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Full Length Research Paper

Molecular characterization of induced mutagenesis through gamma radiation using RAPD markers in *Jatropha curcas* L.

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Genetic variability in *Jatropha curcas* was induced by different doses (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 kR) of gamma-rays. Gamma radiation induced earliness in flowering and the plants set flowers earlier than that of control, which took longer duration of 327 days for flowering. The improved reproductive and yield parameters such as days taken to first flowering, flowering population, male to female ratio and seed yield per plant were recorded in 25 kR dose and seed germination in 5 and 10 kR treated seeds. Molecular characterization of induced mutants (M_1 generation) with 47 Random amplified polymorphic DNA (RAPD) primers showed 65.27% polymorphism. The variability created by gamma rays ranged from 9 to 28%. The 50 kR mutant was found to be the most diverse from control followed by 25 kR mutant. Thus, this integrated approach can be used for carrying out the mutation-assisted breeding and subsequent selection of desired mutants using molecular markers in *J. curcas*.

Key words: *Jatropha curcas*, random amplified polymorphic DNA (RAPD), gamma-rays, induced mutagenesis.

INTRODUCTION

Bio-diesel is a fast-developing alternative fuel in many developed and developing countries of the world. Less availability of edible oil for human consumption in developing countries do not favour its use for bio-diesel production. Hence, non-edible oil from plants like jatropha (*Jatropha curcas*) and karanj (*Pongamia pinnata*) is favoured for bio-diesel production and the trend is expected to continue. *J. curcas* L. commonly known as physic nut or jatropha, a multipurpose, drought resistant, monoecious perennial plant belonging to family Euphorbiaceae has evoked much interest all over the world as potential biodiesel plant. It has gained special attention in tropical and sub-tropical countries and has spread beyond its centre of origin because of its hardiness, easy propagation, drought endurance, high oil content, rapid growth, adaptation to wide agro-climatic conditions and multiple uses of the plant as a whole. It is a non-food crop that produces