leaf lobe explants were established on Murashige and Skoog (MS) based media for initiation of organized embryogenic callus (OES). To produce FEC, resulting OES were established on Gresshoff and Doy based callus induction media with varying levels of sucrose, maltose, tyrosine, tryptophan, naphthalene acetic acid (NAA) under light and dark conditions. Subsequently, FEC was subcultured to MS-based embryo maturation and embryo regeneration media. All genotypes produced OES. All genotypes produced FEC except Bukalasa. The amino acid tyrosine favoured production of FEC in Aladu and Ebwanatereka, but not in 60444, while 20 g/L of sucrose trigged production of FEC in Aladu and 60444, but 40 g/L of sucrose was superior for Ebwanatereka. Media supplemented with 1 ml/L naphthalene acetic acid NAA facilitated embryo regeneration in Ebwanatereka and 60444, while Aladu responded better to 5 ml/L NAA. Light, tyrosine and sucrose were essential for FEC production in Uganda cultivars while NAA was required for regeneration of somatic embryos. Ability to produce FEC in these genotypes lays a foundation for their improvement through genetic transformation for the desired and agronomic traits.

Key words: Cassava (*Manihot esculenta* Crantz), somatic embryogenesis, amino acids, carbon sources.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) of the family Euphorbiaceace, is the second most important staple food crop grown by low income African farmers (FAOSTAT, 2011). In Uganda, annual production of cassava is approximately 4.9 million metric tonnes (MT) (FAOSTAT, 2012), with about 80% of the population depending on the crop as a source of starch and dietary energy (ASARECA, 2002). In addition, the crop is sold locally by small scale farmers to generate income (Otim-Nape et al., 2001) and used for animal feed, production of beverages and various industrial applications such in the manufacture of textiles (Tonukari, 2004).

The area under cassava cultivation in Uganda has increased from an estimated 379,000 ha in 2006 to 426,000 in 2012. However, average yields have dropped from approximately 123,000 Hg/ha to 115,500 Hg/ha over the same period (FAOSTAT, 2012). This decline is in part due to the impact of Cassava brown streak disease (CBSD). CBSD is caused by two Ipomovirus; Cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBVS) (Alicai et al., 2007; Mbanzibwa et al., 2009; Patil et al., 2010) and is now considered to be one of the most important threats to food security in the tropics (Patil et al., 2014). Sources of effective resistance to CBSD within known farmer preferred varieties are limited. In addition, the heterozygous nature and long breeding cycle of the crop, present a challenge to development and delivery of CBSD resistance materials to farmers via conventional breeding systems (Kawano, 2000; Liu et al., 2011).

Recently, transgenic approaches to control CBSD by RNAi technology have been demonstrated under controlled growth conditions (Yadav et al., 2011; Van der et al., 1992) and within confined field trials (Ogwok et al., 2012). In order to exploit these advances for develop-ment of virus resistant Ugandan planting materials, capacity for transgenic modification must be expanded into a wider range of genetic backgrounds. Such ability would also allow application of biotechnology for other traits such as enhanced nutrition value (Sayre et al., 2011) modified starch quality, improved post-harvest shelf life, as well as resistance to pests and diseases (Taylor et al., 2004; Nutti, 2004; Zhao et al., 2011).

Recovery of genetically modified cassava is based on production of totipotent embryogenic tissues that act as the target for transgene integration via Agrobacterium or direct gene transfer technologies (Taylor et al., 2004; Liu et al., 2011). Lack of efficient regeneration systems for cassava has been a limiting factor for application of cassava biotechnology to improve Ugandan cassava genotypes.

Somatic embryogenesis was originally reported for regeneration of cassava plants via zygotic cotyledons (Stamp and Henshaw, 1987), and then from immature leaves, repetitive cycling of somatic embryos (Szabados et al., 1987) and via friable embryogenic callus (FEC) (Taylor et al., 1996, 2001, 2012). The direct use of somatic embryos for recovery of transgenic plants has not been encouraging, because these structures are multi-cellular encouraging, because these structures are

multi-cellular and highly organized, leading to increased chances of recovering chimeras when targeted for transgene integration (Raemaker et al., 1997; Quiroz-Figueroa et al., 2006). Conversely FEC produced from organized embryogenic structures (OES) is highly disorganized and regenerates via a single cell origin (Taylor et al., 2004, 2012). As a result, FEC has proven to be an efficient target tissue for transgene integration (Bull et al., 2009; Taylor et al., 2012). Until recently, most reports were restricted to recovery of transgenic plants in the West African cassava model cultivar 60444. However this capacity has now been expanded into a range of West Africa farmer-preferred germplasm (Sayre et al., 2011; Zainuddin et al., 2012; Nyaboga et al., 2013). As an initial step towards establishing capacity to apply biotechnology to the improvement of Ugandan cassava, we report here, factors affecting induction of somatic embryogenesis, production of FEC and plant regeneration in three Ugandan farmer preferred cassava genotypes. Cultivars like Aladu, Ebwanatereka and Bukalasa were selected for this study representing popular cultivars from major cassava growing regions of Uganda. Ebwanatereka is commonly grown in Soroti, Kaberamaido, Busia and Iganga, districts of the Eastern and North Eastern region while Aladu is grown in Lira and Apac, districts of the Northern region and Bukalasa grown in Masindi, Kibale and Mityana, districts of Western and Central region.

These cultivars have other good attributes that include being sweet, mealy, soft, high dry matter content, produces good cassava flour, good for waragi, ready market for fresh roots, high yielding and stores long in soil (Aladu and Bukalasa) and early maturing (Ebwanatereka). All these cultivars are susceptible to cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) (Orone, Cassava Breeder, NaCRRI, Uganda, personal communication).

MATERIALS AND METHODS

Four genotypes were used in this study; the three Ugandan farmer preferred genotypes Ebwanatereka, Bukalasa and Aladu, and the West African model cultivar 60444. Stem cuttings of the Ugandan genotypes were transported from the National Crops Resources Research Institute (NaCRRI) to the Donald Danforth Plant Science Center (DDPSC), St Louis, MO, USA. Fifty stakes of each genotype were planted in 12 cm pots containing Farad 51, transferred to growth chamber and maintained at 28±2°C for 16 h in light and 8 h dark at 2100 umolm^{-2}s^{-1} for two years.

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Abbreviations: OES, Organised embryogenic structures; **FEC,** friable embryogenic callus; **MS,** Murashige and Skoog Basal media; **GD,** Gresshoff and Doy basal media; **NAA,** naphthalene acetic acid; **BAP,** benzylaminopurine; **CBSD,** cassava brown streak disease; **CBSV,** cassava brown streak virus; **UCBVS,** Ugandan cassava brown streak virus; **UFPCG,** Ugandan farmer preferred cassava genotypes; **GD250P,** Gresshoff and Doy basal medium supplemented with the auxin picloram; **MS2 50P,** Murashige and Skoog basal medium supplemented with the auxin picloram.

Preparation of stock solutions and basal media

Murashige and Skoog (Murashige and Skoog (1962) and Gresshoff and Doy (Gresshoff and Doy (1972) basal media were prepared as described by Taylor et al. (2012). Fifty (50) ml of 10 mM stocks of either tyrosine or tryptophan were added to 1 litre of GD basal media supplemented with sucrose or maltose at 20 g/L or 40 g/L with 50 µM picloram (GD2 50P) after autoclaving. The explants were established and maintained at 28 ± 2°C for 16 h in light and 8 h dark at 2100 umolm^{-2}s^{-1} for 42 days

Initiation of nodal cuttings from the screen house into tissue culture

New stem growth each carrying approximately 10 nodes was removed from soil grown plants. Individual nodes were excised using a single edged razor and placed in 250 ml conical flasks. Approximately 20 individual nodes were added to 100 ml of water containing 15% v/v sodium hypochlorite plus two drops of Tween-20 in a 500 ml conical flask and agitated at 150 rpm on an orbital shaker for 30 min. Individual nodes were then rinsed five times with sterile distilled water and necrotic tissues carefully sliced off using a scalpel in a laminar flow hood. Four individual nodal cuttings were placed on Nunc 25 x 100 mm Petri dish containing MS basal media supplemented with 20 g/L sucrose (MS2) and solidified with 2.3 g/L of phytagel. Approximately 100 nodal cuttings of each cultivar were transferred to the growth-room and maintained for approximately three weeks. Shoots developing from the nodal cuttings were excised above the original nodal tissue and established on MS2 basal media solidified with 8 g/L noble agar at seven shoots per Petri dish.

Induction of embryogenic tissues

Leaf-lobe explants 2 to 6 mm in length were excised from *in vitro* mother plants using a hypodermic needle and placed on MS2 medium supplemented with 50 µM picloram (MS2 50P) in the manner described by Taylor et al. (1996, 2012). After 28 days, organized embryogenic structures (OES) developing from the adaxial surface were excised and sub-cultured onto fresh MS2 50P. The resulting proliferating clumps of OES were then repeatedly sub-cultured onto fresh MS2 50P every four weeks for a total of 12 weeks. Production of OES was assessed as the percentage of leaf explants seen to produce these structures and as the amount of OES produced per responding explant. A scale of 1-5 described an area of the explant surface covered by the embryogenic structures where; 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80%, and 5 = 81-100%. The data obtained was transformed with ARCSINH function using Microsoft Excel 2010 package. Mean values of surface area coverage of OES on leaf lobes and frequencies of OES produced were subjected to analysis of variance (ANOVA) using statistical package Genstat Release 14.1 (2011) at 5% significance level.

OES was used as starting material for FEC production. OES was excised away from associated non-embryogenic callus with the use of a hypodermic needle (Taylor et al., 2012), collected and crushed through a 1 mm^2 pore-sized steel wire mesh using a spatula. One ml of Gresshoff and Doy (GD) liquid medium supplemented with 50 µM picloram was applied over the crushed OES to allow easier manipulation. Nine OES fragments, each 1 mm^2 in size were placed on 15 x 100 cm Petri dish containing GD2 50P medium modified by addition of either an amino acid tyrosine or tryptophan at 500 µM. The sugar type and concentration of sucrose or maltose at 20 or 40 g/L were used to assess production of FEC, from 1 mm^2 OES pieces. Five Petri dishes per treatment were set up each containing nine 1 mm^2 OES fragments. OES pieces were cultured under light

in growth room conditions as described above.

Experiments were conducted three times and recorded for production of non-embryogenic callus (NEC), OES and FEC produced at 28, 35, and 42 days after initiation. In order to quantify FEC production, the FEC was spread to form a monolayer on the agar surface and a grid scale placed under the Petri dish to determine the surface area. Mean values for production of NEC, OES and FEC produced were subjected to ANOVA using statistical package Genstat Release 7.

Regeneration of cotyledon stage embryos into plantlets

To determine its regenerative ability, FEC was transferred as 0.4 x 0.4 cm sized colonies. Five colonies were placed each in 100 × 25 mm Petri dish containing MS2 media supplemented with 5 ml/L naphthalene acetic acid (NAA) with 20 g/L sucrose. Five petri dishes with five colonies each were set up for the cultivar Aladu, Ebwanatereka and 60444. Twenty five (25) colonies per treatment per genotype were established for each experiment. The same was done for the two NAA levels (1 and 0.1 ml/L) with sucrose for the three cultivars. Similar treatments were established for the three cultivars with the three levels of NAA at 20 g/L of maltose. The experiment was repeated three times. Approximately three weeks later developing cotyledon-stage embryos were transferred as individuals to MS basal medium supplemented with 2 ml/L benzylaminopurine (BAP), at six cotyledon-stage embryos per 100 × 25 mm Petri dish (Taylor et al., 2012). Eight weeks later, regenerated plants with well-developed root systems were selected and transferred to MS2 basal media. The number of the embryos produced per cultivar, types of embryos (one cotyledon, two cotyledon and trumpet-shaped embryos) and percentage of embryos regenerating to produce plants were recorded. Mean values for all parameters recorded above were subjected to ANOVA using statistical package Genstat Release 14.1 (2011) at 5% significance level.

Acclimatization of the regenerated plants from *in vitro* to the screen house

A total of 50 plants per cultivar (Aladu, Ebwanatereka and 60444) were transferred to the screen house for acclimatization. The plants were cleaned with distilled water to remove any agar on the roots. To vermiculite, 0.5 g/L of Jacks professional fertilizer containing nitrogen, phosphorus and potassium in the ratios of 9: 45: 15 respectively as well as fungicide called Ridomil (2.5 g/L) were added. The cleaned plants for each genotype were transferred to vermiculite. A total of 150 plants were established in the screen house. A month later, the surviving plants were moved to a composition of vermiculite and soil in the ratio of 2:1 respectively. The number of surviving weaned plants was counted.

RESULTS

Production of embryogenic tissues

In the present study, the capability of three Ugandan cassava genotypes to produce organized embryogenic callus (OES) in the presence of the 50 uM picloram was demonstrated using leaf lobes (Figure 3A). Mean percentages of OES (Figure 3B) production in this study for Aladu was 40.1%, Bukalasa 44.0% and Ebwanatereka 37.9%, compared with control genotype

Table 1. Frequency and mean production of Organised embryogenic structures (OES) from leaf lobes of different cassava cultivars on MS medium supplemented with 50µM Picloram.

Cultivar	Frequency of OES (%)	Mean production of OES (%)
60444	76.84 ± 2.11	36. 42 ± 3.91
Aladu	78.95 ± 1.95	40.1 ± 3.79
Bukalasa	88.95 ± 1.47	44.0 ± 3.38
Ebwanatereka	84.74 ± 1.37	37.0 ± 2.69

Percentages of OES produced from 190 explants. OES production frequencies were recorded by calculating the ratio of OES clusters/cultured explants. Mean OES production were recorded by calculating the amounts of OES produced by each explant/the number of explants. Data represents means ± SD of three independent experiments.
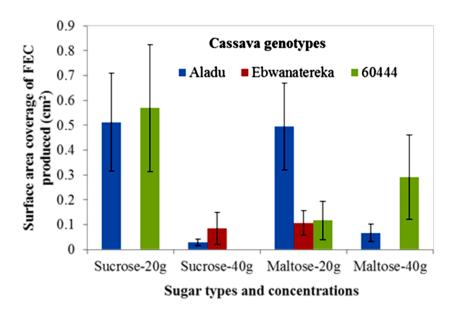
Figure 1. Mean production of friable embryogenic callus (FEC) from cassava genotypes on Gresshoff and Doy (GD) basal medium supplemented with two sugar types at different concentrations

60444 in which had an average of 36.4% (Table 1) produced embryogenic structures under the same conditions. However using the frequencies, the production of OES from the genotypes 60444 was 77%, Aladu 79%, Bukalasa 89% and Ebwanatereka 85% (Table 1). The OES produced was used for production of friable embryogenic callus (FEC) (Figure 3C) as demonstrated in the subsequent experiments using carbon sources and amino acids.

Effect of sugar types and concentration on FEC production

The genotypes responded to production of FEC using the both sucrose and maltose at concentrations of 20 or 40 g/L. Response to these culture conditions varied between cultivars. Significant differences were observed with respect to mean amounts of FEC produced in the presence of sugar types and concentrations for Aladu ($P = 0.019$), while no significant differences were observed for Ebwanatereka ($P = 0.168$) and control 60444 ($P = 0.088$) (Figure 1i). After 42 days, FEC production was greatest in Aladu when OES was cultured on 20 g/L of sucrose ($0.51 ± 0.19$ mm^2) or 20 g/L of maltose ($0.49 ± 0.17$ mm^2) and significantly greater than either sugar type at 40 g/L of maltose ($0.07 ± 0.03$ mm^2) (Figure 1). Conversely, FEC production from Ebwanatereka was highest at 20 g/L of maltose ($0.12 ± 0.05$ mm^2) followed by 40 g/L of sucrose ($0.08 ± 0.06$ mm^2) and no FEC was produced at 20 g/L of sucrose and 40 g/L of maltose (Figure 1). In the case of 60444, highest amounts of FEC were produced on medium containing 20 g/L of sucrose ($0.57 ± 0.26$ mm^2) followed by 40 g/L of maltose ($0.29 ± 0.17$ and lowest at 20 g/L of maltose ($0.12 ± 0.08$ mm^2) (Figure 1).
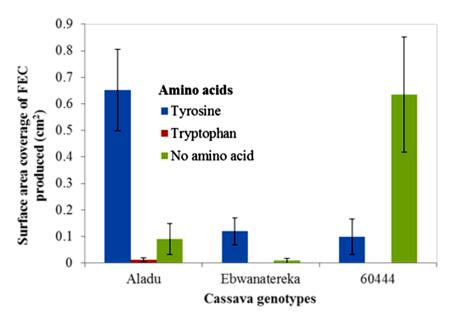
Figure 2. Mean production of friable embryogenic callus (FEC) from cassava genotypes on Gresshoff and Doy basal medium supplemented with amino acids at the same concentration.

Effect of amino acids on FEC production

The Ugandan genotypes responded to production of FEC using the amino acid tyrosine. Recently, Nyaboga et al. (2013) reported the beneficial effects of tyrosine in the induction of FEC from OES tissues in cassava. In the present study the aromatic amino acids tyrosine and tryptophan were investigated for their ability to stimulate FEC production in the Ugandan cultivars Ebwanateraka and Aladu. Significant differences were observed with respect to mean amounts of FEC produced in the presence of amino acids for Aladu (P = <.001), Ebwanatereka (P = 0.030) and control 60444 (P = 0.003) (Figure 2). These results were obtained from tissues after one culture cycle of 42 days. All genotypes produced FEC in the presence of tyrosine. The highest mean amounts of FEC in the presence of tyrosine were produced by Aladu (0.65 ± 0.15 mm^2) followed by Ebwanatereka ($0.0.12 \pm 0.05$ mm^2) and the least by 60444 (0.09 ± 0.07 mm^2) (Figure 2). However, only Aladu (0.01 ± 0.01 mm^2) produced FEC in the presence of tryptophan (Figure 2). In the absence of the amino acids, the control genotype 60444 (0.63 ± 0.22 mm^2) produced the highest mean amounts of FEC followed by Aladu (0.09 ± 0.06 mm^2) and the least by Ebwanatereka (0.01 ± 0.01 mm^2) (Figure 2).

Effect of naphthalene acetic acid (NAA) on germination of FEC into cotyledon embryos

The Ugandan cassava genotypes; Aladu, Ebwanatereka

and the control cultivar 60444, were capable of producing friable embryogenic tissue (FEC) (Figure 3C). The single celled embryo (Figure 3D) formed a torpedo shaped embryo (Figure 3E). Torpedo shaped embryo progressed to form a heart shaped embryo (Figure 3F). Heart shaped embryo formed a cotyledon embryos (Figure 3G). Significant differences in the number of embryos produced were observed among cassava genotypes with respect to levels of NAA (P = 0.003). The highest mean number of germinated embryos was observed at 1 µM of NAA followed by 5 µM and the least at 0.1 µM (Table 2). The highest mean number of germinated FEC into cotyledon embryos was observed at 1 µM of NAA for 60444 (12.24 ± 3.88) and Ebwanatereka (12.34 ± 9.12) while 5 µM of NAA facilitated embryo germination for Aladu (7.44 ± 1.58) (Table 2). The second best option for germination of the FEC into cotyledon embryos was observed at 5 µM of NAA for 60444 (6.58 ± 3.22) and Ebwanatereka (8.35 ± 4.18) while 1 µM of NAA favoured germination of FEC for Aladu (5.82 ± 2.57) (Table 2). In all genotypes used, 0.1 µM of NAA resulted in the least response of germination of the FEC into cotyledon embryos for Aladu (2.94 ± 2.42) followed by Ebwanatereka (3.34 ± 3.46) and 60444 (3.4 ± 2.48) (Table 2).

Effect of sugar types on germination of FEC into cotyledon embryos

The FEC from the three genotypes germinated progressively as explained in the preceding section in the
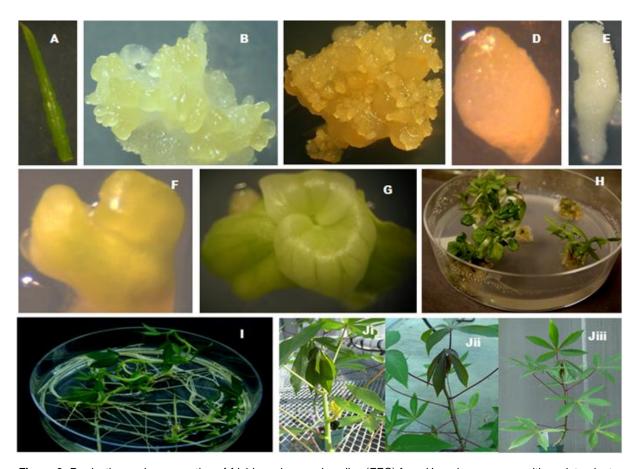
Figure 3. Production and regeneration of friable embryogenic callus (FEC) from Ugandan cassava cultivars into plants demonstrated. A) Leaf lobe established on picloram based induction media, B) Organised embryogenic structures (OES) of cassava genotype 60444, C) FEC produced by the genotype Aladu, D) Bulging single celled embryo, E) Early globular stage of the embryo, F) Late heart stage of the embryo, G) Mature cotyledon embryo H) A mature cotyledon embryo germinating into a plant, I) Fully regenerated plants in vitro and Ji, Jii, Jiii) Acclimatized in vitro regenerated plants for 60444, Aladu and Ebwanatereka respectively.

Table 2. Mean number of embryos produced on media with varying levels of Naphthalene acetic acid (NAA) on two carbon sources.

Cultivar	Mean number of embryos produced on media with different NAA levels			Mean number of embryos produced on media with sucrose or maltose at 20 g/L	
	5 µM	**1 µM**	**0.1 µM**	**Sucrose**	**Maltose**
Aladu	7.44 ± 1.58a	5.82 ± 2.57a	2.94 ± 2.42a	5.72 ± 2.25a	5.08 ± 3.41a
Ebwanatereka	8.35 ± 4.18b	12.34 ± 9.12b	3.34 ± 3.26b	11.85 ± 7.89b	4.17 ± 3.24b
60444	6.58 ± 3.22c	12.24 ± 3.88c	3.4 ± 2.48c	8.0 ± 5.76c	6.81 ± 3.73c

Values are mean ± SD of three independent experiments. Values in a column followed by different letters are significantly different from each other at $p \leq 0.05$

presence of sugars too. Significant differences in the number of embryos that germinated from the FEC as were observed in the presence of sugars (P = <0.001) for the three genotypes. The mean number of cotyledon embryos produced by the different genotypes in media with 20 g/L of sucrose was highest in Ebwanatereka (11.85 ± 2.04) followed by 60444 (8.0 ± 1.49) and least in Aladu (5.72 ± 0.58) (Table 2). In media supplemented with 20 g/L of maltose, the highest number of cotyledon embryos were produced in 60444 (6.81 ± 0.96) followed by Aladu (5.08 ± 0.58) and least with Ebwanatereka (4.17 ± 0.84) (Table 2). Sucrose was a suitable sugar for germination of FEC into cotyledon embryos for all the genotypes.

Table 3. Mean number of embryos produced due to interaction between Naphthalene acetic acid (NAA) and two carbon sources (sucrose and maltose).

Cultivar	Mean number of embryos produced at different NAA levels in media with 20 g/L of Sucrose			Mean number of embryos produced at different NAA levels in media with 20 g/L of Maltose		
	5 µM	1 µM	0.1 µM	5 µM	1 µM	0.1 µM
Aladu	7.52 ± 1.61a	5.12 ± 1.07a	4.52 ± 2.53a	7.36 ± 1.56a	6.52 ± 3.33a	1.36 ± 0.57a
Ebwanatereka	9.42 ± 5.09b	20.24 ± 6.19b	5.88 ± 3.23b	7.28 ± 2.59b	4.44 ± 1.77b	0.8 ± 0.76b
60444	8.72 ± 2.82c	13.72 ± 3.88c	1.56 ± 1.32c	4.44 ± 1.92c	10.76 ± 3.25c	5.24 ± 1.94c

Values are mean ± SD of three independent experiments. Values in a column followed by different letters are significantly different from each other at $p \leq 0.05$.

Figure 4. Types of cotyledons produced during the maturation phase of the somatic embryos. (A) Two cotyledon embryo, (B) one cotyledon embryo and (C) trumpet shaped cotyledon embryo.

Effect of the interaction of sugars and naphthalene acetic acid (NAA) on germination of FEC into cotyledon embryos

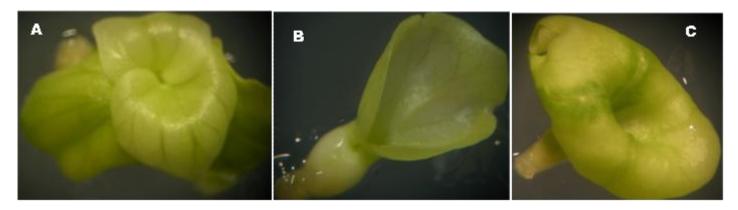The FEC from the three genotypes germinated in interaction of the sugars and the NAA as described in the preceeding sections. Significant differences in the number of embryos that germinated from FEC were observed due to the interaction of the NAA and sugars on each genotype (P = <0.001). In media with 20 g/L of sucrose, the highest mean number of germinated cotyledon embryos were observed in Ebwanatereka (20.24 ± 6.19) with 1 µM NAA followed by 60444 (13.72 ± 3.88) with 1 µM NAA and least were observed in 60444 (1.56 ± 1.32) with 0.1 µM NAA (Table 3). In media with 20 g/L of sucrose, the highest mean number of germinated cotyledon embryos for Aladu were observed in media with 5 µM NAA (7.52 ± 1.61) followed by 1 µM NAA (5.12 ± 1.07) and least 0.1 µM NAA (4.52 ± 2.53) (Table 3). For Ebwanatereka in media with 20 g/L of sucrose, the highest mean number of germinated cotyledon embryos were observed in media with 1 µM NAA (20.24 ± 6.19) followed by 5 µM NAA (9.42 ± 5.09) and least 0.1 µM NAA (5.88 ± 3.23) (Table 3). The highest mean number of germinated cotyledon embryos in 60444 were observed in media with 20 g/L of sucrose with 1 µM NAA (13.72 ± 3.88) followed by 5 µM NAA (8.72 ± 2.82) and least with 0.1 µM NAA (1.56 ± 1.32) (Table 3).

In media with 20 g/L of maltose, the highest number of cotyledon embryos that germinated in media with varying levels of NAA was observed in 60444 (10.76 ± 3.25) with 1 µM NAA followed by Aladu (7.36 ± 1.56) with 5 µM NAA and least in Ebwanatereka (0.8 ± 0.76) with 0.1 µM NAA (Table 3). In media with 20 g/L of maltose, the highest mean number of germinated cotyledon embryos for Aladu were observed in media with 5 µM NAA (7.36 ± 1.56) followed by 1 µM NAA (6.52 ± 3.33) and least was observed with 0.1 µM NAA (1.36 ± 0.57) (Table 3). For Ebwanatereka in media with 20 g/L of maltose, the highest mean number of germinated cotyledon embryos were observed in media with 5 µM NAA(7.28 ± 2.59) followed by 1µM NAA (4.44 ± 1.77) and the least with 0.1µM NAA (0.8 ± 0.76) (Table 3). The highest mean number of germinated cotyledon embryos in 60444 were observed in media with 20 g/L of maltose with 1µM NAA (10.76 ± 3.25) followed by 0.1 µM NAA (5.24 ± 1.94) and the least with 5 µM NAA (4.44 ± 1.92) (Table 3).

Effect of sugar types on regenerative ability of the different cotyledon embryos into plants

The genotypes Aladu, Ebwanatereka and 60444, all produced the three types of cotyledons categorized as two cotyledon embryo (Figure 4A), one cotyledon embryos (Figure 4B) and trumpet shaped embryo (Figure

Table 4. Mean number of cotyledons type embryos produced from the three cassava cultivars and the percentage regeneration.

Cultivar	Two cotyledon		One cotyledon		Trumpet shaped		Mean number of regenerated embryos into plants (%)	
	Sucrose	Maltose	Sucrose	Maltose	Sucrose	Maltose	Sucrose	Maltose
Aladu	1.91 ± 1.14a	2.67 ± 1.90a	1.08 ± 0.83a	1.27 ± 1.0a	0.15 ± 0.22a	0.12 ± 0.19a	46.77 ± 19.08a	51.27 ± 17.85a
Ebwanatereka	5.63 ± 3.99b	1.81 ± 1.72b	1.75 ± 1.41b	1.04 ± 0.79b	0.17 ± 0.31b	0.20 ± 0.31b	67.42 ± 17.07b	56.83 ± 17.62b
60444	4.36 ± 2.50c	3.81 ± 2.24c	2.00 ± 1.47c	0.96 ± 0.60b	0.08 ± 0.09c	0.07 ± 0.12c	74.14 ± 15.85c	68.40 ± 17.51c

Values are mean ± SD of three independent experiments. Values in a column followed by different letters are significantly different from each other at p ≤ 0.05.

4C). Significant differences were observed for two cotyledon embryos (P = <.001) and one cotyledon embryo (P = 0.01) while no significant differences were observed for trumpet shaped embryos (P = 0.856). Generally Ebwanatereka produced the highest (5.63 ± 3.99) and least (1.81 ± 1.72) mean number of two cotyledon embryos (Table 4). The highest mean number of two cotyledon embryos in media with sucrose at 20 g/L were observed in Ebwanatereka (5.63 ± 3.99) followed by 60444 (4.36 ± 2.50) and least in Aladu (1.91 ± 1.14) while in media with maltose at 20 g/L, The highest mean number of two cotyledon embryos were produced by 60444 (3.81 ± 2.24) followed by Aladu (2.67 ± 1.90) and least in Ebwanatereka (1.81 ± 1.72) (Table 4).

Overall, the highest (2.00 ± 1.47) and least (0.96 ± 0.60) mean number of one cotyledon embryos were observed in 60444 (Table 4). The highest mean number of one cotyledon embryos in media with 20 g/L of sucrose were observed in 60444 (2.00 ± 1.47) followed by Ebwanatereka (1.75 ± 1.41) and least in Aladu (1.08 ± 0.83) (Table 4). In media with 20 g/L of maltose, the highest numbers of one cotyledon embryos produced were observed in Aladu (1.27 ± 1.0) followed by Ebwanatereka (1.04 ± 0.79) and least in 60444 (0.96 ± 0.60) (Table 4). The highest mean number of trumpet shaped embryos were produced by Ebwanatereka (0.2 ± 0.31) and the

least number were produced by 60444 (0.07 ± 0.12) (Table 4).

The highest mean number of trumpet shaped embryos in media with 20 g/L of sucrose were observed in Ebwanatereka (0.17 ± 0.31) followed by Aladu (0.15 ± 0.22) and least in 60444 (0.08 ± 0.09) (Table 4). The highest mean number of trumpet shaped embryos in media with 20 g/L of maltose were observed in Ebwanatereka (0.2 ± 0.31) followed by Aladu (0.12 ± 0.19) and the least number were produced in 60444 (0.07 ± 0.12) (Table 4). Among the cotyledonary embryos produced, the two cotyledon embryo (Figure 3A) or those with one cotyledon embryo (Figure 3B) that eventually developed a second cotyledon matured as shown in Figure 3H and these fully regenerated phenotypically normal looking plants (Figure 3I) which were acclimatized as shown in Figure 3J, for the three genotypes; 60444, Aladu and Ebwanatereka represented as Figure 3Ji, Jii, Jiii, respectively. Fully regenerated plants as shown in Figure 3I, were obtained in media with sucrose and maltose. Regenerability of two cotyledon embryos into plants was highest in control genotype 60444 followed by Ebwanatereka and least in Aladu (Table 4). In media with sucrose, the control genotype 60444 had the highest percentage of regenerability (74.14 ± 15.85) followed by Ebwanatereka (67.42 ± 17.07) and least in Aladu (46.77 ± 19.08) (Table

4). In media with maltose, the highest regenerability was in the control genotype 60444 (68.40 ± 17.519) followed by Ebwanatereka (56.83 ± 17.62) and least in Aladu (51.27 ± 17.85) (Table 4). The fully regenerated phenotypically normal looking plants of the genotypes 60444, Aladu and Ebwanatereka were established in the greenhouse as described by Taylor et al. (2012) to ensure that these plants are true to type (Figure 3J).

DISCUSSION

Results indicate that the cassava cultivars investigated were able to produce organized embryogenic structures (OES), friable embryogenic callus (FEC) and regenerate to normal looking plants. The results generally indicated that production of OES was highest in Bukalasa, followed by Ebwanatereka, 60444 and lastly Aladu. Results also indicate that response of cultivars to *in vitro* conditions was cultivar dependent. Previous reports on production of OES from 60444 have resulted in frequencies as high as 80% (Taylor et al., 1996). Lower induction rates in terms of means production of OES reported here could be attributed to the fact that the mother plants used in the experiments were older than 12 weeks old (Taylor et al.,1996).

Bukalasa was not used in subsequent experiments for production of friable embryogenic callus (FEC) because preliminary experiments indicated a failure of the somatic embryos to transit to FEC. The OES produced from Aladu, Ebwanatereka and 60444 were used in experiments to generate FEC.

In *in vitro* manipulation, sucrose is the most common carbon source used (George et al., 2008). .George et al., 2008 stated that sucrose has consistently been found to be the best carbohydrate for *in vitro* growth of cultures. The inversion of sucrose to glucose and fructose is possible in the presence of an enzyme invertase found in the plant cell wall. The degree to which this inversion occurs varies from genotype to genotype, explaining the differences in the way the different genotypes utilize the different sugar concentrations (George et al., 2008). Maltose is known to serve as a carbon source and an osmoticum. It is a reducing sugar that is broken down to give two glucose molecules. In comparison to sucrose, the rate of extracellular hydrolysis is slower. Lower concentrations of maltose favored production of FEC in all the genotypes in comparison to higher concentrations which is in agreement with findings for alfalfa and rice (George et al., 2008, Seul et al., 2013).

In plants, amino acids serve as precursors for a variety of plant hormones such as auxin and salicylate as well as a wide range of aromatic secondary metabolites such as flavonoids, cell wall lignin, phenylpropaniods, anthocyanins and other metabolites (Dixon, 2001; Galili and Tzin, 2010). Ebwanatereka and 60444 did not produce any FEC in the presence of tryptophan while low amounts of FEC were produced by Aladu (Figure 4). Absence of amino acids in the media favoured mean production of FEC in the control genotype 60444 (Figure 4). It has been reported that presence of amino acids in medium is important because it partially replaces the ammonium ions, therefore increasing the levels of reduced nitrogen which is necessary for development of somatic embryos (George et al., 2008; Anthony Ceaser and Ignacimuthu, 2010). For genotype like 60444 which responded in the absence of the amino acid, indicates that the available ammonium ions in that medium were enough to trigger the embryogenic pathway. The ability to produce FEC in Ugandan farmer preferred cassava genotypes (UFPCG) has been made possible by the existence of optimized procedures for genotype 60444, with the adjustment of some conditions to favour UFPCGs.

Tyrosine was noted as more suitable amino acid than tryptophan in triggering FEC production in the UFPCG (Figure 4). Hankoua et al., 2006 also suggested that addition of tyrosine to media facililtated generation of prolific FEC. Galili and Tzin (2010), noted that tyrosine and tryptophan are both involved in the shikimate pathway. Ebwanatereka and Aladu produced FEC in the media with 500 µM tyrosine. This could be attributed to the fact that tyrosine is a precursor of secondary metabo-

lite tocochromanol. Tocochromanols, which include tocopherols and tocotrienols are lipid soluble molecules belonging to the group of vitamin E compounds known to play an essential role as antioxidants in plants (Falk and Munné-Bosch, 2010). Studies on *vte1* and *vte2* A. thaliana and *sxdl* maize mutants indicate that tocopherols may be involved in the regulation of photoassimilate export in leaves, therefore affecting carbohydrate metabolism, source-sink relationships and growth (Sattler et al., 2003; Falk and Munné-Bosch, 2010).

Also tocochromanols are potent hydrogen donors regulating membrane associated signaling pathways and modulation of gene expression (Brigelius-Flohe et al., 2002; Falk and Munné-Bosch, 2010). This may explain why tyrosine facilitated production of FEC. Tryptophan on the other hand, is known as a precussor of auxins such as indole-3-acetic acid (IAA) (Ostin et al., 1998; Galili and Tzin, 2010), indole glucosinolates (Halkier, 1999; Galili and Tzin, 2010) which are beneficial to the plant. However, results in this study indicate that Ebwanatereka and 60444 failed to produce FEC in the media with 500 µM tryptophan while Aladu produced FEC in low quantities. According to Makwarela et al. (2004), two South African cassava genotypes T200 and T400 produced FEC at concentration of 125 µM tryptophan. This may imply that the tryptophan concentration used was too high, or that different genotypes respond differently.

Regeneration potential of FEC investigated by varying the auxin levels while maintaining the cytokinin levels shows that the auxins and cytokinins aid in cell division, differentiation and shoot proliferation. Taylor et al. (2012) demonstrated the ability of FEC to regenerate for the genotype 60444. It is important to note that the callus should not be older than 20 weeks or 4-5 cycles for the regenerative potential of the FEC to be realized (Taylor et al., 2012).

The effect of auxins is remarkable because it induces cellular responses such as cell elongation, which impacts directly on timing and orientation of cell divisions and thus contributes to patterning (Thomas et al., 2004). Results indicate that the carbon sources and auxin levels had a significant impact on the regeneration potential of the genotypes (Table 1). An auxin level of 1 µM of NAA proved suitable for regeneration of the embryogenic tissue from the cassava genotypes, 60444 and Ebwanatereka with sucrose at 20 g/L being the best carbon source. For Aladu, the carbon source maltose at 20 g/L favoured the regeneration process. On the other hand, Aladu required 5 µM of NAA, to produce substantial amounts of embryos, indicating that different genotypes respond differently to the auxin NAA at different concentrations.

The ability of competent embryo cell to go through progress changes in development are closely associated with cell to cell communication, of which extracellular signals and cell surface bound receptors are said to be

involved (Thomas et al., 2004). During maturation process, the competent embryo progressed to globular pattern formation eventually forming heart shaped embryos (George et al., 2008), resulting in the production of phenotypically normal looking plants. Thomas et al., 2004 suggested that cellular auxin response in different parts of the embryo and at different stages play a significant role in its progressive development, because cells are already predetermined therefore the presence of the auxin triggers the preset pathway. The regeneration process took three to four months, with continuous subculture to obtain a fully developed plant as demonstrated in the control genotype 60444 from friable embryogenic callus (Taylor et al., 2012). The percentage regeneration from each genotype varied depending on the carbon source utilized in the MS-based medium. Both sugar types supported plant regeneration from the mature cotyledon embryos to phenotypically normal looking plants.

Conclusion

Production and regeneration of friable embryogenic callus (FEC) has been tested and proven in the Ugandan cassava genotypes Ebwanatereka and Aladu. Tyrosine is important for production of FEC. Naphthalene acetic acid (NAA) and sugars were necessary for maturation and regeneration of FEC into phenotypically normal looking plants. Ability to produce FEC in these genotypes lays a foundation for their improvement through genetic transformation for the desired and agronomic traits.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Dihydrotestostenone increase the gene expression of androgen receptor coregulator FHL2 in human non-transformed epithelial prostatic cells

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The actions of androgens are mediated through an androgen receptor (AR), and AR activity is modulated by coregulators. The aim of this study was to assess the action of androgens in the expression of AR and the coregulators *FHL-2* and *SHP-1* in human non-transformed epithelial prostatic cells (HNTEP) treated with androgens. Prostate tissues were obtained from 12 patients between 60 and 77 years of age. HNTEP cells were grown in basal medium and treated with DHT in different conditions. HNTEP cells under treatment with DHT (10^{-13} M) induced an increase in *FHL-2* expression. In turn, high DHT concentrations (10^{-8} M) induced an increase in the expression *SHP-1*. The present data suggest that the *SHP-1* and *FHL-2* genes play a role in the control of responsiveness and androgen-dose-dependent cell proliferation in HNTEP cells. Further studies are needed to assess the influence of androgens in AR and its coregulators and the implications in the pathophysiology of prostate diseases.

Key words: Androgens, *FHL-2*, *AR*, prostate, proliferation, coregulators.

INTRODUCTION

Androgens are mediators of a wide range of developmental and physiological responses. The effects of androgens occur through the androgen receptor (AR). The androgen interactions with the AR result in transcriptional activation of target genes which are important in male sexual differentiation and puberal sexual maturation (Marker et al., 2003). AR is a ligand inducible transcriptional factor, member of the nuclear

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Abbreviations: AR, Androgen receptor; **HNTEP,** human non-transformed epithelial prostatic cells; **LBD,** ligand binding domain; **DBD,** DNA-binding domain; **NTD,** NH$_2$- terminal transactivation domain; **AF-1,** activation function domain 1; **BPH,** benign prostate hyperplasia; **PCa,** prostate carcinoma; **LUTS,** lower urinary tract symptoms; **ARE,** androgen responsive elements; **SDS,** sodium dodecyl sulfate; T_m, melting temperature; C_T, cycle threshold; **SEM,** standard error of mean; **SPSS,** statistical package for social sciences.

receptor superfamily that mediates the expression of target genes in response to specific stimulus (Aranda and Pascual, 2001). The human AR gene is localized in the chromosome X at the q11-q12 position. There are eight exons and the gene shares a characteristic structure with other nuclear receptors, four domains: the ligand binding domain (LBD) containing activation function domain 2 (AF-2), the zinc-finger-type DNA-binding domain (DBD), a hinge region and the variable NH_2- terminal transactivation domain (NTD) possessing activation function domain 1 (AF-1).

AF-1 acts as a ligand-independent manner and can be more active when in contact with basal transcription factors, while the activity of AF-2 requires the ligand binding. Also, the interaction between NTD and LDB could also be necessary for the activation of AR (Dehm and Tindall, 2007; He et al., 2000).

Androgens play an important role in controlling the growth of the normal prostate gland, and also in promoting benign prostate hyperplasia (BPH) and prostate carcinoma (PCa). Histologic evidence of BPH is found in 50% of men at the age of 50 and up to 90% of males at the age of 80 (Roehrborn et al., 2006). The pathogenesis of BPH is still poorly understood, but there are two accepted permissive factors: the presence of circulating androgens and advancing age (Untergasser et al., 2005). The proliferative effects of androgens in prostate are controversial; some studies demonstrated a biphasic effect of androgen action in prostatic cells proliferation where lower androgen concentrations have a maximum mitogen effect whereas higher concentrations do not (Joly-Pharaboz et al., 2000; Joly-Pharaboz et al., 1995; Lee et al., 1995; Shao et al., 2007; Sonnenschein et al., 1989).

In contrast, other studies were not able to show any effect of androgens, at different concentrations, on cell proliferation of normal, hyperplasic or tumoral prostatic cells (Berthon et al., 1997; Heisler et al., 1997; Krill et al., 1999). Androgen concentrations are determinant to prostate enlargement and dependent on AR activity (Li et al., 2007; Yuan et al., 2006). Prior to androgen binding, AR is held inactive through association with heat shock proteins. Androgen binding releases inhibitory proteins and the AR translocates to the nucleus, where it interacts with DNA sequences, called androgen responsive elements (ARE). After binding to ARE, AR is able to recruit all the compounds of the transcriptional machinery for target genes (Balk and Knudsen, 2008).

Activation of gene expression is one aspect of AR signaling. Repressed or activated androgen-responsive genes appear to play important roles in regulating cell growth and differentiation. The activation of AR by androgens is a complex process involving a large number of activating and repressing proteins called coregulators. Several studies have indicated that altered expression of these molecules may modify transcriptional activity of AR suggesting that these coregulators could also contribute

to the progression of prostatic pathologies (Heemers and Tindall, 2007; Urbanucci et al., 2008, Muramatsu et al., 2013, Toropainen et al., 2015).

SHP-1 (short heterodimer partner) is an orphan nuclear receptor which interacts with a large variety of nuclear receptors and has been shown to be expressed in androgen target tissues (Johansson et al., 1999). Gobinet et al. (2001) demonstrated that *SHP-1* interacts both *in vitro* and *in vivo* with the full-length AR and inhibited both the AR ligand-binding domain and the N-terminal domain dependent transactivation. *SHP-1* could also inhibit AR activity by competing with AR coactivators.

FHL-2 is an LIM-only member of the LIM protein superfamily. It is a selective agonist-dependent coactivator of the AR, but not of other nuclear receptors. *FHL-2* increases the transcriptional activity of the AR in an agonist and AF-2 dependent manner. Also a study showed a FHL2 overexpression in prostatic cells and in the presence of DHT, endogenous FHL2 bind at the ARE to enhance AR-transcriptional activity (Kollara and Brown, 2010). It is expressed in myocardium and in the prostate gland (Muller et al., 2000). The action of androgens in AR is controversial and complex, because it has become clear that the transcriptional activity of AR is regulated by coregulators, including both coactivators and corepressors, by various mechanisms. The aim of the present study was to determine the effect of androgens at different concentrations on AR expression and the coregulators *SHP-1* and *FHL-2* in HNTEP cells.

MATERIALS AND METHODS

Cell culture

Samples of prostatic tissue were obtained from retropubic prostatectomy from 12 patients between 56 and 75 years of age, diagnosed with BPH. Patients with malignant tumors were excluded. The study protocol was approved by the local Ethics Committee (UFRGS, protocol 99001). Informed consent was obtained from all subjects. HNTEP cells were cultured as previously described (Brum et al., 2003). Briefly, after removal of blood clots, prostatic tissue was washed with Hank's balanced salt solution (HBSS, Gibco BRL Grand Island, N.Y., USA) plus kanamycin (0.5 mg/ml) (Sigma Chem Co., St Louis, MO,USA), and then finely minced into 2 to 3 mm pieces. Tissue fragments were treated with type IA collagenase (7.5 mg/g of tissue) (Sigma Chem Co., St Louis, MO, USA) in HBSS. Enzymatic digestion proceeded for 3 h at 37°C with gentle shaking. The enzymatic reaction was interrupted with the addition of warm 199 culture medium (Gibco BRL Grand Island, N.Y., USA) with kanamycin (0.5 mg/ml) and 10% fetal bovine serum (FBS) (Gibco BRL Grand Island, N.Y., USA). Epithelial cells were separated by differential filtration. Cell suspensions were distributed into 35 mm tissue culture dishes (Corning, Glassworks, NY, USA), 1×10^5 cells per dish, or into 24 well tissue culture plates (NUNC[TM], Denmark), 2×10^4 cells/ml per plate, and maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2 (NuAire, Inc., Minnesota, USA). In addition, since it is hard to observe the stimulatory effect of androgen on cell growth *in vitro*, we used a culture medium that was free of growth factors other than those present in FBS. Basal medium consisted of 199 medium containing kanamycin (0.5 mg/ml) enriched with 5%

charcoal-stripped FBS (cFBS). Cultures were kept in the same medium for the first 2 days and then the medium was changed every two days.

Western blot

Protein was obtained from the extraction of RNA with Trizol® (Invitrogen, Carlsbad, CA, USA) reagent following the protocol of the manufacturer. The protein concentration was determined by the Bradford method. Sodium dodecyl sulfate (SDS) polyacrylamide gel eletrophoresis (8%) was carried out using a miniprotein system (Bio-Rad, Hercules, CA, USA) with broad-range molecular weight standards (Bio-Rad, Hercules, CA, USA). Protein (30 μg) was loaded in each lane with loading buffer containing [(0.375 M Tris 6.8 pH), 50% glycerol, 10% SDS, 0.5 M dithiothreitol, and 0.002% bromophenol blue]. Samples were heated at 100°C for 2 min prior to gel loading. After eletrophoresis, proteins were transferred to nitrocellulose membranes using an eletrophoretic transfer system at 110 V for 1 to 2 h. The membranes were then washed with TTBS (20 mM Tris-HCl, 7.5 pH; 150 mM NaCl; 0.05% Tween-20, 7.4 pH) and 8% nonfat dry milk for 90 min. The membranes were incubated overnight at 4°C with the primary antibody diluted in TTBS. A rabbit polyclonal antibody for AR (2 μg/ml) (Upstate Biotechnology) was used. After washing, the membranes were incubated for 2 h at room temperature with secondary antibody (1:20.000) (anti-rabbit IgG peroxidase conjugated; Upstate Biotechnology), washed with TBS (20 mM Tris-HCl; 150 mM NaCl, 7.5 pH), and developed with the chemiluminescence Western Blot system (Amersham Biosciences) followed by apposition of the membranes to autoradiographic films (Kodak X-Omat) exposure for 15 to 60 s. Ponceau S staining was used as protein loading control.

Extraction of RNA and synthesis of cDNA

Cells were grown in serum deprived basal medium for 4 h, and then treated with DHT or ethanol vehicle in different conditions. Cultured Prostatic cells were washed twice with PBS and homogenized in phenol-guanidine isothiocyanate (Trizol, Invitrogen, Carlsbad, CA, USA). Total RNA was extracted with chloroform and precipitated with isopropanol by 12.000 xg centrifugation at 4°C. The RNA pellet was washed twice with 75% ethanol, resuspended in diethylpyrocarbonate-treated water, and quantified by light absorbance at 260 nm. First strand cDNA was synthesized from 1 μg total RNA, using the SuperScript Preamplification System (Invitrogen, Carlsbad, CA, USA). After denaturing the template RNA and primers at 65°C for 5 min, 50 U of reverse transcriptase was added in the presence of 20 mM Tris-HCl (8.4 pH) plus 50 mM KCl, 2.5 mM MgCl$_2$, 0.5 mM dNTP mix and 10 mM dithiothreitol, and incubated at 42°C for 50 min. The mixture was heated at 70°C for interruption of the reaction and incubated with 2 U *Escherichia coli* RNase for 20 min at 37°C for destruction of untranscribed RNA.

Real-time PCR conditions

Amplification and detection were performed with the MiniOpticon Real Time PCR detection system (Bio- Rad Life Sicience Research, USA). Duplicate samples were used. The PCR mixture contained 1.25 μl of SYBR green, 2 ng of cDNA at 1:50 dilution, 3 mM of MgCl$_2$, 20 mM Tris-HCl (8.4 pH) plus 50 mM KCl ,0.2 mM dNTP mix, 1.25 U of taq polymerase and 0.4 μM of each primer in a 25 μl of final volume. The reaction conditions were 94°C for 2 min for initial denaturation and the cycling conditions were designed for each gene. The fluorescence emitted by SYBR green I was measured in every cycle at the end of elongation step. The reaction conditions were 94°C for 2 min for hot-start, and 35 cycles of 94°C

for 30 s, 55°C for 40 s and 72°C for 40 s for the AR gene, 39 cycles of 94°C for 40 s, 58°C for 40 s and 72°C for 40 s for the *SHP-1* gene and 39 cycles of 94°C for 40 s, 59°C for 40 s and 72°C for 40 s for the *FHL*-2 gene. The sequences of primers employed were: AR gene sense 5' CATGGTGAGCAGAAGTGCCCTATC3' antisense 5' TCCCAGAGTCATCCCTGCTTCAT 3' (Taplin et al., 1995), *SHP-1* gene sense 5' CAGCTATGTGCACCTCATCG 3' antisense 5'AGCCACCTCAAAGGTCACAG3' (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and *FHL-2* gene sense 5' AAACTCACTGGTGGACAAGC 3' antisense 5'AGATGAAGCAGGTCTCATGC 3' (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). For normalization of the expression levels, the expression of *β$_2$-microglobulin* (sense: 5' ATCCAGCGTACTCCAAAGATTCAG3', antisense: 5' AAATTGAAAGTTAACTTATGCACGC 3' (Taplin et al., 1995), was used as a housekeeping gene.

Standard curves and efficiency

All samples were automatically processed for melting curve analysis of amplified cDNA. The T_m (melting temperature) is specific to each amplicon. Standard curves were created by plotting the C_T (cycle threshold) values of the real-time PCR performed on dilution series of standard. The real-time PCR assay was analyzed in the linear phase, and a linear function was fitted of the log of relative fluorescence versus cycle number with a typical R^2 value greater than 0.8 (AR R^2=0.91, *SHP-1* R^2=0.881 and *FHL-2* R^2 = 0.89) (Figure 1).

Statistical analysis

Data are reported as means and standard error of mean (SEM). Analysis of the data revealed a normal distribution. Differences between groups were assessed by analysis of variance, followed by Duncan's test. All analysis was performed using the Statistical Package for Social Sciences (SPSS, Chicago, IL, USA). Data were considered to be significant at $P < 0.05$.

RESULTS

The action of DHT treatment on AR, *SHP-1* and *FHL-2* mRNA levels in HNTEP cells was estimated by quantitative analysis. To confirm the presence of the AR in HNTEP cells, we evaluated the protein levels of AR after 1 and 2 h of incubation (Figure 2). The AR mRNA levels were assessed after 4 h of incubation with DHT (10^{-13} and 10^{-8} M) alone or in association with hydroxyflutamide (OH-FLU) at 10^{-6} M and no effect on AR gene expression was observed (Figure 3) in these conditions. The *SHP-1* gene expression was evaluated after different concentrations of DHT after 4h of incubation and we observed an increase in *SHP-1* mRNA levels with the higher dose of DHT (10^{-8} m) in comparison with the control group (data not show). FHL-2 levels in HNTEP cells were analyzed after 4h of treatment with two concentrations of DHT (10^{-8}, 10^{-13} m) alone or in association with the anti-androgen agent hydroxyflutamide (Oh-Flu) at 10^{-6} m. The coactivator of AR, *FHL-2,* showed an increase in mRNA levels in the group treated with the lower dose of androgen (10^{-13} m DHT) in comparison with the other (Figure 4).
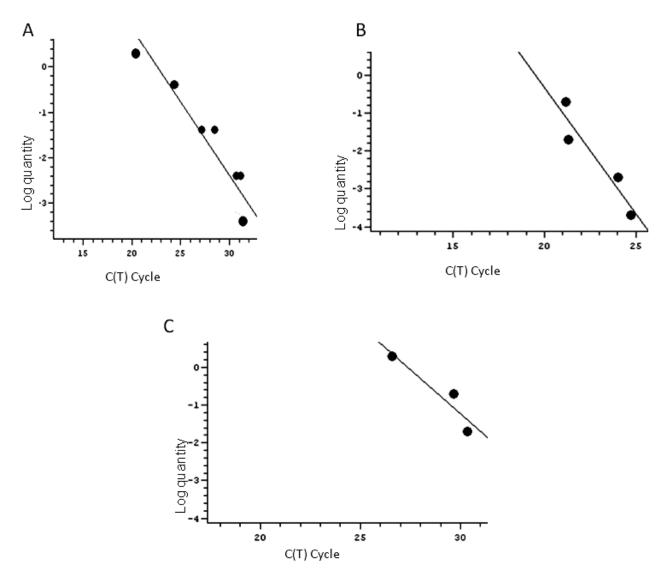
Figure 1. Standard curves of real-time PCR. Standard curves of real-time PCR for AR (A), FHL-2 (B) and SHP-1(C) genes performed on a dilution series of cDNA standard. Real-time PCR assay was analyzed in the linear phase and a fit linear function of the log of relative fluorescence vs. cycle number with a typical R^2 value > 0.8.

DISCUSSION

There is little information about the cell biology of the human prostate and about how hyperplasic and malignant lesions develop. The epithelial prostate cells (HNTEP) were established as an *in vitro* model to study the androgen dependence of human prostate and previously we showed that low concentrations of androgens exerted a positive effect on cell proliferation in HNTEP cells, and high concentrations maintained proliferation similar to that of the control (Pozzobon et al., 2012). In this study, the action of DHT treatment on AR, *SHP-1* and *FHL-2* mRNA levels in HNTEP cells was estimated by quantitative analysis.

HNTEP cells in primary culture expressed a functional AR. The AR mRNA levels were assessed after 4 h of incubation with DHT (10^{-13} and 10^{-8} M) alone or in association with hydroxyflutamide (OH-FLU) at 10^{-6} M and no effect on AR gene expression was observed (Figure 1) in these conditions.

The expression of AR mRNA was detected in several cell types like LNCaP and the androgen-independent cell lines (DU-145 and PC-3) when transfected with AR (Alimirah et al., 2006). In this case, AR gene expression did not change with different treatments of DHT, but the addition of the anti-androgen agent hidroxyflutamide abolished the proliferative effect in HNTEP cells, giving support to the notion that the mitogenic effect of the low dose of DHT in HNTEP cells is regulated by its own receptor, the AR. These results are consistent with those of Mestayer et al. (2003) who reported that AR expression is the same in normal or tumoral prostatic
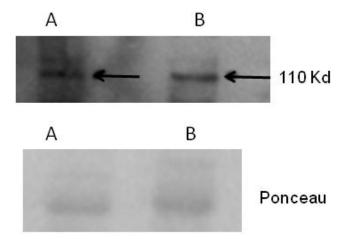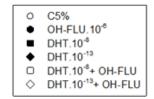
Figure 2. AR Protein. Autoradiogram of AR protein levels of HNTEP cells after 1 h (A) and 2 h (B) of incubation. Samples were separated in 8% SDS-PAGE, transferred to a nitrocellulose membrane and incubated with anti-AR antibody. Ponceau S staining was used as protein loading control.

Figure 3. AR expression in HNTEP cells. Graphic display the relative expression of AR gene. Each bar represents the mean (± SEM) of product amplified (ng) at 4 h of treatment wi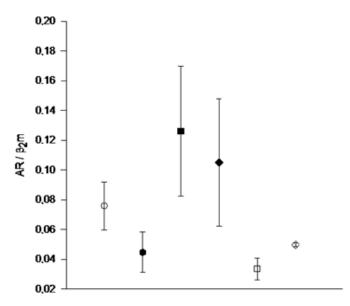th DHT (10^{-8} and DHT.10^{-13} M) alone or in association with the antiandrogen hidroxyflutamide (OH-FLU.10^{-6} M). Comparisons between groups were analyzed by ANOVA followed by Duncan's test.

tissue. However in the immortalized cell lines PNT1A and DU-145, transfected with functional AR other author showed an increased activity in AR expression with DHT (10^{-9}m) treatment (Avances et al., 2001). These controversial results may be explained by the different experimental models employed. The modulation of androgen receptor is a complex phenomenon which involves several mechanisms. It is important to note that the AR may also mediate important cellular functions in the cytoplasm, independent of its role as a transcriptional factor. For example, AR has been shown to participate in rapid signaling cascades which involve the activation of the MAPK pathway and thereby potentially induce a mitogenic reponse (White et al., 2005). The mechanisms underpinning the capacity of AR to induce a mitogenic program may be diverse and dependent on cell context. A study demonstrated in LNCaP that protein levels of AR were unaffected during 5 days of treatment with 5.10^{-11} M R1881, a very similar dose to the one used in our study (Nesslinger et al., 2003).

The AR expression is influenced by several coregulator molecules, which facilitate domain interactions and consequently AR transativation. As a general definition, AR coregulators are proteins that are recruited by the AR and either enhance or reduce its transativation. More than 200 nuclear receptor coregulators have been identified since the isolation of the first nuclear receptor coactivator, SRC-1 in 1995 (Onate et al., 1995). The isolation of a multitude of proteins with AR coregulatory properties leads to speculation about the manner in which the formation of the AR transcriptional complex is orchestrated. The balance of corepressors and coactivetors in the AR complex determines AR transcriptional activity. In the present study, we observed an increase in the mRNA levels of *SHP-1* when HNTEP cells were treated with a high dose of androgens. *SHP-1* was described as an inhibitor of AR activity, and this result can indicate a negative modulation of AR with higher doses of androgens and consequently a lower proliferation of HNTEP cells. The repression of AR activity by androgen treatment with 10^{-9} M R1881 was demonstrated in a monkey kidney cell line CV1 and in CHO cells, cotransfected with a functional AR and *SHP-1* (Gobinet et al., 2001). It is possible that the mechanism of SHP-1 repression could be through competition with coactivators in AF-2 region, and this process depends on a higher concentration of *SHP-1* or greater affinity (Jouravel et al., 2007).

In this article, we examined the role of the AR coregulator *FHL-2*. This gene does not bind to DNA, but it has an intrinsic transactivation domain and interacts with AR in a ligand-dependent manner (Kahl et al., 2006). Moreover, *FHL-2* interacts with several AR-associated coactivators (Johannessen et al., 2006; Nair et al., 2007). However, the molecular mechanism by which *FHL-2* modulates AR transactivation remains unclear. *FHL-2* expression occurs in the cytoplasm of normal prostate
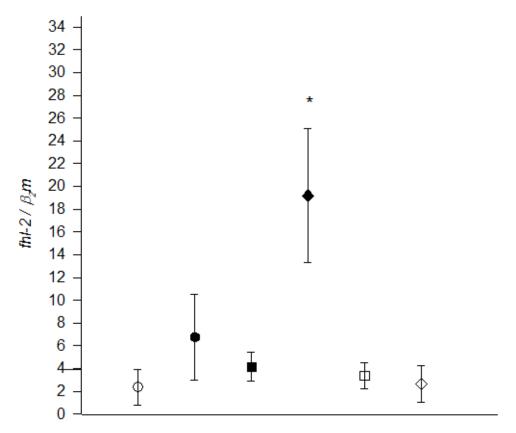
Figure 4. FHL2 expression in HNTEP cells. Graphic display the relative expression of *FHL2* gene. Each bar represents the mean ($\pm$ SEM) of product amplified (ng) at 4 h of treatment with DHT (10^{-8} and DHT.10^{-13} M) alone or in association with the antiandrogen hidroxyflutamide (OH-FLU.10^{-6} M). Comparisons between groups were analyzed by ANOVA followed by Duncan's test. *$p < 0.05$ for all groups analyzed.

cells, and the degree of nuclear translocation increases in less-differentiated cancer cells (Muller et al., 2002). In our primary cell culture, we found an increase in mRNA levels of *FHL-2* after incubation with a lower concentration of androgens, the same concentration that stimulated cellular proliferation. This effect was abolished by the anti-androgen agent OH-flutamide suggesting a positive modulation of androgen levels by *FHL-2* in HNTEP cells. A study also described an increase in AR activity in CV1 cells cotransfected with AR and *FHL-2* and treated with androgens (Gobinet et al., 2001). Another study showed that a coregulator of multiple nuclear receptors, PELP1

interacts with *FHL-2* and synergistically enhances the transcriptional activity of *FHL-2* in PC-3 cells cotransfected with MMTV-luc, β-Gal reporter gene and AR when incubated with androgen R1881. The same study has shown that *FHL-2* also enhanced AR-mediated transativation of PSA promoter activity (Nair et al., 2007). Some authors did not show an *FHL-2* expression in LNCaP cells (Nessler-Menardi et al., 2000), but the androgen induction of *FHL-2* was demonstrated by Heemers (Heemers et al., 2007). The androgen exposure led to a marked increase in *FHL-2* expression, both at the mRNA and protein levels in LNCaP cells. They also demon-

strated a time dependent androgen stimulation of *FHL-2*, reaching a maximum at 48 h. Finally, they also demonstrated the influence of *FHL-2* in other androgen dependent genes containing androgen responsive elements (ARE) in their promoter regions.

There are few studies regarding *FHL-2* and *SHP-1* expression in the normal prostatic gland, and most of the data used immortalized cell lines. The dependence of AR coregulators to form a functional transcriptional complex suggests an important role in the development and maintenance of androgen-responsive tissues and can be involved in pathologies like benign prostatic hyperplasia and prostatic carcinoma.

This is the first article that shows a relation between *FHL-2* and *SHP-1* expressions and androgen levels in human non-transformed prostatic cells. In summary, we showed that the expression of the AR coactivator *FHL-2* is stimulated by lower androgen concentrations whereas the corepressor *SHP-1* is inhibited by this concentration and stimulated by higher androgen concentrations. We speculate that an increase in *FHL-2* may lead to enhanced AR signaling or sensitize HNTEP cells to low levels of androgens and thus proliferate. This finding provides insights into the regulation of AR in prostatic diseases and identifies a possible mechanism by which the AR is able to assure its aberrant activity in prostatic diseases.

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Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Effects of rumen digesta on the physico-chemical properties of soils in Nsukka, Southeastern Nigeria

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In tropical and subtropical areas, the importance of organic manure in improving soil physico-chemical properties and crop production for food security cannot be overemphasized. A study was conducted to investigate the effects of rumen digesta on the physical and chemical properties of soils in Nsukka, Enugu State, Nigeria. The soil samples collected from Opi, Nsukka were treated with four rates of rumen digesta (viz. 0, 50, 100 and 150 g/kg soils). Physical and chemical properties of the soil were determined pre- and post-experiment. The results obtained reveal that rumen digesta significantly ($p = 0.05$) increased the mean weight diameter (0.49 to 1.75 mm), aggregate stability (54.7 to 75.3%), soil pH (3.8 to 7.8), total nitrogen (0.01 to 0.02%), exchangeable sodium and potassium (0.22 to 4.39 cmol/kg for Na^+ and 0.30 cmol/kg to 4.31 for K^+), CEC (7.2 to 14.9 cmol/kg) and organic matter content (0.97 to 4.29%). It had no significant effect on the texture, micro-aggregate stability (measured as dispersion ratio), exchangeable calcium and magnesium content of the soils. The study found a significant reduction in the exchangeable aluminum (1.5 to 0.0 cmol/kg) and hydrogen content (3.7 to 2.2 cmol/kg) of the soils. It is recommended that farmers can improve the physical and chemical properties of soils by using rumen digesta as an alternative liming material.

Key words: Rumen digesta, soil properties, evaluation, rural land, Nsukka-Nigeria.

INTRODUCTION

The use of organic manures (especially ruminant dung, poultry droppings, household refuse and effluents) for crop production is an age-old agricultural practice among subsistence farming communities in the West African sub-region (Lombin et al., 1991). In many developing countries (for example Nigeria), the likelihood of obtaining enough synthetic fertilizers to meet the food crop requirements of the farming population is remote. The ever-increasing demand for food has intensified the quest for more production per unit area and for an increase in land under arable cultivation. Farmers in the tropics and the subtropics have been forced to eliminate fallow

periods and rely on synthetic fertilizers. These practices (reduction in fallow periods and increased use of synthetic fertilizers) have led to increased land degradation and declining crop productivity. Odu and Mba (1991) stated that inorganic fertilizers supply nutrients alone, while organic manures not only supply nutrients, but also help improve the soil physico-chemical properties through microbial activities. The inventory of urban and industrial wastes in Nigeria, as compiled by Sridar (2006), showed that millions of tons of industrial, domestic and animal wastes produced annually are not effectively used, whereas they could be effectively used for agriculture. Evidence indicates that judicious application of these wastes on agricultural fields could maintain a high level of soil fertility. Investigations into the possible use of organic waste to improve the productivity of soils have been carried out by Agbim (1981); Anikwe (2000); Asadu and Igboka (2014); Hellal et al. (2014); Mbagwu (1985); Mbah, (2008); Nyamangara et al. (2001); Omaliko and Agbim (1983); Omaliko (1985).

Many studies have evaluated the fertilizer value of organic waste products (poultry dropping, cow dung, sewage sludge and swine wastes) and determined their potential for improving soil fertility. However, there is little information available about the effect of rumen digesta on the physical and chemical properties of soils in the study area. Rumen digesta are part of abattoir organic wastes or manures, and are obtained from the paunch content (rumen) of ruminant animals. A total of 194 kg of solid (rumen/stomach) waste is generated daily in Nsukka abattoir (Nwanta et al., 2010). The objective of this study was to determine the effects of rumen digesta on the physico-chemical properties of soils from Opi in Nsukka local government area (L.G.A) in Enugu State.

MATERIALS AND METHODS

Study area

The experiment was carried out at the greenhouse of the Faculty of Agriculture, University of Nigeria, Nsukka. Nsukka is located by latitude 06°52 N and longitude 07°24 E at approximately 400 m above sea level. The climate of Nsukka is characterized by mean annual total rainfall of about 1600 mm and mean annual evapotranspiration (ET) of about 1560 mm, the ET exceeds total rainfall in most months of the year (Igwe, 2004). Rainfall distribution is characteristically biomodal, with peaks during July and October. The entire wet season lasts from April to October, whereas the dry season lasts from November to March. During the wet season, there is a soil moisture decline of 104 mm and a moisture surplus of about 260 mm which depletes to an average deficit of about 650 mm in the dry season (Mbagwu, 1987). Temperature is uniformly high throughout the year, with mean minimum and maximum annual values of 21 and 31°C, respectively. Temperatures rarely exceed 35°Cduring the hottest months (Asadu and Akamigbo, 1990; Obi and Salako, 1995). Grassland vegetation is predominant in the study location which is within the forest-savanna transition vegetation zone (Mbagwu, 1991). The area has an ustic soil moisture regime and the soils are described as being well-drained with very low total exchangeable base, cation exchange capacity (CEC) and base saturation (Asadu and Akamigbo, 1990).

The soil is deep, coarse textured and low in organic matter with perennial leaching risks (Igwe, 2004). The soils mostly belong to the orders of Ultisols and Vertisols.

Sample collection

Auger soil samples were collected at a depth of 0 to 30 cm from randomly selected positions in farmer's fields (5 ha) at Opi, inNsukka LGA of Enugu State. The samples were bulked to form composite samples. The soil samples were air-dried and sieved through a 2 mm mesh and stored in 12 plastic containers (1564 cm^3) in the greenhouse before amendments were applied. The rumen digesta was collected from the paunch content of cattle, from Ikpa-Nsukka abattoir, Enugu State, and air dried. The soil type used for this experiment is a sandy clay loam ultisol.

Experimental design

The experiment was conducted during the months of April and May, 2014 and repeated at the same period in 2012. One kg of soil was measured into plastic containers and treated with different rates of air dried rumen digesta (0, 50, 100, and 150 g). Experimental design was completely randomized design (CRD) and treatments were replicated 3 times giving a total of 12 samples. The treated soil samples were kept moist with distilled water (200 ml daily) during the 5 week duration of the experiment. The quantity of water applied corresponds to actual quantity of rainfall in the field during the experiment. The soil samples were analyzed for physical and chemical properties before and after amendment, while the rumen digesta was analyzed for its chemical properties before application.

Laboratory studies

The laboratory analysis was conducted at the Soil Science laboratory, Faculty of Agriculture, University of Nigeria, Nsukka. The physical properties analysed were as follows:

i. Particle size distribution, determined using the hydrometer method (Bouyoucos, 1962)
ii. Dispersion ratio, clay dispersion ratio and water stable aggregation, determined by wet–sieving method of Kemper and Rosenau (1986). Aggregate stability was calculated as:

$$\frac{weight\ of\ stable\ aggregate - weight\ of\ sand}{weight\ of\ sample - weight\ of\ sand} \times 100 \tag{1}$$

Chemical properties analysed were:

i. Soil pH, determined using glass electrode pH meter in water in the ratio of 1:2.5 (Maclean, 1982).
ii. Organic carbon content, determined by wet dichromate acid oxidation method (Nelson and Sommers, 1982).
iii. Total nitrogen, determined using Kjeldahl apparatus (Bremner and Mulvaney, 1982).
iv. Exchangeable base, determined by ammonium acetate leaching.
v. Exchangeable acidity by titration (MaClean, 1982).
vi. Effective cation exchangeable capacity (ECEC), determined by summation of exchangeable bases and exchangeable acidity.

Statistical analysis

All data collected were statistically analyzed using Genstat 9.2 edition. A t-test was used to verify whether there were statistically

Table 1. Chemical properties of dry weight rumen digesta (RD).

Parameter	Value
pH H_2O	8.0
OC %	28.15
TN %	0.023
Na^+ (cmol/kg)	24.21
K^+ (cmol/kg)	28.68
Ca^{2+} (cmol/kg)	3.2
Mg^{2+} (cmol/kg)	4.6
Al^{3+} (cmol/kg)	0.00
H^+ (cmol/kg)	33.46

OC = Organic carbon; TN = total nitrogen.

Table 2. Initial soil analysis.

Parameter		Value
Physical properties	Clay (%)	20
	Silt (%)	4
	Total sand (%)	76
	Textural Class	Sandy Clay Loam
	WD Clay (%)	10
	WD Silt (%)	10
	DR	0.77
	CDR	0.5
	MWD (mm)	0.47
	AS (%)	53
Chemical properties	pH	3.1
	OC (%)	0.54
	OM (%)	0.93
	TN (%)	0.011
	Na^+ (cmol/kg)	0.22
	K^+ (cmol/kg)	0.39
	Ca^{2+} (cmol/kg)	1.2
	Mg^{2+} (cmol/kg)	2.4
	Al^{3+} (cmol/kg)	0.8
	H^+ (cmol/kg)	4.4
	CEC (cmol/kg)	6.4

WD clay = Water dispersible clay; WD silt = water dispersible silt; DR = Dispersion ratio; CDR = clay dispersion ratio; MWD = mean weight diameter; AS = aggregate stability; OC = organic carbon; OM = organic matter; TN = total nitrogen; CEC = cation exchange capacity.

significant differences ($LSD_{0.05}$) according to Steel and Torrie (1980).

RESULTS

The chemical properties of the rumen digesta are shown in Table 1. The pH value was 8.0 and alkaline in character. The physical and chemical properties of the soil before application of rumen digesta are presented in Table 2. The soil has sandy clay loam texture. The percentage of water dispersible (WD) clay and silt is 10%. Chemically, the soil is highly acidic (pH 3.1) with a low percentage of organic carbon (0.54%) and total nitrogen content (0.11%). Generally, the values of exchangeable cations (Ca^{2+}, Mg^{2+}, K^+ and Na^+) are low according to Mbagwu (1992).

Physical properties

The results of the soil particle size distribution (PSD) at different rates of rumen digesta application are presented in Table 3. The results show that there were no significant differences (p=0.05) between the %clay, %silt, and %total sand content and textural class of the treated soils and the untreated soils. The water-dispersible particle size distribution, dispersion ratio and clay dispersion ratio at different rates of rumen digesta application are presented in Table 4. There was no significant difference between the DR of the treated (0.76) and untreated (0.75 to 0.84) soils. The soil weight at >2, 2 to 1, 1 to 0.5, 0.5 to 0.25 and < 0.25 mm, mean weight diameter and aggregate stability at different rates of rumen digesta application are shown in Table 5. There were significant differences (p=0.05) between the mean weight diameter (MWD) of the treated and untreated soils.

Chemical properties

The soil pH in water, exchangeable acidity (Al and H), exchangeable bases (Ca, Mg, K, and Na), ECEC, total nitrogen, and organic matter at different rates of rumen digesta application are presented in Figure 1. As the rate of rumen digesta application increased, the soil pH, exchangeable sodium (Na), exchangeable potassium (K), effective cation exchange capacity (ECEC), and organic matter content increased, while the exchangeable acidity decreased. The result shows that untreated soil had high acidity (pH = 3.8) while application at 50 g rumen digesta gave a pH of 6.6 (Figure 1). At application rate of 150 g/kg, soil pH increased by 15.4% relative to at 50 g/kg. There were significant differences (p = 0.05) between the organic matter contents of untreated and treated soils. Organic matter content of the untreated soil was 0.97% and ranged from 2.38 to 4.29% for treated soils (Figure 1). The organic matter content was highest in soils with a rumen digesta treatment level of 150 g/kg soil. The total nitrogen obtained in the untreated soil was significantly different (p=0.05) from that of treated soils. The total nitrogen content of the untreated soil was 0.010% but

Table 3. Particle size distribution of the soils of Opi-Nsukka.

Application rate (g/kg)	Clay (%)	Silt (%)	Total sand (%)	Textural class
0	20	4	76	Sandy clay loam
50	21	5	74	Sandy clay loam
100	20	6	74	Sandy clay loam
150	19	5	76	Sandy clay loam
LSD$_{0.05}$	n.s	n.s	n.s	

n.s = Not significant.

Table 4. Water-dispersible particle size distribution of the soils of Opi-Nsukka.

Application rate (g/kg)	WD Clay %	WD Silt %	DR	CDR
0	9.0	12	0.89	0.47
50	9.7	12.3	0.85	0.48
100	10.3	11.3	0.72	0.47
150	10.0	10.3	0.72	0.50
LSD$_{0.05}$	1.331	2.491	0.0182	0.038
SD	0.577	1.080	0.0463	0.0645

WD clay = Water dispersible clay; WD silt = water dispersible silt; DR = dispersion ratio; CDR = clay dispersion ratio; SD = standard deviation.

Table 5. Percent water-stable aggregates, mean-weight diameter (MWD) and aggregate stability of the soils of Opi-Nsukka.

Application rate (g/Kg)	>2 mm	2-1 mm	1-0.5 mm	0.5-0.25 mm	<0.25 mm	MWD (mm)	AS (%)
0	0.28	6.15	25.19	33.61	34.77	0.49	54.7
50	7.98	15.29	28.25	25.59	22.89	0.84	69.3
100	26.55	18.65	18.53	16.77	19.49	1.40	72.7
150	34.26	13.99	14.49	12.79	20.24	1.75	75.3
LSD$_{0.05}$	8.60	4.875	3.919	3.764	6.99	0.2816	6.54
SD	3.73	2.114	1.699	1.632	3.03	0.1221	2.84

MWD = Mean weight diameter; AS = aggregate stability; LSD = least significant difference; SD = standard deviation.

ranged from 0.014 to 0.020 for treated soils. The highest nitrogen content (0.020%) of treated soils was obtained from the application of 150 g/kg of rumen digesta and it was higher than that of 100, 50 and 0 g/kgby 10, 30 and 50%, respectively. However, there were no significant differences between the total nitrogen obtained from the applications of 50, 100 g/kg soil rumen digesta and the control. Exchangeable cations in the soil (Ca^{2+}, K^+ and Na^+) apart from Mg^{2+} increased significantly (p=0.05) with increases in treatment application rate (Figure 1). Mg^{2+} content at application rates of 0, 50 and 100 g/kg were not significantly different (p=0.05) but was significantly different (p=0.05) from that of the control at 150 g/kg Mg^{2+} content. Exchangeable K^+ increased by 93.0% in application rate of 150 g/kg relative to that of 0 g/kg. The untreated soil contained 1.5 cmol/kg of Al^{3+} but after

amendment the Al^{3+} content was significantly reduced to 0.0 cmol/kg (Figure 1). Rumen digesta significantly reduced the H^+ content of the soils. The H^+ content of the untreated soils was 3.7 cmol/kg, while that of the treated soils were between 2.2 and 2.3 cmol/kg. Effective cation exchange capacity in the treated soils was significantly different (p=0.05) from that of the untreated soil. The ECEC in the controls was 7.2 cmol/kg while in the treated soils it varied from 11.1 to 14.9 cmol/kg (Figure 1).

DISCUSSION

The texture of the soils was determined before and after treatment with rumen digesta. Rumen digesta had no effect on the sandy clay loam texture of the soil (Table 3).
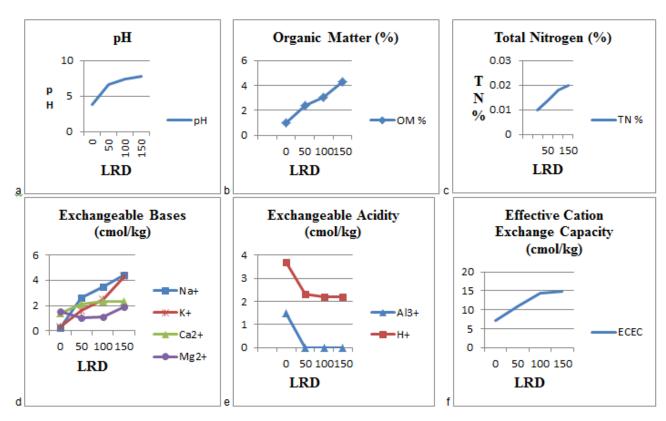
Figure 1. Effect of rumen digesta on the soil pH (a), organic matter content (b), total nitrogen (c), exchangeable bases (d), exchangeable acidity (e) and cation exchange capacity (f).

This could be due to the fact that texture is believed to be a fixed property. The non-significant difference between the DR of treated and untreated soils in Table 4 is similar to the result obtained by Mbah and Onweramadu (2009) which states that addition of soil organic amendments failed to significantly improve micro-aggregation (measured as dispersion ratio). In Table 5 there were significant differences (p=0.05) between the mean weight diameter (MWD) of the treated and untreated soils, especially at higher rate (150 g/kg) of rumen digesta application. The changes in water-stable aggregate, mean-weight diameter and aggregate stability could be due to application of the rumen digests. The results also show that there were significant (p=0.05) increases of the aggregate stability (AS) of the treated soils when compared to untreated soils at higher rates of rumen digesta application. This is probably due to the binding power of organic matter on soil particles to form stable aggregates. This result accords the research findings by Chaney and Swift (2006), El Hadj et al. (2013), Mbah and Onweremadu (2009) and Nwite (2013).

The results in Figure 1 show that application at rumen digesta at different rates correspondingly increased soil pH. This could be associated to a significant (p=0.05) increase in exchangeable cation contents in the soil colloidal complex. Low pH of 3.8 and 6.6 obtained in application rates of 0 and 50 g/kg might be associated

with the loss of exchangeable bases resulting from displacement reactions in the soil colloidal complex. Soil acidity has been blamed on excessive rainfall that causes eluviation and leaching losses of cations under field conditions. The rise in pH of the treated soils above 6 may have an effect on nutrient availability in the soil. A pH range of 6 to 7 is generally most favourable for plant growth because most plant nutrients are readily available in this range (NRCS, 1998). The improvement of soil pH in the treated soils confirms the liming effect of rumen digesta. Similar reports on the liming effect of organic materials were shown by Anon and Ubochi (2007); Duruigbo et al. (2006); Ekpe (2013); Okonkwo et al. (2009); Osemwota (2010). The application of rumen digesta at the rate of 150 g/kg soil gave the highest organic matter content relative to untreated soils (control). The increase in organic matter of the treated soils can be attributed to the increase in organic carbon and mineralization of the rumen digesta. This finding is in agreement with that of Ekpe (2013) and NRCS (1996) who noted that applying animal manure increases the supply of organic matter in the soil. The total nitrogen content of the untreated soil was found significantly different from that of treated soils. The highest nitrogen content of treated soils was obtained from the application of 150 g/kg of rumen digesta.

These findings are in agreement with that of Awodun

(2008) and Okonkwo et al. (2009) who noted that mineralization of organic wastes results in the release of organic bound nutrients in the soil notably N.P.K. Differences between the total nitrogen obtained from the applications of 50, 100 g/kg soil rumen digesta and the control were not significant. This may be related to the lower quantity of nitrogen at these application rates that can easily volatilize. A report by Prasad and De Datta (1979) had shown that a pungent smell emanating from organic waste indicate loss insubstantial amount of nitrogen through volatilization. Significant increase in exchangeable cations (Ca^{2+}, K^+, and Na^+) of the soil apart from Mg^{2+} due to increases in treatment application rates (Figure 1), suggest that rumen digesta improved the exchangeable base contents in the soil. The values of exchangeable cations from application rates of 50 to 150 g/kg being higher than the critical values of 0.16 to 0.20 cmol/kg implies that they may not be limiting crop production in the subtropical area (Isirima et al., 2003; as cited in Ezeaku, 2011). Findings are in agreement with those reported by Osemwota (2010). The increases in values may be as a result of increased soil pH, which invariably has a liming effect on the soil. NRCS (1998) noted that increase in soil pH increases the availability of exchangeable bases. Increases in exchangeable bases due to application of organic residues have also been reported by Mbagwu (1992). The application of rumen digesta significantly reduced the exchangeable acidity of the treated soils when compared to untreated soils. This result is in accordance with other works where organic manure reduced the Al^{3+} and H^+ content of the soil (Agboola and Odeyemi, 1972; Charreau, 1975; Enueke et al., 2013; Nwite et al., 2012).

Effective cation exchange capacity in the treated soils was found significantly different (p=0.05) from that of the untreated soil (Figure 1). This result is similar to other reports (Asadu and Nweke, 1999; Egawa, 1975; Nwite et al., 2012) that showed increases in ECEC of the soil due to increase in organic matter content. This is also in agreement with NRCS (1996) which noted that organic matter retains nutrients by providing cation and anion exchange capacities. For the soils of the tropics, values of ECEC between 6 and 8 cmol/kg was regarded as low, 8 and 11 cmol/kg as medium and >12 cmol/kg as high (Enwezor et al., 1990; cited in Ezeaku, 2011). Based on these limits, the ECEC obtained in application rates of 0 g/kg (control) and 50 g/kg was low and medium, respectively (Figure 1). The values obtained at application rates of 100 and 150 g/kg were high, suggesting that higher application of rumen digesta in the soil would significantly improve the effective cation exchange capacity of the soil.

Conclusion

Results of the study reveal that rumen digesta significantly increased the mean weight diameter (MWD), aggre-

gate stability (AS), soil pH, total nitrogen (TN), exchangeable sodium (Na), exchangeable potassium (K), effective cation exchange capacity (ECEC) and organic matter content of a soil. It had no significant effect on the texture, exchangeable calcium (Ca) and exchangeable magnesium (Mg) content of the soil, although it significantly reduced the exchangeable aluminum (Al) and hydrogen (H) content. Rumen digesta is a source of organic manure. For improved soil sustainability and crop productivity, it is recommended that 100 g of rumen digesta/kg soil should be applied to improve the physico-chemical properties of the soils of Opi. This level gave the best results, and at levels higher than this, the soil would become alkaline/saline, which would have a negative effect on both the soil and crop productivity. Rumen digesta is not always available in the sufficient amount of quantity needed by the large scale farmers; therefore a complementary use of rumen digesta and inorganic fertilizers is recommended. This study also recommends the optimal use of rumen digesta as a form of liming material, but with caution to avoid making the soil too alkaline.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Estimation of heritability and genetic gain in height growth in *Ceiba pentandra*

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Ceiba pentandra (L.) has been selected for plantation establishment in Ghana under the National Forest Plantation Development Programme of Ghana (NFPDP). This has led to a rise in demand for its seedlings for large scale commercial plantation establishments with seed of *C. pentandra* being collected from the wild and used to raise seedlings to meet the demands of the programme. The primary purpose of most of these plantations is timber with height growth as one of the key economic traits. However, there is relatively inefficient information available on the heritability and genetic gain in height growth in *C. pentandra* based on which selection and subsequent breeding could be made. This poses a major challenge to the production of new cultivars for the forestry industry of Ghana. The current study looked at the estimation of narrow sense heritability (h^2) and genetic gain in height growth in *C. pentandra* using 37 accessions from Ghana. Narrow sense heritability was 0.56 and genetic gain in height ranged from -52.82 to 37.30 cm/yr. Twenty-two (22) accessions (59.46%) had mean height increment above the overall mean performance and were recommended for conservation as seed trees. The results were discussed in relation with the conservation of *C. pentandra* genetic resources and the potential of accessions for high genetic gains.

Key words: *Ceiba pentandra*, narrow sense heritability, genetic gain, height growth, stem dieback disease.

INTRODUCTION

Heritability is the measure of the degree to which parents transfer heritable characteristics to their offspring (Jansson, 2005). The differences in the degree of transfer of these characteristics result in variation in genotypes among the offspring. This variation is usually referred to as genetic variance (additive and non-additive variance) and that of the environment as environmental variance (Lavi et al., 1993; Suzuki et al., 1986). Additive genetic variance is responsible for the similarities between relatives and the population in response to selection (Lavi et al., 1993) and mostly transmissible by seed (Wright, 1976). Generally, heritability is useful, among other things, in predicting genetic gain from selection (Paikhomba et al., 2014; Holland et al., 2003) and in

selecting superior phenotypes on the basis of the phenotypic performance of quantitative characters (Vasudeva et al., 2004). In trees, several traits are known to be under genetic influence including growth rate, fibre yield, photosynthetic efficiency and pest resistance (Namkoong et al., 1980). Selection of genotypes based on the phenotypes becomes difficult when heritability is low (Jansson, 2005) whereas genetic gain increases with increase in heritability (Mckeand et al., 2008). Further, characters with high heritability also has high additive effect and respond effectively on phenotypic selection (Lewis et al., 2010). Several methods of estimation of heritability have been proposed, including the use of the variance components from the analysis of variance tables and from parent-offspring regression lines. The later is practice when heritable traits from both parents and offsprings are measured which in most cases is difficult to achieve due to the long regeneration time of forest trees. The use of half-sib, full-sib or clonal tests allow the partitioning of the phenotypic variances into genetic and environmental components using analysis of variance (Jansson, 2005).

Ceiba pentandra (L.) has been selected for plantation establishment in Ghana with the onset of the National Forest Plantation Programme in Ghana (NFPDP) (Ghana Forest Service Division, 2005). The wood is used for making stools in the Ashanti Region of Ghana, domestic utensils, boxes, wooden figures, quivers, drums, dugout canoes, plates, trays and coffins, especially, in the Ada area of Ghana (Burkill, 1985; Irvine, 1961). It is also suitable for wooden sandals, heels, rafts, floats, lifeboats, models, particle board and for papermaking. The seeds are very rich in unsaturated fatty acids such as palmitic, linoleic, oleic, and stearic acids (Burkill, 1985). The fibre, commonly called kapok, is used for stuffing cushions, pillows and mattresses, insulation, as an absorbent material and tinder. The gum is eaten to relieve stomach upset, whereas the leaves and fruits are used as a laxative and infusion from the leaf is used for colic treatment in human and livestock (Irvine, 1961). Therefore, demand its seedlings for large scale commercial plantation establishment has become inevitable. As such, seeds of *C. pentandra* are being collected from the wild and are being to raise seedlings to meet this demand. However, there is relatively inefficient information available on the heritability and genetic gain in height growth in *C. pentandra* base on which selection and subsequent breeding could be made. Also, dieback of *C. pentandra* seedlings and saplings has been observed in both the nursery and plantation (Apetorgbor et al., 2003) and it poses a great threat to large scale commercial establishment of this species and puts high investment in this direction at risk. There is therefore the need for the identification and use of resistant stock. The estimation of genetic parameters, such as prediction of breeding values, is also essential for the selection of genetic resistant genotypes.

Height is a key economic trait in plantation development of this species since growth in height depends on the level of resistance to dieback or recuperative ability after fungal infection. Also, height is a composite trait with different growth components and its variation is under different degrees of inheritance. A better understanding of the genetics of height growth components will result in a better understanding of it and simplifies selection in tree breeding (Rweyongeza et al., 2003).

The rationale of the study was that wild, undomesticated, out-bred forest trees are highly variable and their natural populations retain a wide genetic diversity that helps them resist pests and diseases. The objective of the study was to estimate the level of narrow sense heritability and genetic gain in height growth of C. *pentandra*.

MATERIALS AND METHODS

Study site

The field test was done at Abofour, one of the research plots of the Forestry Research Institute of Ghana of the Council for Scientific and Industrial Research. Abofour is located between latitude 6.15º N and longitude 1.10º W in the Afram Headwaters Forest Reserve. It falls within the Dry Semi-Deciduous Forest Zone with mean annual precipitation in the range of 1250 to 1500 mm (Hall and Swaine, 1981). The reserved was created in 1908 and it is about 123.3 sq-km (47.6 sq-miles). Several factors influenced the choice of this site: It is one of the sites which is most likely to be reforested with the end products of the planting stock that is being assessed and it is neither too dry (compared to the Savanna Zone) nor too wet (compared to the Moist/Wet Evergreen Zones).

Progeny trials

Seeds from 37 half-sib families (open-pollinated trees in which only one parent; the female, is known) were used in this study. The seeds were germinated and screened maintained under shed made from palm branches. The seedlings were transplanted to the field at the age of five months. The design for field testing was done using 30 seedlings per accession in randomized complete block design (RCBD) with three replications each. RCBD was used to control variation in spatial effects in the field so that observed differences are majorly due to true differences between treatments. Ten seedlings were planted in each block in two rows with 5 seedlings per row. The planting distance was 2.5 m × 2.5 m within and between rows. Weed controlled was done by manual weeding at least once every month after planting.

Data collection and analysis

Growth in height was estimated as y_2-y_1, where y_1 and y_2 are height at time of planting and 12 months, respectively. Data were subjected to analysis of variance using Microsoft Excel 2007 at a P-value of 0.05. Mortality data were arc-sine transformed (Snedecor and Cochran, 1980) before subjecting the data to analysis of variance. Standard errors of mean height were also computed to depict the variation about the means. The procedure proposed by Zobel and Talbert (1991) was used to estimate the narrow sense heritability (h^2), selection differential and the genetic gain. This

Table 1. Analysis of variance.

Source of variation	Df	MS	F
Between accessions	f-1	MS_1	MS_1/MS_3
Blocks	r-1	MS_2	
Within accessions	f (r-1)	MS_3	

Table 2. Analysis of variance in height growth.

Source of variation	df	SS	MS	F	P-value	F crit
Between accessions	36	163693.80	4547.05	2.26	0.00	1.58
Blocks	2	27205.45	13602.73	6.77	0.20	8.12
Within accessions	72	144644.50	2008.95			
Total	110	335543.70				

procedure is as described below:

$$h^2 = V_G/V_P \quad (1)$$
$$V_P = V_G+V_E \quad (2)$$

Where, h^2 = Narrow sense heritability, V_G = Variations arising from differences in genetic constitution (between accessions variation), V_P = Variations in phenotypic characters, V_E = Variation due to environment (within accessions variation).

From the analysis of variance table (Table 1),

$$V_G = (MS_1-MS_3)/r \quad (3)$$

Where, MS_1 = Mean square between accessions =

$$V_E + rV_G \quad (4)$$

MS_2 = Mean square of blocks, MS_3 =Mean square among accessions = V_E, r =Number of replications = number of blocks.

The selection differential (S) was estimated as shown below.

$$S = Xs - X\mu \quad (5)$$

Where, S = Selection differential (difference between mean of selected individual and the population mean (Jansson, 2005)), $X\mu$ = Mean of population, Xs = Mean phenotypic value after selection (sample mean), Genetic gain (G) was estimated as:

$$G = \text{Percentage gain} *h^2 \quad (6)$$

Where, h^2 = Narrow sense heritability.

RESULTS

Mean height increment was significantly different (P < 0.001) among the 37 accessions studied (Table 2). Height increment, selection differential, narrow sense heritability and genetic gain varied among the accessions (Table 3). Narrow sense heritability (h^2) in height growth was 0.56 indicating that 56% or more of the observed variation in height growth was genetically controlled.

Genetic gain varied from 20.89% (DNY 1) above mean performance to as low as -29.58% (TAN 1) below mean performance. Twenty-two accessions (59.46%), out of the 37 accessions screened, had mean height increment above the overall mean height growth of 106.24 cm/yr. The genetic gain in height growth for selecting a particular accession is shown in Table 3.

Estimation of selection differential, narrow sense heritability and genetic gain in individual accessions

From equation 3 above, V_G was estimated as:

$$V_G = MS_1 - MS_3/r$$

From the analysis of variance table (Tables 1 and 2)

$MS_1 = 4547.05$, $MS_3 = 2008.95$ and r = 3

$$V_G = (4547.05 - 2008.95)/3 = 846.03$$

From the analysis of variance (Tables 1 and 2)

$$V_E = MS_3/r = 2008.95/3 = 669.65$$

From equation 2, V_P was estimated as:

$$V_P = V_G+V_E = 846.03 + 669.65 = 1515.68$$

From equation 1, h^2 was estimated as:

$$h^2 = V_G/V_P = 846.03 /1515.68 = 0.56$$

Taking accession DNY 1 for instance. From equation 5, selection differential/deviation (S) was estimated as:

$$S = Xs - X\mu$$

Xs = sample mean (mean height increment of accession

Table 3. Height increment and genetic gain in height growth among 37 accessions of *C. pentandra*

Accession	Mean height (cm/yr)	Deviation	Percentage deviation	Percentage gain	Genetic gain	Rank
DNY 1	177.00	70.76	66.61	37.30	20.89	1
KON 8	163.27	57.03	53.68	30.06	16.83	2
AYE 1	157.62	51.38	48.36	27.08	15.16	3
SOF 1	157.39	51.15	48.15	26.96	15.10	4
ODO 6	150.82	44.58	41.96	23.50	13.16	5
SKD 1	148.87	42.63	40.13	22.47	12.58	6
BUF 1	140.73	34.49	32.46	18.18	10.18	7
NKIN 2	138.65	32.41	30.50	17.08	9.56	8
POK 1	131.78	25.54	24.04	13.46	7.54	9
KWA 1	129.61	23.37	22.00	12.32	6.90	10
MAM 1	129.61	23.37	22.00	12.32	6.90	11
KUE 2	127.33	21.09	19.85	11.12	6.23	12
NKA 2	124.92	18.68	17.58	9.84	5.51	13
ABF 1	123.14	16.90	15.91	8.91	4.99	14
ACH 1	118.48	12.24	11.53	6.46	3.62	15
EDW 1	117.79	11.55	10.87	6.09	3.41	16
GYE 1	116.80	10.56	9.94	5.57	3.12	17
NKA 1	115.98	9.74	9.16	5.13	2.87	18
DAN 1	115.30	9.06	8.53	4.78	2.68	19
NKIN 1	113.07	6.83	6.43	3.60	2.02	20
KEC 10	109.59	3.35	3.15	1.76	0.99	21
KUE 1	109.38	3.14	2.96	1.66	0.93	22
ASE 1	104.38	-1.86	-1.75	-0.98	-0.55	23
AYE 2	96.48	-9.76	-9.18	-5.14	-2.88	24
KUE 3	91.47	-14.77	-13.91	-7.79	-4.36	25
KEC 9	88.97	-17.27	-16.26	-9.11	-5.10	26
BAW 10	83.20	-23.04	-21.69	-12.15	-6.80	27
KON 9	82.37	-23.87	-22.47	-12.58	-7.04	28
MSH 1	75.17	-31.07	-29.25	-16.38	-9.17	29
BAW 1	73.49	-32.75	-30.83	-17.26	-9.67	30
MAA 1	59.38	-46.86	-44.11	-24.70	-13.83	31
BAW 4	55.28	-50.96	-47.96	-26.86	-15.04	32
PAW 5	52.09	-54.15	-50.97	-28.54	-15.98	33
KON 13	49.30	-56.94	-53.59	-30.01	-16.81	34
WOA 1	48.92	-57.32	-53.96	-30.22	-16.92	35
BAW 5	47.15	-59.09	-55.62	-31.15	-17.44	36
TAN1	6.03	-100.21	-94.33	-52.82	-29.58	37
Mean	106.24					

DNY 1)

X_μ = population mean (mean height increment of all the 37 accessions)

S = 177.00 -106.24

= 70.76 cm/yr (See Table 3).

$$\text{Percentage deviation} = \frac{\text{Deviation} \times 100\%}{\text{Mean}}$$

$$= \frac{70.76 \times 100\%}{106.24}$$

= 66.60%

Percentage gain = percentage deviation x heritability = 66.60 x 0.56
= 37.3 % (Table 3)

Genetic gain = percentage gain x heritability = 37.3 x 0.56

= 20.89

This procedure was repeated for all the accessions. The results produced are as shown in Table 3.

Estimation of selection differentials and genetic gain in the 22 selected accessions (accessions with mean height performance above the overall mean. These are ranked 1 to 22 in Table 3).

$$S = X_s - X_\mu.$$

Where, X_s = sample mean (mean height increment of the 22 selected accessions), X_μ = population mean (mean height increment of all the 37 accessions).

$$S = 132.60 - 106.24$$
$$= 26.36 \text{ cm/yr}$$

$$\text{Percentage deviation} = \frac{26.36 \times 100\%}{132.60}$$
$$= 19.88 \%$$

Percentage gain = percentage deviation x heritability = 19.88 x 0.56
$$= 11.13 \%$$

DISCUSSION

The narrow sense heritability estimated was 0.56. Since heritability is a measure of the degree to which parents transfer heritable traits to their offspring (Jansson, 2005; Zobel and Talbert, 1991), the variation in height that is genetically controlled is higher or equal to 56**%**. This value is similar to results from other studies in forest trees. For instance, heritability values of 0.74 and 0.51 were recorded in the total height growth of two-year old *Khaya anthotheca* and *Khaya ivorensis* respectively (Ofori et al., 2007). McKeand et al. (2008) also reported narrow sense heritability in height growth of Loblolly pine to range from 0 to 0.62 at different planting sites. A positive correlation is known to exist between phenotype and breeding value, increased potential for natural selection and high heritability (Paikhomba et al., 2014; Jansson, 2005). According to Santos et al. (2014), high heritability enables greater dynamism in the breeding program, allowing recombination of the best individuals in a shorter period of time. The high heritability obtained in the current study, therefore, suggests that selection of individuals based on height increment has the potential to retain high productivity in future generation of the species. Heritability estimates are known to be population, trait and test environment specific (Gonçalves et al., 2009; Jansson, 2005). Also, tree characteristics vary in the degree of genetic versus environmental influence and

that genetically inferior trees may appear phenotypically desirable if planted in an unusually favourable micro-environment. Likewise, genetically superior trees may appear phenotypically undesirable due to poor environmental conditions (Frampton, 1996). Hence, the accessions which performed below average could possibly do well if planted in a different test environment and those which performed above the mean could possibly perform poorly if planted in another environment. It is therefore, suggested that this study be repeated in all the ecological zones and the differences in genetic gain be established.

The values of the genetic gain (-29.58 to 20.89 cm/yr) obtained in the study indicate the presence of different levels of resistance to dieback and, hence, a high potential for selection of dieback tolerant individuals. The 22 accessions that had mean height above the mean performance (Table 3) were, therefore, recommended for selection, protection and use as seed trees. Selection is preferred, in a decreasing order, from DNY1 to KUE 1. This followed the recommendations of Namkoong et al. (2000) that only genotypes whose phenotypes approximate the population mean are good for selection while those below should be considered as selection disadvantage. *C. pentandra* is known to have a pan tropical distribution (Lobo et al., 2005) and to be a widespread rainforest tree species (Dick et al., 2007). In Ghana, it is found in all the forest types (Hall and Swaine, 1981). This suggests that the species has a wide environmental adaptability. However, the 44% variability attributable to the growth environment suggests that some environments may be more suitable for the growth of the species than others.

Selection becomes a more effective tool in genetic improvement when all traits of economic importance are evaluated (Silva et al., 2008). Therefore, this study should be repeated and heritability estimated based on other economic traits, such as seed size, seed weight, seed colour, number of seeds per pod, leaf size, leaf length, leaf width, petiole length, petiole colour, degree of spines, stem colour and stem diameter. This will enable the accurate prediction of genetic gain for efficient selection in *C. pentandra* and also enhances its use as a multipurpose species.

Conclusion

At 12 months, there was significant variation in tree heights among accessions across the country and this is important for tree breeders who would like to carry out selection as early as possible. It could be concluded that greater gains in plantation yield of *C. pentandra* could be realized by selecting fast growing accessions. Therefore, based on these results, 22 accessions were selected from a total of 37 accessions to advance their generations. Though the genetic gains estimated in the study were encouraging, higher values could be obtained when

full-sib progenies are used. It is therefore, suggested that a breeding program for *C. pentandra* be initiated to enhance its profitability in the future. This is the first study to demonstrate the existence of variability in narrow sense heritability and genetic gain in *C. pentandra* and it has important practical implications for genetic resource management and tree improvement programs in Ghana. The detection of genetic differences at an early age would therefore be very useful for agroforestry tree improvement programs in Ghana.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of genetic diversity in barley (*Hordeum vulgare* L.) from Wollo high land areas using agro-morphological traits and hordein

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This study aimed to determine the genetic diversity and relationships among barley varieties (*Hordeum vulgare* L.) growing at Wollo Highland areas by using hordein and agro-morphological traits. Twenty (20) varieties were laid down in randomized complete block design (RCBD) design with three replications; they were planted by irrigation at Wollo University, Dessie Campus from January to May 2014. The genetic analysis using hordein was done in the laboratory of Ethiopian Biodiversity Institute (EBI) in July 2014. Ten (10) competitive random plants from the rows of the experimental plots were taken for recording their agro-morphological characters. Electrophoretic separation of barley storage proteins or hordeins was done using acid polyacrylamide gel electrophoresis (A-PAGE). The traits: day of heading, day of maturity, grain yield (kg/ha), plant height, spike length, number of spiklet per spike, kernel number per spike, weight of seed per spike and biomass yield (g/plot) were highly significant for the diversity of barely, whereas thousand seed weight was less significant. The results reveal positive correlation between spike length and number of spiklet per spike (the highest correlations from the agro morphological traits); the next highly correlated traits were kernel weight per spike and thousand seed weight. The A-PAGE analysis showed limited variation among the analysed accessions. The Nei's genetic distance for all varieties of barely varied from 0.0000 to 1.6094. It is found that the 20 genotypes of barely investigated in this research were having a gene diversity (h) of overall populations (0.138) using hordein. The cluster analysis grouped the 20 barely genotypes into three different clusters using agro-morphological traits and into four clusters using hordein. This indicates the presence of wide diversity among the tested genotypes. From cluster mean values of agro-morphological traits, genotypes in cluster III deserve consideration for directly developing high yielding barely varieties. The result of the principal components analysis revealed that the first three principal components having greater than 1 eigenvalue contributed 84.22% of the total variation. From this study, it can be concluded that the presence of high morphological variation indicated the potential of Wollo Highland areas in contributing to barley improvement and conservation activities of land areas.

Key words: Acid polyacrylamide gel electrophoresis, agro-morphological traits, hordein, genetic distance, hordeum vulgare, variability.

INTRODUCTION

Evaluation and assessment of genetic diversity in crop species is fundamental for their improvement. The study of genetic diversity is the process by which variation among varieties or groups of individuals or populations is analysed by a specific method or a combination of methods. Genetic diversity assessment with different methods and their comparison could provide complementary information on improvement and conservation

programmes.

Criteria for the estimation of genetic diversity can be different: pedigree records, morphological traits, biochemical markers and molecular markers (Eshghi and Akhundova, 2010). In order to obtain a good insight into the available variation and into the structure of landraces, it is necessary to assess the level of variation between as well as within a representative set of landraces. This information is also important for the maintenance and future use of the varieties.

According to Muhie and Assefa (2011), knowledge of the genetic diversity and agronomic potential of barley landraces in variable environments is an important task to design strategic utilization, targeted collections and introductions of germplasms. In line with this, Tefera (2012) indicated that barley is cultivated by smallholders in every region of Ethiopia, since it is able to grow in all elevations; though it performs best at higher elevations in the northern and central regions of the country.

Among the cereal crops, barley is a species with the greatest adaptability to a wide range of environments. In terms of the area and production worldwide, barley is the fourth most important cereal after wheat, rice and maize (Abebe, 2010). At the global level, the most important cereal crops are maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare* spp. *vulgare*), with a total of 2.4 billion tons produced annually at a value of more than 446 billion Int. $ (FAOSTAT, 2012). All four of these crops are members of the Poaceae family.

Barley is cultivated from arctic latitudes to tropical areas, and it is grown at the highest altitudes. It is cultivated from 1,400 metre above sea level (m.a.s.l.) to over 4,000 m.a.s.l, and it has adapted to specific sets of agro-ecological areas (Alemayehu and Parlevliet, 1997). Barley is adapted to a broad range of agro-ecological environments and it is tolerant to soil salinity, drought and frost to a considerable level. The crop successfully grows in the arid climates of the Sahara, the Tibetan plateaus, the highlands of the Himalayas, and the Andean countries, the tropical plains of India and the mountains of Ethiopia (Abebe et al., 2010). As barley is one of the major cereals grown in wide agro-ecology of the country like Ethiopia, it has immense economic and social importance for Ethiopians.

In its ambitious five-year growth and transformation plan, the Government of Ethiopia aimed to double the production of grains by 2015 (Tefera, 2012). However, the land in the densely-populated highlands and semi-highlands is fully utilized; therefore, there is little chance for increased area planted with highland crops, especially wheat and barley (Tefera, 2012). It is the most important crop with total area coverage of 1,129,112 ha and total

annual production of about 1.7 million tons in main season (CSA, 2010). As a highland crop, there is little to increase in the area planted with barley; the small increase in 2011/12 and forecast for 2012/13 are due to heightened interest by local breweries and local malt producer (Tefera, 2012). Therefore, based on this, there is a need to identify the variety of better traits leading to better production of Wollo Highland areas.

According to Alemayehu and Parlevliet (1997), landraces of such crops are expected to consist of more or less homozygous plants. The observed variation on and within landraces was very large for all traits and the magnitude of variation was so large that most, if not all, plants within a landrace had a different genotype. The landraces also varied from the degree of variation and they added that in order to obtain a good insight into the available variation and the structure of Ethiopian landraces, it is necessary to assess the level of variation between as well as within representatives of landraces. Variation between the Ethiopian barley landraces has been observed by Bekele (1983) and Asfaw (1988, 1990). The increase in barley yields over the recent period has largely affected the introduction of more productive cultivars into farming practice.

In determining the productive varieties analysis of storage protein, hordein and monomeric prolamins (having a great inter-genotypic variation) have been used as marker in cultivar identification, genetic diversity studies, and phylogeny origins in barley (Eshghi and Akhundova, 2009). Potential grain yield of a given cultivar depends on various characters including the plant growth habit. However, there is a lack of information on different responses of new barley cultivars to delayed sowing date as expressed by plant morphological characters and grain yield (Noworolnik, 2012).

In contrast to the genetic uniformity of modern cultivars, landraces show variation both between and within populations. Landraces represent a very interesting model for studying the processes of adaptation and identifying genes and genomic regions that have adaptive roles in a crop species (Hadado et al., 2010). Though there are studies on Ethiopian barley, such as phenotypic genetic diversity in relation to altitude (Engels, 1994), eco geographical distribution of isozyme, allozyme and hordein alleles (Bekele, 1983; Demissie and Bjornstad 1996), and the relationships between hordein and morphotype variation (Asfaw, 1989), the information obtained is still limited. The experiment is done mainly in the limited region of the country (Assefa and Labuschagne, 2004; Abay et al., 2009; Hadado et al., 2009) and out of the area where the farmers try to grow the crop. On the other hand, Engels (1994) reported that only a small fraction of the total phenotypic diversity was

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present among administrative regions, while almost all of the characters were considerably influenced by difference in altitude within regions.

Therefore, the objectives of this study are to assess and evaluate the barley accessions that are grown at the highlands of Wollo Province using agro-morphological and protein variation (hordeins) and to identify groups of accessions with desirable and major traits that contribute to the overall observed diversity as well as barley improvement and conservation in the study area.

MATERIALS AND METHODS

Experimental site

The field experiment was carried out at Wollo University, Dessie Campus from January to May 2014 using irrigation. The research station has 38°, 39´ E longitude and 8°, 11´N latitude with an elevation between 2470 and 2550 meters above sea level; the mean annual maximum and minimum temperatures are 24 and 8°C. The storage protein (hordein) analysis was done at Ethiopian Biodiversity Institute (EBI), Addis Ababa, Ethiopia, Laboratory in July 2014.

Plant material and design

A total of 20 barely genotypes, representing 15 landraces and five released varieties were used for the study. From the 15 landraces, eight were obtained from EBI (202802, 217126, 217125, 202793, 202813, 217119, 225241 and 202799) and the remaining seven (Arusigebs, Anbedat, Enatgebs, Workye, Temaj, Nechita, Wogera) were collected by the researcher; the five released varieties (Estayish, Trit, Yedogit, Shedeho and Agegnehu) were obtained from Sirinka Agricultural Research Centre.

The genotypes were planted in January 2014 in randomized complete block design (RCBD) with three replications. Seed rate of 85 kg/ha was used and sown by hand drilling. Each treatment was planted in a plot area of 2 m^2 consisting of five rows 2 m long, spaced 0.2 m apart between rows and 0.5 m between plots, respectively. An approximate distance of 10 cm was maintained between plant to plant by hand thinning. Ten (10) competitive random plants from the middle rows of the experimental plots were taken for recording the agro-morphological characters. The first weeding was carried out at 21 days after crop planting and the second weeding was performed 15 days after the first weeding. A total of four weeding was done. The experiment was regularly prevented from bird damage. Generally, maximum care was taken in the experiment to minimize the possible occurrence of yield limiting factors which could affect yield potential performance of the varieties. The DAP was applied during planting at the rate of 60 kg/ha. Total nitrogen was applied at rate of 60 kg/ha as urea in two splits: first split (½) and the second split (½) of the total dose during planting and mid-tillering stages, respectively.

Data collection

Agro-morphological data

Data were recorded for quantitative characters using barley descriptors (IPGRI, 1994). The descriptions used in the study were according to IPGRI (1994) including plant based characters in which 10 plants from each plot were selected randomly and then tagged with threads. Data were collected for spike length (SL), number of seeds per spike (NSpS), plant height (PH), number of

spikelet per spike (NSKPS), and weight of seed per spike (WSPS). Whereas on plot bases, the traits that were recorded were days to heading (DH), days to maturity (DM), thousand seed weight (TSW), biomass yield (BY) (g/plot), and grain yield (GY) (g/plot).

Methods used in Hordein extraction and identification

Hordein representing major group of storage proteins was extracted from each dry seed in buffered alcohol according to Shewry et al. (1985), with some modifications. Identification of several proteins extracted with various solvents from barley grains was performed by acid polyacrylamide gel (acid-PAGE).

Sample preparation for gliadin (hordein) analysis of barely

For the analysed accessions, 16 seeds per accession and for the released varieties five seeds from each variety were used. Seeds were mechanically crushed into powder and transferred into a capped eppendorf tube. Propanol (55%) containing 2% B - mercaptoethanol was used for extraction and 250 micro litre of extraction buffer was added to each eppendorf tube containing barely powder and vortex and kept at room temperature overnight. 180 µl of loading buffer was added before electrophoresis and vortex, and kept at room temperature until use .

Single barley seed was crushed and ground individually to fine powders with a pestle in a mortar. The powders were extracted with 250 µl extracting solvent in test tubes by mixing vigorously on a vortex mixer at 15-min intervals over a 1-h period.

Gel preparation

Twenty micro litre of hydrogen per oxide (15%) was added to 150 ml gel solution (acrylamide gel); it was mixed well and poured in the gel cast. Combs were inserted and allowed to polymerize for about 10 min.

Sample loading and running

Extra gel was removed from the surface and 250 ml of aluminium lactate buffer was added. Then the combs were removed. Thirty five microliter of sample was transferred into each well with a micropipette. The upper gel cast was kept in the lower buffer tank and connected to power supply. Finally, it was run for about 2 and half hours at 50, 150 and 250 v for 10 min each, 350 v for 30 min and 550 v for one and half hour. At the end of the run, the power supply was switched off and the gel cast was taken out. The side clips were removed. The side of the gel was cut from the starting position to mark the orientation of the samples.

Staining and destaining

Gel was removed from the glass and kept in staining boxes after the addition of tri-chloro acetic acid (TCA) at room temperature for 20 min. The fixing solution was poured back into another material from staining boxes and staining solution was added and kept for one or two days. Finally, the gel was de-stained using tap water. Gel pictures were taken using digital camera and scoring was done by scoring band as present (1) and absent (0).

Analysis of data

Agro-morphological traits analysis

Each variable was subjected to cluster analyses and principal

Dendrogram using Average Linkage (Between Groups)
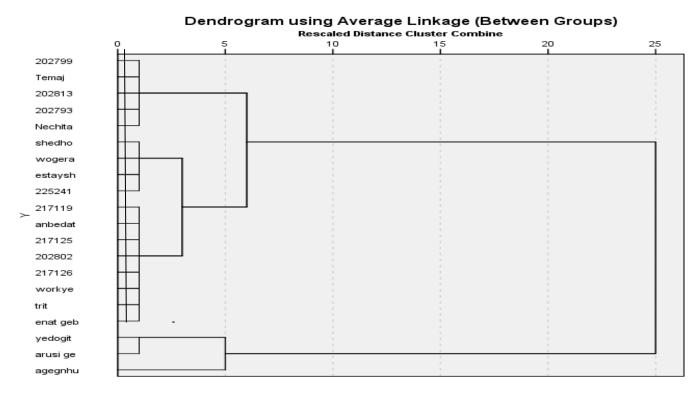Rescaled Distance Cluster Combine

Figure 1. Dendrogram of barley genotypes constructed using agro-morphological traits.

component analysis, and the test of comparison between mean was done. For all traits, cluster mean analysis was used to compare and classify the observed variation in the varieties. For all the traits assessed on individual sample plant basis, the means of the sample plants from each row were used for analyses. The mean and variance of agro-morphological traits was done using GENStat (13th edition) and for the principal component, SAS software (SAS, 9.01) (SAS, 2004) was used. Dendrogram of 20 barley genotypes was done using hierarchical analysis, between–groups linkage methods of SPSS version 20 statistical software based on the agro-morphological traits (Figure 1).

Analysis of barley storage proteins

In order to obtain a grouping by quantitative estimate of variation based on data of the major hordein bands genetic distance matrix was done using Popgene 1.31 and cluster analysis was done using TFPGA 1.3. Hordein bands were scored as either present (1) or absent (0), and these bands were used to calculate genetic similarity and distance among all the twenty varieties. Nei (1978)'s Unbiased Measures of Genetic Identity and Genetic distance generated for the twenty barely varieties was employed to see the genetic distance and genetic identity using pop gene software 1.31.The genetic distance analysis was employed to assess the pattern of genetic diversity among the 20 barely varieties using the standard genetic distances.

RESULTS AND DISCUSSION

Analysis of variance

A wide range of values were observed in agro-morphological

traits of the studied barley genotypes (Table 1). Grain yield exhibited the widest range (2258 to 6202 kg/ha) followed by biomass yield per plot (1483 to 2733 gm), plant height (82.9 to 118.1 cm) and days to maturity (110.3 to 137). Derbew et al. (2013) reported that grain yield exhibited the widest range (436 to 3752.5 kg/ ha) followed by plant height (44.95 to 94.1 cm), days to maturity (92 to 131) and heading (57 to 94). In this study, the genotypes had broad range of grain yield, narrow ranges in plant height and days to maturity compared with the work of Derbew et al. (2013). The early maturing genotypes reached physiological maturity in 110.3 days while the late maturing genotype took 137 days to mature. Anbedat and Temaj were early maturing geno-types and Nechita was late maturing genotype. With regard to grain yield, Arusi gebs was the highest yielding genotype and 202799 was low yielding genotype (Table 2).

With regard to days of heading and plant height, the barely varieties used in this research show narrow range of variation compared to the varieties used by Alemayehu and Parlevliet (1997) in which they reported 62 to 97 days and 70.5 to 112.2 cm for heading and plant height, respectively. Lakew et al. (1997) obtained a wide range of variation for days to heading (96 to 116), maturity (137 to 174), plant height (80 - 140 cm) and grain yield (4202 to 5705 kg/ha). In this study, wide range of variation was observed with grain yield and days of heading and narrow range of variation in days to maturity and plant height than the genotypes used by Lakew et al. (1997).

Table 1. Mean, minimum and maximum values of and range of agro morphological traits.

Agro morphological trait	Range (min to max)		Range unit	Mean
	Min	**Max**		
Days to heading	75 (Temaj)	100 (Nechita)	25	80.78
Days to maturity	110.3 (Temaj & Anbedat)	137(Nechita)	27	121.10
Thousand seed weight(g)	29.66 (202802)	40.05 (Anbedat)	10.41	35.2533
Plant height (cm)	82.9 (Temaj)	118.1(217119)	35.2	101.3197
Number of Spikelet per spike	14.06 (Nechita)	22.62(202793)	8.56	18.1938
Kernel number per spike	28.07 (Temaj)	59.54 (202793)	31.43	46.0983
Spike length (cm)	3.823 (Enat gebs)	9.38(202793)	5.557	6.9300
Biomass per plot (g)	1233 (Nechita)	2733 (Arusigebs)	1250	1786
Grain yield per plot kg/ha	2258 (202799)	6202 (Arusigebs)	3944	3493
Weight of kernel per spike	1.117 (Temaj)	1.94 (Yedogit)	0.823	1.586

Table 2. Average agro-morphological data for barely.

Genotype	BY	GY	KNPS	NSPS	PH	SL	TSW	DH	WSPS	DM
Shedho	1700^{cdef}	3820^{cde}	48.89^{abcd}	18.58^{bcd}	100.7^{bcde}	6.577^{efghi}	34.92^{abc}	79^{bcde}	1.62^{abcd}	121.3^{def}
Estaysh	1783^{cdef}	3998^{cd}	53.89^{abc}	19.6^{abc}	102.6^{bcde}	6.783^{cdefghi}	36.04^{abc}	78.33^{abcde}	1.87^{a}	121.7^{def}
Trit	2100^{bcd}	3403^{def}	37.62^{de}	15.33^{de}	100.7^{bcde}	6.453^{efghi}	37.5^{abc}	85^{f}	1.343^{bcde}	126.3^{ef}
Agegnhu	2133^{bc}	4851^{bc}	43.69^{cd}	17.52^{bcde}	106.5^{bc}	6.247^{fghi}	37.86^{abc}	77^{abcd}	1.69^{abc}	118.3^{d}
Yedogit	2000^{bcde}	5602^{ab}	50.86^{abcd}	18.86^{abcd}	101.1^{bcde}	6.707^{defghi}	39.11^{ab}	74.33^{a}	1.94^{a}	122.7^{def}
202802	1483^{ef}	2889^{def}	44.24^{bcd}	19.94^{abc}	96.1^{cde}	7.963^{abcde}	29.66^{c}	81^{def}	1.213^{de}	122^{def}
217126	1667^{cdef}	3371^{def}	52.83^{abc}	20.91^{ab}	104.8^{bc}	8.993^{ab}	34.66^{abc}	80.33^{cde}	1.727^{ab}	121.3^{def}
217125	1483^{def}	2995^{def}	47.39^{abcd}	19.44^{abc}	101.7^{bcde}	8.177^{abcd}	33.06^{abc}	81^{def}	1.52^{abcde}	119.7^{de}
202793	1967^{bcde}	2523^{ef}	59.54^{a}	22.62^{a}	93.3^{de}	9.38^{a}	30.96^{bc}	94.67^{g}	1.72^{ab}	128^{f}
202813	1700^{cdef}	2335^{f}	45.44^{bcd}	17.59^{bcde}	103.7^{bcd}	7.38^{cdefg}	31.45^{abc}	81^{def}	1.243^{cde}	121.7^{def}
217119	1733^{cdef}	3029^{def}	47.67^{abcd}	19.37^{abc}	118.1^{a}	7.63^{bcdef}	35.43^{abc}	79.67^{cde}	1.653^{abcd}	126^{ef}
225241	2433^{ab}	4124^{cd}	50.82^{abcd}	19.25^{abc}	109.9^{ab}	7.077^{cdefgh}	35.56^{abc}	80.67^{def}	1.783^{ab}	122^{def}
202799	1517^{def}	2258^{f}	43.31^{cd}	17.09^{bcde}	92.3^{e}	7.16^{cdefgh}	33.41^{abc}	79.33^{bcde}	1.54^{abcde}	121.7^{def}
Arusigebs	2733^{a}	6202^{a}	56.91^{ab}	20.82^{ab}	109.4^{ab}	8.313^{abc}	38.82^{ab}	82.67^{ef}	1.927^{a}	126.3^{ef}
Anbedat	1667^{cdef}	3039^{def}	39.49^{de}	16.74^{cde}	105.3^{bc}	5.623^{hi}	40.05^{a}	76^{abc}	1.623^{abcd}	110.3^{a}
Enatgebs	1617^{cdef}	3509^{def}	38.25^{de}	14.69^{e}	96.6^{cde}	3.823^{j}	39.65^{ab}	74.33^{a}	1.52^{abcde}	112^{abc}
Workye	1933^{bcde}	3316^{def}	49.36^{abcd}	19.05^{abcd}	98.6^{cde}	6.53^{efghi}	35.2^{abc}	79.67^{cde}	1.697^{abc}	117^{cd}
Temaj	1267^{f}	2259^{f}	28.07^{e}	14.68^{e}	82.9^{f}	5.327^{i}	31.41^{abc}	75^{ab}	1.117^{e}	110.3^{ab}
Nechita	1233^{f}	2457^{f}	39.42^{de}	14.06^{e}	102.7^{bcde}	6.01^{ghi}	36.3^{abc}	100^{h}	1.507^{abcde}	137^{g}
Wogera	1567^{cdef}	3871^{cd}	44.27^{bcd}	17.75^{bcde}	99.4^{bcde}	6.447^{efghi}	34.02^{abc}	76.67^{abcd}	1.467^{abcde}	116.3^{acd}
Mean	1786	3493	46.098	18.19	101.3	6.93	35.25	80.78	1.586	121.1
DMRT (5%)	***	***	***	***	***	***	NS	***	*	***
CV (%)	17.5	19.2	14.6	10.7	5.4	11.7	12.5	2.9	15.2	2.8
S.E	58.32	154.4	1.169	0.360	1.103	0.188	0.614	0.843	0.0399	3.45

*,**,***Significant at P=0.05, P= 0. 01and P= 0. 001 level and Non-significant at P>0.05, respectively; DH=days to head, DM=days to maturity, TSW=thousand seed weight; BY =biomass yield; GY=grain yield; SL=spike length; KNPS=kernel number per spike; PH=plant height, NSPS=number of spiklet per spike; WSPS = weight of seed per spike.

Among traits under this study, a considerable diversity was observed for grain yield per plot, biomass per plot, plant height, kernel number per spike, days of maturity, day of heading and 100 seed weight, respectively.

It is shown that Temaj and Yedogit show early heading (74.33 days) and Nechita shows late heading (100 days); and with regard to day of maturity, Anbedat and Temaj were early maturing varieties (110.33 days) and Nechita was late maturing variety (137 days). With regard to grain yield per plot in hectare Arusi gebs shows better perfor-

mance (6202 kg/ha) of all of the varieties used, and from the checked varieties, Yedogit had better yield (5602 kg/ha); from the accessions collected EBI 225241 was better in yield per plot, which was 4851 kg/ha. The variety 202799 shows the least yield from the observed varieties, which was 2258 kg/ha followed by Temaj 2259 kg/ha. Variety 202813 was 2335 kg/ha respectively. Regarding plant height, variety 217119 had the highest height (118.1 cm) followed by variety 225241 (109.9cm); Temaj had the lowest height (82.9 cm).

In the case of spike length, variety 202793 had higher length than the other varieties (9.38 cm), had the best number of spiklet per spike, which was 22.62 and contained 59.54 seeds per spike; whereas, Enat gebs had the smallest spike length (3.823 cm), which was farmers' new collection. In the case of biomass yield per plot, Arusi gebs had the highest value of 2733 g/plot and varieties Nechita and Temaj had the smallest values of 1233 g/plot and 1267 g/plot, respectively. With regard to kernel number per spike, variety 202793 had the highest value of 59.54 kernel per spike and Temaj had the least kernel number per spike (28.07). The variety with the highest number of spiklet per spike was 202793, which was 22.62 spiklets per spike. Nechita had the least number of spikelet per spike (14.06).

Concerning the weight of thousand seed, Anbedat variety had 40.05 g, and 202802 had least values of 29.66 g. In the case of kernel weight per spike, the released variety, Yedogit showed the highest measure of 1.94 g per spike and Temaj was the variety with 1.17 g of kernels per spike. Generally, from Table 2, it is observed that varieties Temaj and Nechita had the least results in the observed agro-morphological traits, which were new collections of the researcher and from varieties collected from farmers. Arusi gebs had better results in the observed agro-morphological traits. The released variety Yedogit was also a better variety in showing better results in the observed traits.

The traits day of heading, day of maturity, grain yield (kg/ha), plant height, spike length, number of spikelet per spike, kernel number per spike, weight of seed per spike and biomass yield (g/plot) were highly significant in showing the diversity of barely. Whereas seed weights per spike were significant and thousand seed weight was less significant.

Cluster analysis

If the cutting is done as shown with lines on the dendrogram with 90% similarities, the 20 varieties are divided into four hierarchical cluster groups and Agegnhu remains solitary. The four clusters observed were: cluster I containing the varieties 202793, 202813, 202799, Temaj and Nechita; cluster II containing the varieties 225241, Estaysh, Shedho and Wogera; cluster III containing 217119, Anbedat, Enatgegn, Workye, 217125, 217126

and 202802; and cluster IV contains Arusigebs and Yedogit. This clustering of varieties indicates that barley varieties included in those clusters are variable for the traits considered.

In cluster I, 40 and 60% of the genotypes were from farmers' collection and collections from EBI, respectively; they contain both the early maturing variety, Temaj and the late maturing variety, Nechita- both of which are collections from farmers. All of them were with higher altitudinal locations of more than 2800 based on the information of collected site, and this cluster also contains variety having maturation and day of heading less than the average. Early matured accessions and short plant height containing genotypes were clustered under cluster I. While cluster II included four genotypes in which two of them were released varieties of Sirinka Agricultural Research Institute which were Shedho and Estaysh (50%); the remaining 50% were landraces. All the cluster members from cluster II contain yield per hectare more than the total mean, and variety 225241 was a better yielding variety than the other three varieties, giving a yield of 4851 kg per hectare.

Cluster III contains eight genotypes, four obtained from EBI accounting for 50% of the cluster, three farmers' collections accounting for 37.5% of the cluster and 1 (12.5%) which was the released variety. All of the variety in this cluster contains a yield per hectare less than the total mean (3493 kg/ha) except Enat gebs which yields more than the average (3509 kg/ha), and this variety had the least spike length (3.823 cm). Cluster IV contains a total of two varieties in which 50% was released variety, that is, Yedogit and the other was farmers' collection, Arusi gebs (50%). This cluster contains the highest yielding varieties, Arusi gebs and and Yedogit that yield 6202 kg/ha 5602 kg/ha, respectively. The variety that remains ungrouped, Agegnehu, has got high yield of 4851 kg/ha which was above the mean. Therefore, it is recommended to use variety Arusi gebs from farmers' collection and Agegnehu and Yedogit which were released varieties having the highest yield and highest biomass per plot. So it is preferable both for its yield and biomass which increases the amount of straw and yield for both human and animal use. Significant variations were observed among genotypes in the agro-morphological traits investigated.

Correlation analysis

Based on the Pearson correlation, grain yield was positively correlated to kernel number per spike (0.858), biomass yield (0.656), plant height (0.846) and negatively correlated to thousand seed weight (-0.795), spike length (-0.769), kernel weight per spike (-0.814), number of spikelet per spike (-0.755), day of maturity (-0.760) and day of heading (-0.816). Thousand seed weight was highly positively correlated with spike length, kernel

Table 3. Correlation (Pearson correlation) between agro-morphological traits.

	GY	TSW	KNPS	SL	WSPS	NSPS	BY	PH	DM	DH
GY	1									
TSW	-0.795	1								
KNPS	0.858	-0.925	1							
SL	-0.769	0.939	-0.858	1						
WSPS	-0.814	0.973	-0.948	0.933	1					
NSPS	-0.755	0.955	-0.864	0.983	0.946	1				
BY	0.656	0.411	0.523	0.207	0.457	0.434	1			
PH	0.846	-0.953	0.959	-0.919	-0.980	-0.926	0.414	1		
DM	-0.760	0.963	-0.882	0.901	0.939	0.928	0.177	-0.911	1	
DH	-0.816	0.920	-0.889	0.928	0.933	0.914	0.016	-0.922	0.881	1

DH=Days to heading, DM=Days to maturity, TSW=Thousand seed weight, PH=Plant height, WSPS = Weight of seed per spike, SL=Spike length, NSPS=Number of spikelet per spike, KWPS= Kernel weight per spike, KNPS=Kernel number per spike.

weight per spike, number of spikelet per spike, day of maturity and day of heading, biomass yield, kernel weight per spike and negatively correlated with kernel number per spike (-0.925), grain yield and plant height (Table 3).

Kernel number per spike is positively correlated with grain yield, biomass yield and plant height, and negatively correlated with kernel weight per spike, number of spikelet per spike, day of maturity and day of heading and spike length. Biomass yield is positively correlated with all the traits investigated.

Thousand seed weight is highly positively correlated with spike length, kernel weight per spike, number of spikelet per spike, days of maturity and days of heading, respectively and negatively correlated with grain yield, kernel number per spike and plant height. Kernel number per spike is positively correlated with plant height and grain yield and negatively correlated with other traits. Spike length is highly correlated with thousand seed weight, kernel weight per spike, number of spikelet per spike and day of maturity and day of heading and negatively correlated with kernel number per spike, grain yield and plant height. Spike length and number of spikelet per spike were the traits showing the highest correlations from the agro morphological traits and the next highly correlated traits were kernel weight per spike and thousand seed weight from the ten agro morpho-logical traits investigated. The highest diversity among the genotypes under study was those of grain yield per plot and thousand seed weight.

Similar results for grain yield and plant height in barley were reported by other studies (Bhutta et al., 2005; Kisana et al., 1999; Samarrai et al., 1987). Akdeniz et al. (2004) observed positive and significant correlations between grain yield and yield components such as plant height, spike length and spike number per m[2] but found negative and non-significant correlations between grain yield and kernel number per spike. Positive and significant correlations of grain yield with spike number per m[2] and 1000-kernel weight were reported by Ataei

(2006).

Principal component analysis (PCA)

Principal component analysis was used to observe the general pattern for variation of traits. The first three principal components with eigenvalues greater than unity (1) together extracted about 84.22% of the total variation. According to Johnson and Wichern (2002), based on the Eigen values and vectors, it is possible to indicate which traits are mainly responsible to explain the variation. Accordingly, the first principal components which contributed about 42.03% of total variation were due to kernel number per spike, kernel weight per spike and number of spikelet per spike, respectively (Table 4). Similarly, about 26.54% of the variation, accounted for by the second principal component, was due to contributions of thousand seed weight, spike length, yield per plot and days to heading followed by days to maturity.

On the other hand, the third principal component which explained about 15.65% of the variation was mainly through days to heading, days to maturity followed by number of spikelet per spike, thousand seed weight and plant height. In the work of Abebe et al. (2010), the first three principal components (PCs), with eigenvalues greater than unity, explained about 73% of the total variation among accessions for the nine quantitative traits. Hence, even if the genotypes and the number of traits used vary, the value of the first three principal components greater than unity shows a better percentage in this study than the genotypes investigated by Abebe et al. (2010). According to the work of Zaheer et al. (2008), the variation studied through Principal Com-ponent Analysis revealed that five principal components having greater than 1 eigenvalues contributed 83.40% of the total variation. In this study, three principal components greater than 1 Eigen values contributed 84.22% of the total variation.

Table 4. Eigenvectors and eigenvalues of four principal components of quantitative traits of 20 barley genotypes.

Quantitative trait	Pc1	Pc2	Pc3	Pc4
Days to head	0.0996	-0.37607	0.5622	0.2585
Days to mature	0.2371	-0.2747	0.5586	0.0674
Plant height	0.3093	0.1644	0.2267	-0.865
Spike length	0.3148	-0.4119	-0.1496	-0.137
No of spikelet per spike	0.3734	-0.2425	-0.3886	-0.039
Kernel no per spike	0.4389	-0.1568	-0.1816	0.1009
Kernel weight per spike	0.4050	0.1991	-0.0201	0.2100
Thousand seed weight	0.1079	0.5136	0.3290	0.0429
Biomass yield per plot	0.3632	0.2318	0.0227	0.1831
Grain yield per plot	0.3154	0.3819	-0.0706	0.2647
Eigen value	4.2026	2.6539	1.56472	0.5858
Difference	1.54864	1.0892	0.9789	0.1147
Proportion	0.4203	0.2654	0.1565	0.0586
Cumulative	0.4203	0.6857	0.8421	0.9007

Storage protein analysis

Nei (1978) reported that the formula for obtaining unbiased estimates of average heterozygosity and genetic distance can be applied to any sample size and are superior to sample average heterozygosity and genetic distance, as long as many loci are used. However, the difference between the biased and unbiased estimators is very small when the number of individuals used is large, say more than 50.

In this study, most of the bands were common for all the analysed samples that show very limited variation among the analysed accession. Due to this, the gene diversity estimate was done for all populations. In this work, Nei's (1978) gene diversity was computed with pop gene software 1.31. This procedure was used by Hailu et al. (2005, 2010) using Nei's (1973) with popgene software 1.31 to see the genetic distances of tetraploid wheat germplasm (Table 5).

Regarding the genetic identity and genetic distance of the 20 genotypes of barely using hordein, it is found that the highest genetic diversity was observed in varieties 217119 and 202793 and 217125 with genetic distance of 1.6094; the second genetic distance observed was found between varieties 217119 and 225241, and 217126, 202813, Temaj, Nechita, Wogera, Shedho, Estaysh, Trit, Agegnehu and Yedogit with a genetic distance of 0.9163. The third genetic distance observed was between variety 217119 and Anbedat, Workye with a genetic distance of 0.8417. The fourth genetic distance found between the varieties was between varieties 217119 and 202799, Arusi gebs, 225241, Enat gebs, Temaj, with a genetic distance of 0.6931. In genetic identity found between the genotypes used, it is found that variety 202799, Arusi gebs, Enat gebs, Temaj were totally identical to each other and varieties 225241, 217126, 202813 were

identical to each other. Finally, varieties Wogera, Shedho, Estaysh, Trit, Agegnehu and Yedogit, except Wogera which was new farmers' collection were genetically identical, using hordein.

It is found that the 20 genotypes of barely investigated in this research were having a gene diversity (h) overall populations (0.138). Eshgehi and Akundova (2010) found that the average of genetic diversity index for the proteins investigated was calculated as H = 0.856. With this regard, the average genetic diversity obtained in this study compared with the work of Eshgehi and Akundova (2010) shows a minimum genetic distance between the populations using hordein. However, in the genetic distance observed between individual accessions, 217119 and 202793 and 217125, with a genetic distance of 1.6094 showed higher genetic distance compared to the works of Eshgehi and Akundova (2010).

Variety 217119, a collection of EBI, had the highest genetic distance (1.6094) compared to the other investigated genotypes, using Hordein. According to Nei (1978), when a dendrogram for a group of species is constructed from genetic distance estimates, the reliability of the topology of the dendrogram depends on the differences in genetic distance among different pairs of species.

The 20 barely varieties were clustered into three distinct groups and two of the germplasm (Nechita and 217119) remain solitary based on hordein. The dendrogram shows the relation among the different genotypes used in the study based on Hordein analysis (Figure 2). Cluster I consisted of 11 genotypes whereas clusters two and three consisted of four and three genotypes, respectively. Workye, Ambedat, Wegera, Shedho, Stayish, Tirit, Agegnhu, Yedogit 225241, 217126 and 202813 were grouped together under cluster one. The second cluster consists of 202799, Arusi gebse, Enat

Table 5. Nei's Unbiased Measures of Genetic Identity and Genetic distance of 20 genotypes using hordein.

Pop ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	****																			
2	0.0000	****																		
3	0.1054	0.1054	****																	
4	0.3567	0.3567	0.2231	****																
5	0.3567	0.3567	0.2231	0.0000	****															
6	0.3567	0.3567	0.2231	0.0000	0.0000	****														
7	0.6931	0.6931	0.9163	1.6094	1.6094	1.6094	****													
8	0.1054	0.1054	0.0000	0.2231	0.2231	0.2231	0.9163	****												
9	0.1054	0.1054	0.0000	0.2231	0.2231	0.2231	0.9163	0.0000	****											
10	0.0608	0.0608	0.0030	0.2319	0.2319	0.2319	0.8417	0.0030	0.0030	****										
11	0.0000	0.0000	0.1054	0.3567	0.3567	0.3567	0.6931	0.1054	0.1054	0.0608	****									
12	0.0608	0.0608	0.0030	0.2319	0.2319	0.2319	0.8417	0.0030	0.0030	0.0040	0.0608	****								
13	0.0000	0.0000	0.1054	0.3567	0.3567	0.3567	0.6931	0.1054	0.1054	0.0608	0.0000	0.0608	****							
14	0.1054	0.1054	0.0000	0.2231	0.2231	0.2231	0.9163	0.0000	0.0000	0.0030	0.1054	0.0030	0.1054	****						
15	0.1054	0.1054	0.0000	0.2231	0.2231	0.2231	0.9163	0.0000	0.0000	0.0030	0.1054	0.0030	0.1054	0.0000	****					
16	0.1054	0.1054	0.0000	0.2231	0.2231	0.2231	0.9163	0.0000	0.0000	0.0030	0.1054	0.0030	0.1054	0.0000	0.0000	****				
17	0.1054	0.1054	0.0000	0.2231	0.2231	0.2231	0.9163	0.0000	0.0000	0.0030	0.1054	0.0030	0.1054	0.0000	0.0000	0.0000	****			
18	0.1054	0.1054	0.0000	0.2231	0.2231	0.2231	0.9163	0.0000	0.0000	0.0030	0.1054	0.0030	0.1054	0.0000	0.0000	0.0000	0.0000	****		
19	0.1054	0.1054	0.0000	0.2231	0.2231	0.2231	0.9163	0.0000	0.0000	0.0030	0.1054	0.0030	0.1054	0.0000	0.0000	0.0000	0.0000	0.0000	****	
20	0.1054	0.1054	0.0000	0.2231	0.2231	0.2231	0.9163	0.0000	0.0000	0.0030	0.1054	0.0030	0.1054	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	****

1, 202799; 2, Arusi gebs; 3, 225241; 4 , 202802; 5, 202793; 6, 217125; 7, 217119; 8, 217126; 9, 202813; 10, Anbedat; 11, Enat gebs; 12, Workye; 13, Temaj; 14, Nechita; 15, Wogera; 16, Shedho; 17, Estaysh; 18, Trit; 19, Agegnhu; 20, Yedogit.

gebs, and Temaj. And the third cluster consists of 202802, 202793 and 217125. The analysis of hodein helps to group the released varieties together under cluster I which was not possible to cluster together based on the agro-morphological criteria.

In general, the results of this study provide information about diversity of barley which should be of particular interest for the further collecting of genetic resources and show a wide agro morphological variability between the genotypes investigated. The results obtained have shown that sets of agro morphological data are very useful. The study of inter relationship between different variables showed that the yield is significantly and positively correlated with kernel number per spike, biomass yield and plant height. It can be concluded that genotypes, Arusi gebs of farmers' collection and local checks of Agegnehu and Yedogit, collection of EBI 225241 had overall good performance in the experimental fields. As indicated in the correlation analysis above, spike length had a strong positive correlation with number of spikelet per pike.

In the future, selection needs to be conducted, that is, each spike needs to be planted for comparison. The best performed materials need to be promoted to the next step and tested across locations in Wollo high land areas. Finally, after the varieties are selected and approved for the better yield and yield related traits on both research site and on local farmers land with full involvement of farmers living in the research areas proper collection, storage and usage of the varieties in the selected area should be the next activity to be investigated.

Conflict of interests

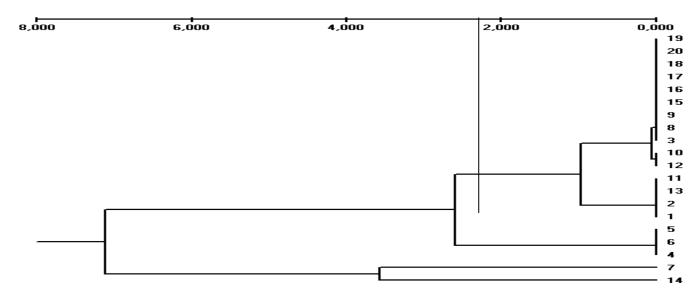The author(s) did not declare any conflict of interest.

Figure 2. Dendrogram of 20 barley genotypes constructed based on Hordein bands. 1, 202799; 2, Arusigebs; 3, 225241; 4, 202802; 5, 202793; 6, 217125; 7, 217119; 8, 217126; 9, 202813; 10, Anbedat; 11, Enat gebs; 12, Workye; 13, Temaj; 14, Nechita; 15, Wogera; 16, Shedho; 17, Estaysh; 18, Trit; 19, Agegnhu; 20, Yedogit.

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Full Length Research Paper

Purification and characterization of a new cold active lipase, *En*L A from *Emericella nidulans* NFCCI 3643

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A mesophilic fungi producing an extracellular cold-active lipase was isolated from the soil samples of palm oil mill effluent dump sites, Pedavegi, West Godavari Dist, A.P. India and was identified as *Emericella nidulans*. The enzyme was purified by ammonium sulfate fractionation followed by hydrophobic interaction chromatography using phenyl sepharose. The enzyme was 35 fold pure compared to crude with a specific activity of 1494.51 U/mg. SDS PAGE analysis revealed that the protein is monomeric with a MW of ˜54 kDa and zymogram analysis showed that the purified protein was active. Characterization studies revealed that the temperature optimum was at 30°C and an optimum pH of 5. The K_m and V_{max} values were found to be 0.61 mM and 322.58 mM/min.mg, respectively. Sequencing of the purified protein by MALDI TOF-MS analysis followed by BLAST P analysis indicated that the protein is a putative secretary lipase from *E. nidulans*. Search of lipase engineering data base (LED) revealed that this protein belongs to a newly introduced super family of *Candida antarctica* lipase A like and to the homologous family of *Aspergillus* lipase like.

Key words: Cold active lipase, *Emericella nidulans*, hydrophobic interaction chromatography, *Candida antarctica* lipase A like.

INTRODUCTION

A large part of the earth's biomass is occupied by lipids; hence the role of lipolytic enzymes in the turnover of these compounds became significant. Lipases (EC 3.1.1.3) (triacylglycerol acyl hydrolases), are α/β hydrolases and are mainly involved in the hydrolysis and synthesis of esters from glycerol and long chain fatty acids (Gilbert, 1993). The general reaction catalyzed by the lipases is shown in Figure 1. The applicability of lipases in various bioprocess applications mainly depends upon their availability and stability in both organic and as well as in aqueous media (Aulakh and

Prakash, 2010; Kumar and Kanwar, 2012). Microbial lipases are receiving much attention of the industrialists due to the advancements in enzyme technology (Hasan et al., 2006). They possess broad substrate, pH, temperature specificities and exhibit good chemo-, regio- and enantio selectivity. All these features make lipases as versatile biocatalysts (Kademi et al., 2005). Lipases as biocatalysts can be effectively used for biodiesel production (Delos Rios et al., 2011; Yan et al., 2011) and also for various other industrial applications. Most of the fungal lipases that were reported so far are derived from

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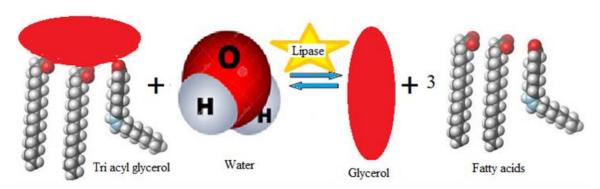
Figure 1. General reaction catalyzed by the lipases, depicting the lipase catalyzed reversible hydrolysis of triacyl glycerol to glycerol and fatty acids.

the genus *Aspergillus* (Contesini et al., 2010). Lipases purified from *Aspergillus* spp. were reported to have good thermal stability, specificity and stability in organic solvents etc.

Cold active enzymes with their unique kinetic and molecular properties along with their ability to serve as catalysts for enthalpy deficient conditions are widely employed in the organic synthesis of potentially useful compounds. These enzymes with their marked thermo lability and activity at low temperatures (Cavicchioli et al., 2002) have found numerous applications (Lo Giudice et al., 2006). Hence cold active lipases can be employed in various industrial applications which require substrate/ product stability and energy savings (Gerday et al., 1997; Marshall, 1997).

New lipases isolated from novel sources with potential industrial applications are paving way for the use of these enzymes for various bioprocess reactions (Islam et al., 2008). In the present study, a new cold active lipase, *Emericella nidulans* Lipase A (*En*L A), belonging to the new super family of *Candida antarctica* lipase A like was purified and characterized from a mesophilic fungus *E. nidulans* NFCCI 3643, screened and isolated from Palm Oil Mill Effluent (POME) dump sites.

MATERIALS AND METHODS

Microorganism and culture conditions

The fungal culture in the present study was screened and isolated from Palm Oil Mill Effluent (POME) dump sites, Pedavegi, West Godavari District. The strain was identified as *E. nidulans* DAOM 222012 by National fungal culture collection of India (NFCCI), Agarkhar Research Institute, Pune and was also deposited at NFCCI with an accession number 3643. Since then the strain was called *E. nidulans* NFCCI 3643.

Lipase production medium

Purification of extracellular lipase from *E. nidulans* NFCCI 3643 was carried out with the lipase production medium optimized using Response surface methodology (Suseela and Naveena Lavanya

Latha, 2015) consisting of (gm/L), Olive oil, 18.98 mL; ammonium sulphate, 14.38; KH_2PO_4, 1; $MgSO_4$, 0.5; Gum Arabic, 5; and pH 5.17. The Fermentation was carried out at 25°C for a period of 4 days at 150 rpm.

Extracellular lipase assay

The culture filtrate after 4 days of growth was collected by centrifugation for 15 min at 10,000 *g*. The supernatant (crude enzyme) was tested for enzyme activity. The assay mixture (1 ml) consisted of 100 µl of sample and 900 µl of substrate solution containing 10 mg of pNPP in Tris–HCl pH 7.0 containing 40 mg of Triton X-100 and 10 mg of gum arabic. The mixture was incubated at 30°C for 30 min and the p-nitrophenol released was measured at 410 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µ mol of p-nitrophenol per min under the assay conditions (Maia et al., 2001).

Purification of extracellular lipase

Crude enzyme extract was subjected to ammonium sulphate precipitation (40 to 60% saturation) at 4°C, and dialyzed against three changes of same buffer for overnight. This was followed by centrifugation at 10,000 x *g* for 15 min to collect the precipitated protein which was then dissolved in 50 mM phosphate buffer, pH 6.5. The sample was applied to phenyl sepharose (Pharmacia Biotech) column pre equilibrated with 3 bed volumes of 50 mM phosphate buffer (pH 6.5). The column was washed with 2 bed volumes of 1 M ammonium sulfate in the equilibration buffer and then with 2 bed volumes of 50 mM Tris-HCl buffer (pH 7.5) until the absorbance of the eluent at 280 nm was zero. Finally, lipase was eluted with 2 bed volumes of 5 mM Tris-HCl buffer (pH 7.5) containing 20% 2-propanol (Mayordomo et al., 2000). The eluent was collected in 5 ml fractions and concentrated by freeze-drying. The fractions were assayed for lipase activity by using pNP-palmitate as substrate. The protein content of the collected fractions was estimated using the method of Bradford (Bradford, 1976).

Gel electrophoresis and zymography

The molecular weight of the purified lipase was determined by SDS-PAGE using Laemmli method (Laemmli, 1970). Zymographic analysis was done following native PAGE (Davis, 1964). For zymographic analysis, the gel was rinsed three times with distilled water and equilibrated in 50 mM citrate phosphate buffer (pH 6.0)

for 30 min at room temperature. The gel was overlaid with molten chromogenic substrate prepared by using phenol red (0.01%) along with 1% lipidic substrate (olive oil), 10 mM CaCl$_2$, and 2% agar. The pH was adjusted to 7.3 to 7.4 by using 0.1 N NaOH. The over laid gel was then allowed to solidify and incubated at 30°C. The lipase activity can be observed within 5 to 15 min as yellow band over a pink background (Rajni et al., 2006).

Effect of temperature and pH on enzyme activity and stability

The effect temperature and pH on enzyme activity and stability was studied by using pNP-palmitate as substrate. The temperature optima was determined by incubating the purified lipase at different temperatures ranging from 0 to 70°C in 50 mM citrate phosphate buffer of pH 6 followed by enzyme estimation. The temperature stability of the enzyme was determined by incubating the assay mixture at respective temperatures for 1 h followed by enzyme estimation. For the optimum pH, the enzyme was incubated with substrate at 30°C over a varied pH range (from 2 to 10.0) followed by estimation of enzyme activity. The pH stability was determined by incubating the purified enzyme in the buffers of different pH. The reaction mixtures were then incubated at 30°C for 1 h and the residual enzyme activities were then determined.

Effect of organic solvents on enzyme activity and stability

The effect of organic solvents on enzyme activity and stability was studied by incubating the purified enzyme with substrate in the presence of various organic solvents viz., methanol, ethanol, isopropanol, diethyl ether, DMSO, butanol and acetone at a concentration of 10 to 50% (10, 30 and 50%) for a period of 24 hrs followed by enzyme estimation.

Kinetic studies

The kinetic parameters of the purified lipase were determined by incubating the enzyme with different concentrations of pNP-palmitate (0 to 50 µM), pH 5 for 30 min at 30°C followed by enzyme estimation. The K_m and V_{max} for the purified enzyme were determined by using Line weaver-Burk equation plot.

Bioremediation of simulated oil effluents using partition gravimetric method

The potential of the purified lipase in the bioremediation was studied by incubating the enzyme (1%v/v) with simulated oil effluents containing 10% each of palm oil, dalda, grease, butter, olive oil, ghee etc. at 30°C for 24 h followed by the estimation of oil and grease content using partition gravimetric method (Kirschman and Pomeroy, 1949).

Biodiesel production

Analysis of FAME's (fatty acid methyl esters)

FAME's production was carried out by incubating the purified lipase (2.6 mL) with Olive oil (7.89 mL) and methanol (0.99 mL) at 30°C with shaking at 150 rpm for 48 h (Yoo et al., 2011). After incubation, 200 µL of sample was taken from the reaction mixture and diluted with 1 mL of n hexane for 2 min followed by centrifugation at 10,000 rpm for 15 min. The upper layer separated was used for FAME's analysis using GC-MS and Fourier transform infrared (FTIR) spectroscopy.

GC-MS analysis for FAME's

Fatty acid methyl esters (FAME) content in the reaction mixture was analyzed using GCMSQP2010, SHIMADZU instrument equipped with a ZB-5ms column with 30 m length, 0.25 µm thickness and 0.32 mm diameter. The column temperature was held at 150°C for 2 min and then raised to 300°C at the rate of 8°C /min and maintained at this temperature for 10 min. The injector temperature was set at 250°C and that of detector at 1000 kV. Helium was used as carrier gas and the total GC scan time was 44 min. The FAME's produced were identified using GC-MS library.

FTIR analysis for FAME's

The infrared absorption spectrum of the sample was obtained in a Fourier transform infrared spectrometer (Attenuated Total Reflectance (ATR), Bruker) using KBr tablets in the range of 4000 to 400 cm^{-1}.

Determination of the amino acid sequence of the purified lipase

The amino acid sequence of the purified lipase was determined by MALDI TOF-MS analysis of the tryptic digested peptides using MALDI-TOF/TOF MS Bruker Daltonics, ULTRAFLEX III instrument and further analysis was done with Flex analysis software for obtaining the peptide mass fingerprint. The masses obtained in the peptide mass fingerprint were submitted for Mascot search engine for the identification of the protein.

EnL A and lipase engineering database (LED)

Since LED (http://www.led.uni-stuttgart.de/) is a repository of lipase sequences from all possible sources, it was searched for the presence of EnL A sequence.

RESULTS

Purification of extracellular lipase

The extracellular lipase from E. nidulans NFCCI 3643 was purified using ammonium sulphate precipitation followed by Hydrophobic Interaction Chromatography (HIC) using Phenyl Sepharose. The elution profile of the protein by HIC was shown in Figure 2. A 35 fold purified lipase with a specific activity of 1494.5 U/mg was achieved with this method (Table 1).

Gel electrophoresis and zymogram analysis

The apparent molecular weight of the purified lipase as determined by SDS PAGE was found to be around ~54 kDa (Figure 3a). A single band appeared on native PAGE gel and the purified enzyme showed activity on Zymogram (Figure 3b).

Effect of temperature, pH and organic solvents on enzyme activity and stability

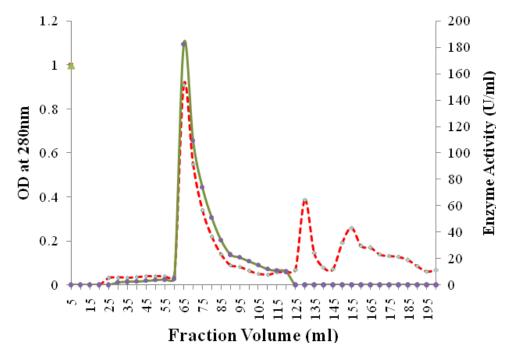The purified lipase exhibited temperature optima at 30°C.

Figure 2. Elution profile of Enzyme fractions obtained from HIC using Phenyl sepharose. Elution of proteins from the column was followed by measuring absorbance at 280 nm using UV absorbance at 280 nm and by spectro photometric estimation of the enzyme activity using pNP-palmitate as substrate.

Table 1. Purification table.

Sample	Volume (ml)	Total protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield of protein (%)	Yield of Activity (%)	Fold purification
Crude extract	700	980	61,789	63.05	100	100	1
Ammonium sulphate precipitation (40-60%)	15	12	15055.16	1254.59	1.22	24.36	19.96
Hydrophobic Interaction Chromatography (HIC)	60	4.2	9266	1494.51	0.428	14.996	35.037

Table showing the summary of the fold purification of extracellular lipase from *E. nidulans* NFCCI 3643.

The enzyme was active over a wide temperature range between 10 to 30°C and the enzyme even exhibited ~40% activity at 0°C. Further, the temperature stability of the purified lipase was determined by incubating the enzyme with substrate at different temperatures for a period of 1 h and found that the enzyme is relatively stable between 4 to 30°C (Figure 4a). The optimum pH for pure lipase was found to be 5, however; the enzyme is relatively active over a broad pH range from 5 to 7. The lipase was stable in the pH range of 4 to 7 after incubation for 1 h suggesting that the lipase is acidic lipase (Figure 4b). Enzyme was active and stable towards all the organic solvents tested at 10% concentration and even the enzyme retained more than 70% of its activity at higher concentrations (30 and 50%) with an exception to butanol (Figure 4c).

Determination of kinetic parameters

The Michaelis-Menten constant (K_m) and the maximum velocity of the reaction (V_{max}) were determined from the Lineweaver-Burk plot (Figure 5). The K_m was found to be 0.61 mM and V_{max}, the maximal velocity was found to be 322.58 mM/min.mg.

Bioremediation of simulated oil effluents using purified lipase from *E. nidulans*

From the results obtained (Figure 6), it can be inferred that there was 84.84% reduction in oil/grease content after treatment of simulated oil effluent containing olive oil with *E. nidulans* lipase, followed by palm oil, grease,
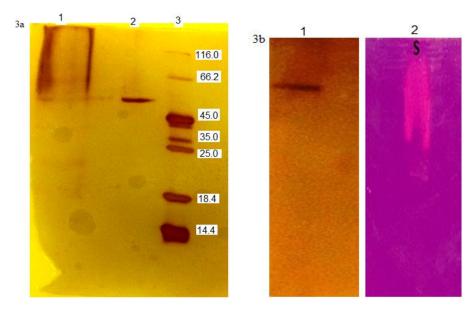
Figure 3. SDS-PAGE, Native PAGE and Zymographic analysis of purified lipase from *Emericella nidulans* NFCCI 3643. **a.** SDS PAGE of purified lipase from *E. nidulans.* The 40-60% ammonium sulphate precipitate and the purified lipase from HIC and molecular weight marker were electrophoresed on 12% SDS-PAGE followed by silver staining to visualize the bands. Lane 1, 40 to 60% ammonium sulphate precipitate; lane 2 Purified protein from HIC; lane 3 molecular weight markers (the makers are β-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp 981 (25.0 kDa), β-lactoglobuin (18.4 kDa) and lysozyme (14.0 kDa)). **b.** Native PAGE (Silver stained gel) and Zymogram of purified lipase. The purified lipase was electrophoresed on 10% Native page gel. Zymography of the purified lipase on native PAGE gel was done by overlaying the gel with the molten chromogenic substrate, which was then allowed to solidify and incubated at 30°C. The lipase activity can be observed within 5 to 15 min as yellow band over a pink background. Lane 1**,** Purified Lipase on Native PAGE Gel; lane 2, Activity stained Native Gel.
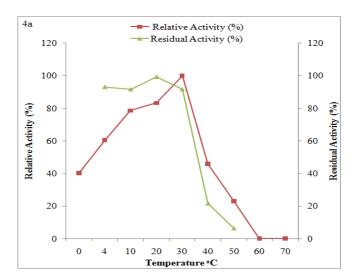

Figure 4 Characterization of purified lipase from *Emericella nidulans* NFCCI 3643. **a,** Effect of Temperature on lipase activity and stability. The effect of temperature on lipase activity was determined by incubating the purified lipase with substrate at different temperatures ranging from 0 to 70°C followed by enzyme estimation. The temperature stability of the enzyme was determined by pre incubating the enzyme extract at indicated temperatures for a period of 1 h followed by estimation of remaining activity.
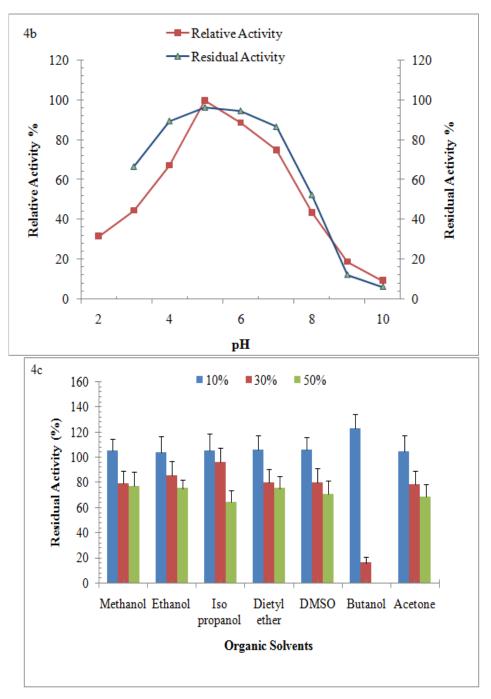
Figure 4 Contd. b, Effect of pH on enzyme activity and stability. The effect of pH on lipase activity was studied by incubating the enzyme with substrate at 30°C over different pH ranges followed by estimation of enzyme activity. The pH stability of the lipase was determined by incubating the enzyme at each desired pH for 1 h at 30°C followed by enzyme estimation. **c,** Effect of organic solvents on enzyme activity and stability. The effect of organic solvents on enzyme activity was studied by incubating the purified enzyme with substrate containing each of various organic solvents Viz., methanol, ethanol, isopropanol, diethyl ether, DMSO, butanol and acetone at a concentration of 10 to 50% (10, 30 and 50%) for a period of 24 h followed by enzyme assay.

ghee, butter and dalda with 71.82, 57.2, 47.5, 41.7, 29.8% reduction in oil/grease content suggesting that the purified lipase from *E. nidulans* can be effectively used for bioremediation of oil effluents.
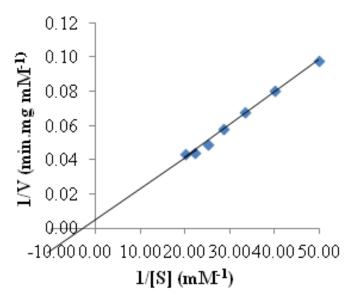
Figure 5. Lineweaver-burk plot of purified lipase from *E. nidulans* NFCCI 3643. The K_m and V_{max} values were determined by incubating the purified enzyme with different concentrations of the substrate (pNP-palmitate).
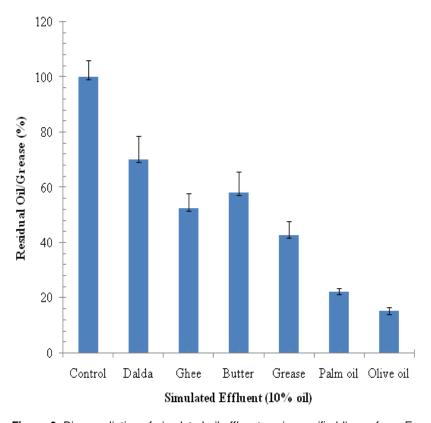

Figure 6. Bioremediation of simulated oil effluents using purified lipase from *E. nidulans* NFCCI 3643. Pure enzyme (1% v/v) from *E. nidulans* NFCCI 3643 was incubated with simulated oil effluents containing 10% each of Palm oil, Dalda, Grease, Butter, Olive oil, Ghee etc at 30°C for 24 h followed by the estimation of oil and grease content using partition gravimetric method.
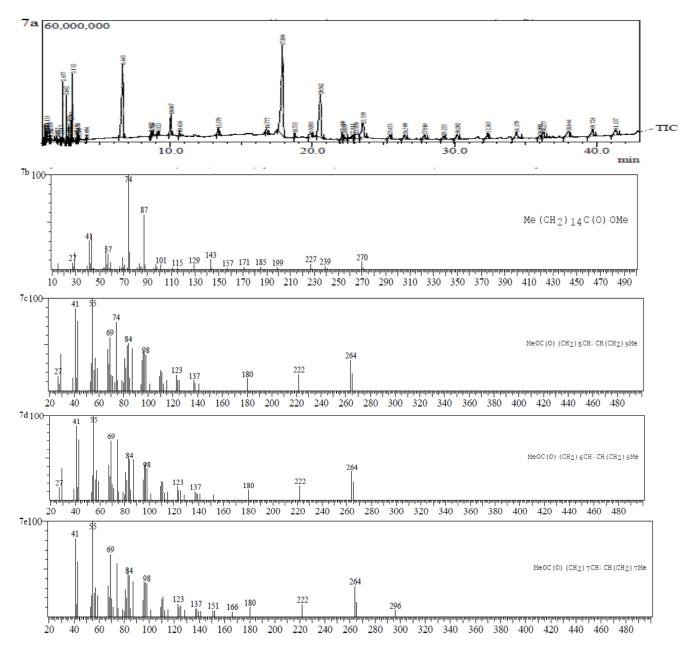
Figure 7. FAME's (Biodiesel) production using purified lipase from *Emericella nidulans*. **a,** Full scan GC-MS chromatogram of FAME's synthesized by incubating purified lipase with olive oil and methanol at 30°C with shaking at 150 rpm for 48 h followed by extraction of products (methyl esters) with n-hexane. **b,** mass spectra of hexadecanoic acid ethyl ester. **c,** 7-octadecenoic acid methyl ester. **d,** 8-octadecenoic acid methyl ester. **e,** 9-octadecenoic acid methyl ester.

Biodiesel (FAME's) production

GC-MS analysis

GC-MS analysis of the n-hexane extracted products of the olive oil, purified lipase and methanol reaction mixture showed peaks related to the retention time (RT): 18.735 min, for the production of hexa decanoic acid methyl ester, the peak with RT: 22.100 min, for the production of 7-Octadecenoic acid methyl ester and the peak with

relation to the RT: 22.250 min, for the production of 9-Octadecenoic acid methyl ester, indicating the potential of the purified lipase in the biodiesel production (Figure 7a to e).

FTIR spectroscopic analysis of FAME's

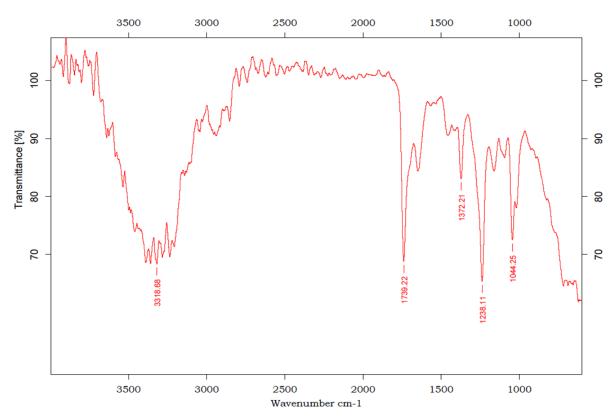The Fourier transform infrared spectrum of the biodiesel from the olive oil is shown in Figure 8. The spectrum

Figure 8. FTIR spectrum of transesterified olive oil. The n-hexane extracted products were used for spectral analysis which were obtained by incubating the purified enzyme with olive oil and methanol at 30°C with shaking at 150 rpm for 48 h followed by extraction with *n*-hexane.

showed a peak at 3318 cm^{-1}, assigned to the -OH stretch of carboxylic acid; the strong peak at 1739.22 cm^{-1}, due to –C=O stretch of ester; the peak at 1372.21 cm^{-1}, due to –C=C- stretch of alkenes; a peak at 1238.11 cm^{-1}, due to C-O single bond; and the peak at 1044.25 cm^{-1}, due to C-O stretch of ester indicating the formation of FAME's.

Determination of the amino acid sequence of the purified lipase

MALDI TOF-MS analysis followed by mascot search with the obtained sequence revealed that the sequence of the purified lipase showed high score with the sequence from *Aspergillus nidulans* with accession no gi|67522685|XP_659403.1 (Figure 9). The peptide mass spectrum is shown in Figure 9a and mascot search engine results with the obtained peptide sequences is shown in Figure 9b. Search of UNIPROT KB revealed that the protein is a putative secretary lipase from *E. nidulans* (Figure 10).

EnL A and lipase engineering database (LED)

Lipase engineering database, a database of all lipases

including putative ones from various organisms placed *En*L A under the *Aspergillus* lipase like homologous family of *Candida antarctica* lipase A like super family (Table 2a and 2b).

DISCUSSION

Cold active lipases are attracting industrial biotechnologists owing to their specific applications in various fields. Although a number of cold active lipase producing sources are available, only a few bacteria and yeasts have been exploited for the production of cold adapted lipases (Joseph et al., 2006). A very few reports regarding cold active lipases from fungal sources were available (Joseph et al., 2007). These include *C. antarctica* (Patkar et al., 1993); *Candida lipolytica, Penicillium roqueforti* and *Geotrichum candidum* (Alford and Pierce, 1961), *A. nidulans* WG 312 (Mayordomo et al., 2000), *Rhizopus* sp. and *Mucor* sp. (Coenen et al., 1997) etc. There were also some recent reports regarding cold active lipases from *Pichia lynferdii* Y- 7723 (Hak-Ryul Kim et al., 2010); *Yarrowia lipolytics* NCIM3639 (Yadav et al., 2011); *Penicillium expansum* (Suja et al., 2013). In addition, all the cold active lipases that were reported till date were mainly screened and
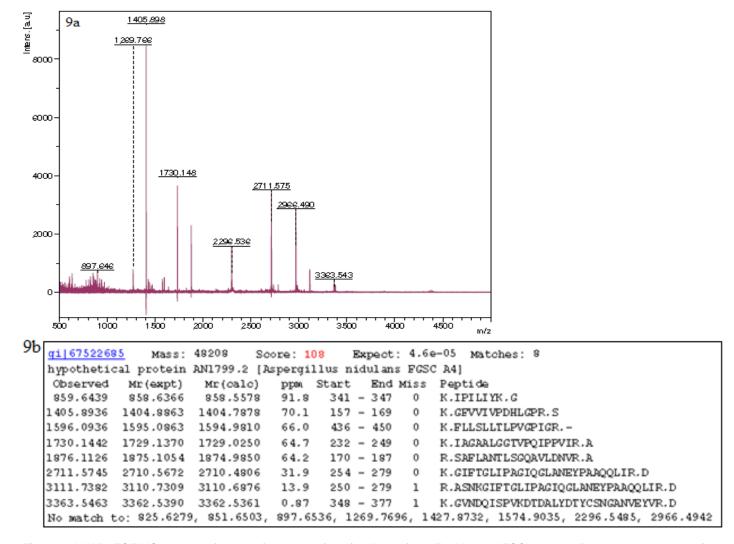
Figure 9. MALDI TOF-MS analysis of peptide fragments of purified lipase from *E. nidulans* NFCCI 3643. **a,** Peptide mass spectra of the tryptic digested peptides obtained from MALDI TOF-MS. **b,** MASCOT search engine results for the obtained peptides.

MASLLYQLLFLLVPLLAAGLPANPVKKAGPQPPGEDPFYTPPDGWESTEPGAILRHRTPP

YPIAAFGLAEVNLDASYQILYRTTDSFGEPIATVTTILIPHNADYTKVLSYQVAQDAADP

NCSPSFAIQQFSDAGEALALVMPQLEYLFMSSALNKGFVVIVPDHLGPRSAFLANTLSGQ

AVLDNVRAALASTDITGISSQATVALWGYSGGSLASGFAAELQPSYAPELKIAGAALGG

TVPQIPPVIRASNKGIFTGLIPAGIQGLANEYPAAQQLIRDAILPDKWAEFNKTQELCLTG

NLIEYLGKDIYTYVNDPNVFESPLANSLTEPNAMGHNTPKIPILIYKGVNDQISPVKDTDA

LYDTYCSNGANVEYVRDLLAEHALMTITGAPDAFMWLTERLSGVPVKKGCRRKTQLT

GLQDPKALAALGTTVVKFLLSLLTLPVGPIGR

Figure 10. Amino acid sequence of the purified lipase from *E. nidulans* NFCCI 3643 obtained from the Uniprot KB based on annotation and gi number of the protein that showed high scores with peptide sequences analyzed by MALDI TOF-MS. Uniprot KB results (Q5BCD1_EMENI) indicated that the sequence is a putative secretary lipase from *Emericella nidulans*.

Table 2a. *Candida antarctica* Lipase A like super family and Aspergillus lipase like homologous family of LED (Lipase Engineering Database) containing *EnL* A sequence. Table showing number of proteins, their sequences and structures belonging to *Aspergillus* lipase like homologous family of *C. antarctica* superfamily.

Homologous family abH38.03 (*Aspergillus* lipase like)	
Superfamily	abH38 - Candida antarctica lipase A like
Proteins	9
Sequences	10
Structures	0 Alignment [annotated clustalW] Tree [rooted] Sequences [FASTA] HMMER [profile]

*Source: Lipase engineering database (LED)

Table 2b Table showing a hypothetical protein (AN 1799.2) from *A. nidulans* belonging to *Aspergillus* lipase like homologous family and *Candida antarctica lipase* A like super family.

Organism	Protein	Source DB	NCBI accession code	Description	LED/3D
Aspergillus nidulans	Hypothetical Protein	gi	67522685	Hypothetical protein (AN 1799.2)	No 3D structure

*Source: Lipase engineering database (LED)

and isolated from organisms of deep sea sediments, frozen food samples and Antarctic habitats etc. (Joseph et al., 2007). In the present study, we reported the production of a new cold active lipase, *EnL* A from a mesophilic fungus *E. nidulans* NFCCI 3643, screened and isolated from POME effluent dump sites of Pedavegi palm oil industry, West Godavari Dist, Andhra Pradesh, India. A cold active lipase from *A. nidulans* WG 312 was already reported by Mayordomo et al. (2000), but that lipase was different from the one we are reporting now with respect to molecular weight and as well with other biochemical characteristics.

The extracellular lipase from *E. nidulans* NFCCI 3643 was purified by HIC using phenyl sepharose. Several different lipases were purified from *Aspergillus niger* using hydrophobic supports (Fernandez-Lorente et al., 2005). The apparent molecular weight of the purified lipase from *E. nidulans* NFCCI 3643 separated by SDS PAGE was found to be around ˜54 kDa whereas the molecular weight of the lipase sequence obtained from MALDI-TOF analysis of tryptic digested peptides of the purified lipase was 48 kDa. Similar differences in the molecular weights were reported by Tsuchiya et al. (1996) for *A. oryzae* lipase, Bihong et al. (2010) for *Aspergillus tamari* lipase, Yamaguchi et al. (1991) for *Penicillium camembertii* lipase. This could be due to glycosylation of the purified protein separated by SDS PAGE. Most of the lipases isolated from *Aspergillus* spp. were found to have molecular weights in the range of 25 to 70 kDa (Sharma et al., 2001; Contesini et al., 2010).

The optimum temperature of the purified lipase was found at 30°C. The enzyme is active over a broad temperature range between 10 to 30°C and the enzyme even showed up to 40% of its activity at 0°C indicating that the purified enzyme is a cold active enzyme. The enzyme was stable between the temperature range 4 to 30°C. The lipase exhibited pronounced heat lability above 30°C and this property is similar to many other cold adopted enzymes (Marshall, 1997; Gerday et al., 1997; Feller and Gerday, 1997). Microorganisms adapted to cold environment in general requires lower temperatures for their growth and metabolism which necessitates the use of energy consuming cooling systems to maintain lower temperatures during their fermentative production. Such problem will not arise with *E. nidulans* NFCCI 3643 as it is a mesophilic organism with optimum growth temperatures between 25 to 30°C. The optimum pH of the purified enzyme is 5 and the enzyme exhibited greater stability over a broad pH range 4 to 7. An optimum temperature and pH of 40°C and 6.5, respectively, were reported for the cold active lipase from *A. nidulans* WG 312 by Mayordomo et al. (2000) and 37°C, pH 8, respectively, for *Serratia marcescens* by Abdou et al. (2003).

The K_m and V_{max} for the cold active lipase *En*L A was found to be 0.61 mM and 322.58 mM/min.mg, respectively. The low K_m of the enzyme indicates that the enzyme is having good affinity with its substrate. K_m and K_{cat} values of 0.28 mM and 494 s^{-1} were reported for cold active lipase purified from *A. nidulans* by Mayordomo et

al. (2000). The enzyme exhibited pronounced activity and stability in the presence of various organic solvents and also proved its potential in biodiesel production. Biodiesel production by immobilized *Rhizopus oryzae* fungal cells was reported by Nagaraj et al. (2010) using GC-MS and FTIR analysis. Sunil Kumar et al. (2015) also reported biodiesel production by immobilized lipase from *Bacillus aerius* employing GLC and FTIR analysis. Biodiesel production from rice bran oil via immobilized lipase catalysis was reported by Ying et al. (2013) using GC-MS and FTIR analysis. Use of lipases is a new path in the field of bioremediation for waste disposal. Cold active lipases have also found numerous applications in the field of bioremediation, a technique of waste management involving the use of microorganisms to remove or neutralize pollutants from the harmful wastes disposed at contaminated sites. Cold-adapted lipases play significant role in the field of bioremediation especially in fat contaminated cold environment and waste water treatment (Buchon et al., 2000). Bioremediation studies of simulated oil effluents by the cold active lipase from *E. nidulans* revealed that this enzyme can be effectively used for the removal of oil from oil contaminated soils and water bodies.

Protein sequence determination using MALDI-TOF/MS analysis following search of protein databases with the sequence obtained revealed that the purified protein is a hypothetical protein from *E. nidulans* with a gi no. 67522685. Search of Lipase engineering database, a database that maintains information about all the lipases including putative lipases from various sources revealed that this protein belongs to newly introduced super family of *C. antarctica* lipase A like and to the homologous family of *Aspergillus* lipase like of Class Y lipases. This super family was introduced in the new release 3.0 (December 2009) of the Lipase Engineering Database (Widmann et al., 2010) based on the differences in the cap region of CAL-A (*C. antarctica* lipase A) which is unique among all other proteins of the α/β hydrolase fold. CAL -A was found to have wide spread applications in various fields (Joseph et al., 2008). The purified enzyme from *E. nidulans* was named as *En*L A (*E. nidulans* Lipase A) as this enzyme belongs to *C. antarctica* lipase A like super family.

Conclusion

Taking all the above characteristics into consideration, we conclude that the *En*L A from *E. nidulans* NFCCI 3643 reported in our study is a new cold active enzyme purified from a POME isolate and this enzyme could become a novel biocatalyst to suit for various industrial applications where cold active enzymes are needed.

Conflict of interests

The authors did not declare any conflict of interest.

Abbreviations: SDS PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; **MALDI TOF-MS,** Matrix-Assisted Laser Desorption Ionization Time of Flight-Mass Spectrometry; **BLAST,** Basic Local Alignment Search Tool; **LED,** Lipase Engineering Data base; *En*L A, *Emericella nidulans* Lipase A; **NFCCI,** National Fungal Culture Collection of India; **POME,** Palm Oil Mill Effluent; **pNPP,** para-nitro phenyl palmitate; **DMSO,** Dimethyl sulfoxide; **FAME's,** Fatty Acid Methyl Esters; **GC-MS,** Gas Chromatography–Mass Spectrometry; **FTIR,** Fourier Transform Infrared Spectroscopy; **ATR,** Attenuated Total Reflectance; K_m, Michaelis-Menten constant; V_{max}, Maximum velocity of the reaction **RT,** Retention time; **CAL -A,** *Candida antarctica* lipase A.

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Full Length Research Paper

Evaluation of methane production features and kinetics of *Bougainvillea spectabilis* Willd waste under mesophilic conditions

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The objective of this study was to investigate and evaluate the biomethane potential (BMP) of *Bougainvillea spectabilis* Willd waste in Yunnan, China, when subjected to mesophilic anaerobic digestion (AD). Three different categories of plant waste investigated were as follows: the flowers, leaves and stems of *B. spectabilia*. These three portions were assessed for their BMP in a laboratory-scale batch anaerobic digester for a period of 60 days at 30±0.1°C temperature. The results show that maximum daily methane yield of *B. spectabilia's* flowers, leaves and stems were 65.95, 56.29 and 18.8 mL/(g.VS), respectively. Moreover, the research used substrate kinetics, including the Gompertz equation, the logistic equation and the transference function to analyze the AD process. All models fit the experimental data with $R^2 > 0.993$. However, the Gompertz equation presented the best agreement in the fitting progress.

Key words: *Bougainvillea spectabilis* Willd, biomethane potential, anaerobic digestion, mathematical model.

INTRODUCTION

Bougainvillea spectabilis Willd (BSW) is a flowering, ornamental plant and is economically important to tropical and subtropical regions (Mohammad et al., 2013). In China's subtropical Yunnan Province, the *Bougainvillea* plant can be seen everywhere. In fact, it is one of the main urban plants, particularly in Yunnan's capital, Kunming. In the course of natural growth and pruning, BSW produces a great deal of leaves, branches and other plant material that accumulates and must be removed. The Chinese Government presently regards the greenery as waste and has taken some measures to treat it. At present, large cities, for instance Beijing, Shanghai and Guangzhou, have started to manage this waste with formal classification as green waste, grinding and com-posting. After being mulched, this waste can become organic fertilizer for soil improvement and landscaping.

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However, in Kunming, green waste is chiefly landfilled and does not receive any effective treatment.

Green waste management systems in developing countries like China must address many challenges, including limited technical experience and financial resources that frequently cover only collection and transfer costs and leave no resources for safe and sustainable treatment (Tadesse et al., 2014). If we take measures to deal with the great deal of green waste by burning it, it will generate significant greenhouse gas (GHG) emissions. Meanwhile, the escalating prices of conventional energy sources and global warming issues have compelled to promote sustainable renewable energy. Among the many options available for treatment of municipal green waste, the anaerobic digestion treatment process is sustainable and cost-effective because of its capacity to capture not only nutrients but also energy production (biogas) and anaerobic digestate as agricultural fertilizer. In recent years, the conversion of municipal green waste to biogas has become increasingly popular in industrialized nations as a means of turning a liability into an asset. Various relatively advanced designs have evolved at different scales, but significant potential for increased biogas use still exists.

Biogas production depends on a number of factors. Chief among them are the volatile solids (VS) content of the feedstock and the parameters of the biological community that inhabits the digester (Macias et al., 2008). Additionally, according to Kayhanian (1995) and Hartmann et al. (2004), the type of plant waste – particularly the amount of lingocellulose material (composition of lignin, cellulose and hemicellulose) it contains – influences biological activity and hence the biodegradability of the waste. Biogas production rates and the activity of anaerobic microorganisms are also influenced by the balance of carbon and nitrogen in the feedstock. The C/N ratio should range between 25:1 and 30:1 for proper anaerobic digestion (Fricke et al., 2007).

Environmental factors such as high or low temperature and pH also determine the efficiency of biogas production (Mashad, 2004). This suggests that the BMP of green waste has to be determined according to local environmental conditions and any unique characteristics of local waste. However, from the literature survey, the scientific study on this topic currently scarce in, particularly with regard to the BMP of the BSW that pervades Kunming. Considering this fact, this study, therefore, aimed to determine the biogas and biomethane production potential of the different components of BSW waste in Yunnan and comparable subtropical regions using a proximate analysis method. We used different model for instance, the Gompertz equation, the logistic equation and transference function to analyze the AD process stability and explore the biodegradability parameters of BSW.

Several researchers have used mathematical models in their studies to obtain kinetic parameters of the anaerobic digestion of energy crops. The first-order kinetic model used by Massè et al. (2010) and Mähnert et al. (2009) evaluated the BMP of switchgrass, maize silage and so on. The model fit the experiment data, and the coefficients of determination were higher than 0.99.

Based on existing knowledge, the main parameter considered in the previous studies is the final amount of biogas produced (total cumulative biogas yield); only a few studies investigated the biogas production rate. In the current paper, anaerobic digestion tests were conducted to determine the biogas production of BSW, mainly focusing on the biogas production. Additionally, the three above mentioned mathematical models were used to evaluate biogas production rate using obtained experimental data.

MATERIALS AND METHODS

Feedstock and inoculum

Flowers, leaves and stems of fresh BSW were collected from Yunnan Normal University's Chenggong campus. Flowers and leaves were cut into small fragments and the stems into 0.5 cm pieces. This feedstock did not receive any chemical pretreatment. Total solids content (TS) of BSW flowers, leaves and stems were 24.02, 28.71 and 24.83%, respectively. The volatile solid content (VS) of flowers leaves and stems were 20.77, 23.39 and 21.20%, respectively.

Inoculum was taken from a mesophilic anaerobic digestion reactor fed with swine manure (30°C well-run anaerobic digester). Before use, inoculum was sieved through a sieve of 1 mm mesh to remove large particles and grit. The pH, TS content, and VS content, of the sieved inoculum were 7.3, 32.31 and 27.62%, respectively.

Experimental set up and procedure

Anaerobic digestion experiments were carried out in 500 ml glass bottles at 30±0.1°C with a working volume of 400 ml. Firstly, inoculum (120 g) was added to each bottle, followed by the addition of substrate with a substrate to inoculum (S/I) ratio of 0.75 (flowers), 0.85 (leaves), 0.77 (stems) based on VS content. Then, all bottles were filled with 400 ml water. Finally, 3 mol/L sodium hydroxide (NaOH) was added as a buffering agent. The headspaces of the digesters were flushed with N_2-gas for 2 min to remove the residual oxygen and ensure anaerobic condition. After then, the bottles were sealed with butyl rubber stoppers. Biogas potential tests were performed under mesophilic conditions (30±0.1°C) controlled by a water bath. Bottles were mixed automatically once per hour. The experiment period was about 60 days. Biogas production was measured in mL and later adjusted to normal (standard) conditions: 273 K and 1,013 mbar. Bottles with inoculum only were used to determine the methane produced from it. The methane produced from inoculum only was subtracted from the bottles containing substrate and inoculums when calculating the biogas yield from the substrates alone. The instrumental set-up consists of a sample incubation unit (A), a CO_2-fixing unit (B) and a gas volume meter (C) (Figure 1).

A pH indicator (that is, thymolphthalein) was added to each bottle to control the acid-binding capacity of the solution. The volume of CH_4 released from the CO_2-fixing unit was measured using a wet gas flow measuring device with a multi-flow cell arrangement (15 cells). This measuring device can monitor low gas flows and works
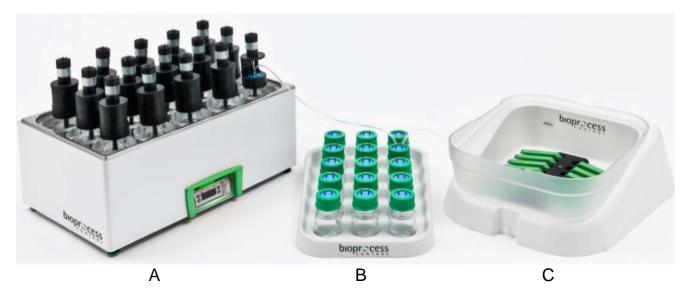
Figure 1. Photograph of AMPTS II.

Table 1. Model and Equations.

Model	Equation
Modified Gompertz equation	$M = P \times \exp\left\{ -\exp\left[\dfrac{R_m \times e}{P}(\lambda - t) + 1 \right] \right\}$
Transference function	$M = P \times \left\{ 1 - \exp\left[-\dfrac{R_m(t - \lambda)}{P} \right] \right\}$
Logistic function	$M = \dfrac{P}{1 + \exp[4R_m(\lambda - t)/P + 2]}$

according to the principle of liquid displacement and buoyancy; a digital pulse is generated when a defined volume of gas (that is, 5 mL) flows through the device. An integrated embedded data acquisition system is used to record, display and analyze the results.

Models for data fit

Three models were used to estimate performance parameters. The logistic function corresponds to established trends of biogas production kinetics: an initial exponential increase and a final stabilization at a maximum production level. Moreover, the logistic function is based mainly on four assumptions and is designed to be as simple as possible in order to avoid unidentifiable parameters (Bhatta et al., 2015). Similarly, the modified Gompertz equation can be used to analyze methane production; however, the three parameters of this model are restricted to specific experimental conditions and cannot be used in a predictive mode (Ye et al., 2015). The transference function predicts maximum gas production solely based on CH_4 production (Pommier et al., 2007).

In this study, after obtaining cumulative methane production curves over time from the AD tests, the modified Gompertz

equation, logistic function and transference function (Table 1) as presented by Donoso-Bravo et al. (2010) were used to determine methane production potential (P), maximum rate of methane production (R_m) and duration of the lag phase (λ).

RESULTS AND DISCUSSION

Methane production

Cumulative methane production and daily methane pro-duction as a function of time for BSW flowers, leaves and stems are presented in Figures 2 and 3, respectively. From the Figure 2, the specific methane yield of BSW flowers increased from 0.6 to 1369 mL. Similarly, the specific methane yield of BSW leaves increased from 0.3 to 1351 mL, achieving the same production trend as like flowers. The final outputs are almost identical in flowers and leaves. However, the specific methane yield of BSW stems merely increased from 0.3 to 960 mL. Specific
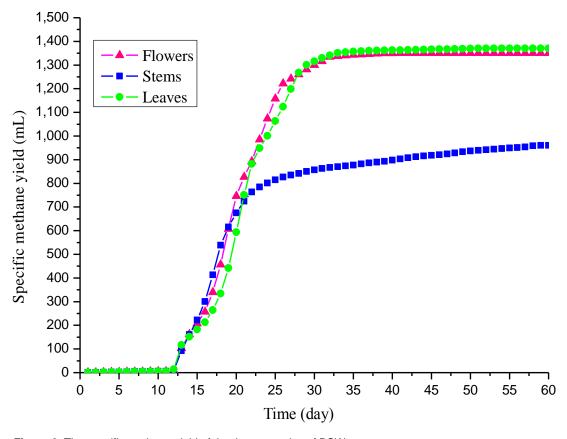
Figure 2. The specific methane yield of the three samples of BSW.

methane yields obtained in the current study conformed to the range of results obtained from other plants and straw (Redzwan and Banks, 2004; Donoso et al., 2010) (Figures 4 to 6).

The maximum daily methane production peak of flowers (154 mL) and leaves (151 mL) occurred on the 23rd day; however that of stems (120 mL) was on the 17rd day (Figure 3). The achieved cumulative methane yield of stems (120 mL) was much lower than that of flowers (154 mL) or leaves (151 mL). This could be primarily due to the fact that stems contain significant amounts of complex lignocellulose structure which limits the anaerobic biodegradability.

The parameters obtained in the optimization process are summarized in Table 2. There was an overall agreement between the models and the experimental data. Among the performance models, the best fit was obtained using the Gompterz equation, which achieved the highest regression of coefficients in all cases (> 0.993). In case of BSW flowers, methane production potential (P, in mL) was ranked as follows: transference function (1388)> modified Gompertz equation (1355)> logistic function (1351). Maximum specific biogas production rate (R_m, in mL) of flowers was ranked as follows: logistic function (287.6)>Gompertz equation (266)> transference function (141.2). For BSW leaves, biogas production potential and

maximum specific biogas production rate in different models were almost same as those of the flowers (Table 2). Similarly, biogas production potential (P, mL) and maximum specific biogas production rate (R_m, in mL/(g·VS·day) of stems ranked as follows: transference function (929.4)> modified Gompertz equa-tion (907)> logistic function (903.2) for P; and logistic function (244.8) > modified Gompertz equation (241.7)> transference function (127.4) for R_m.

The difference in lag time (λ) was negligible in the cases of the logistic function and modified Gompertz equation, varying from 0.1 to 0.3 days for flowers, leaves and stems. Calculated lag time difference was found to be less than 1 day for transference function, lag time ranging between 2.062 to 2.379 for flowers, leaves and stems. However, the lag time of 2 to 5 days were observed among three models, because the readily biodegradable components of each feedstock were broken down at different rates (Table 2). Furthermore, the correlation coefficients (R^2) of nonlinear analysis for flowers, leaves and stems were above 0.990 in all models except for the transference equation in which it ranged from 0.906 to 0.935. The best consistency was obtained with the modified Gompertz equation.

All the models consisted of the experimental data with regression coefficients above 0.90, however among the
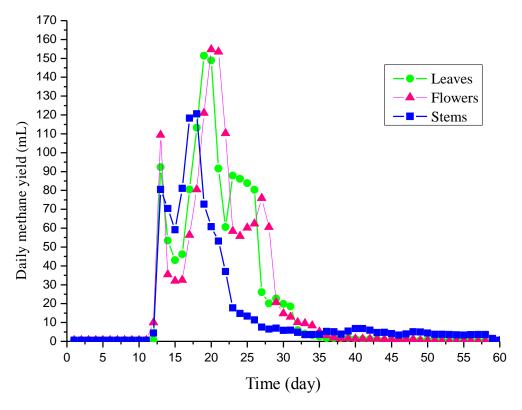
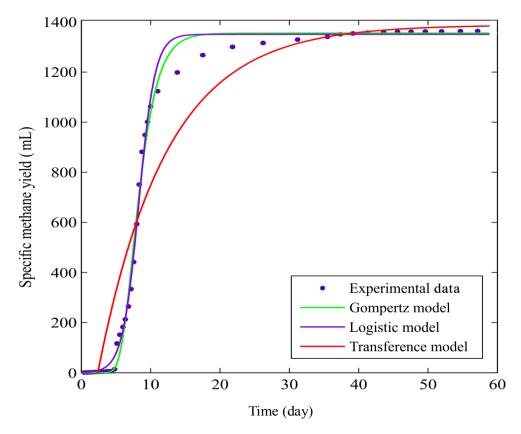**Figure 3.** The daily methane yield of the three samples of BSW.

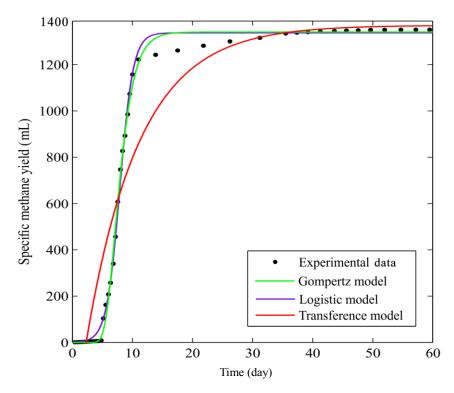**Figure 4.** Model fit with methane yield of BSW flowers.
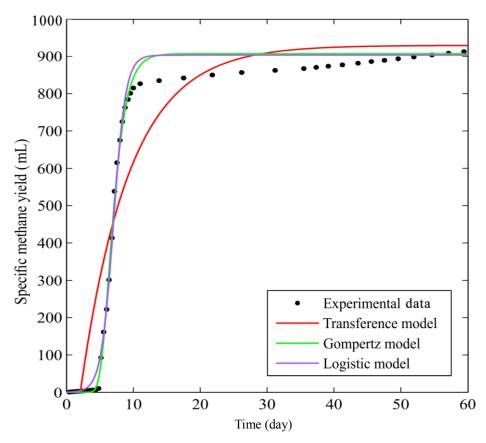
Figure 5. Model fit with methane yield of BSW leaves.

Figure 6. Model fit with methane yield of BSW stems.

Table 2. Parameters and conformance to the evaluated models.

Sample	Model	P(mL)	R_m(mL/(g·VS)	λ(day)	R^2
Flowers	Gompertz equation	1355	266.1	5.635	0.9967
	Logistic function	1351	287	5.934	0.9964
	Transference function	1388	141.2	2.379	0.9359
Leaves	Gompertz equation	1341	289.3	5.445	0.9982
	Logistic function	1338	301.2	5.674	0.9979
	Transference function	1370	155	2.285	0.9261
Stems	Gompertz equation	907	241.7	5.012	0.9936
	Logistic function	903.2	244.8	5.156	0.9916
	Transference function	929.4	127.4	2.062	0.9066

models; the modified Gompertz equation presented the best agreement. A comparison between the modified Gompertz, logistic, and Richards's models was performed by Altas (2009), who fit these models to biogas production from granular sludge to describe the deactivation of anaerobic microbial activity by heavy metals.

Economic benefit analysis

On one hand, AD is known as a more environmental friendly and energy saving approach for organic waste treatment than other disposal methods like landfilling, incineration and composting (Hosseini et al., 2014). On the other hand, this experimental study was carried out on the purpose of feasibility evaluation towards heat and power generation in large-scale anaerobic digestion system.

Compared with other similar fermentation raw material, the methane production potential of *B. spectabilis* is better (Luo et al., 2013). In view of the fact that *B. spectabilis* waste can produce biogas through anaerobic digestion approach, anaerobic digestion is not only one of the feasible ways to treat *B. spectabilis* waste, but also an ideal method with high energy efficiency.

Additionally, our researching team is going to implement further studies on economic benefits analysis of *B. spectabilis*'s biogas engineering. What's more, a new-installed biogas project with *B. spectabilisas* main fermentative material is about the set-up at present.

Conclusions

1. Methane gas production peaks ranged from the 13th to the 27th day; the maximum rates of biogas production occurred during this period. BSW flowers, leaves and stems were observed to produce 65.95, 56.29 and 18.8 mL/(g·VS), respectively. BSW is an excellent feedstock for AD. The results obtained from this experiment can be useful for the further development of BSW biogas projects in Kunming City.

2. A significant difference between the models was observed for the value of maximum methane production rate. Among the models, the modified Gompertz equation showed better consistency with the experimental data than the transference model or logistic model.

Conflict of interests

The authors did not declare any conflict of interest.

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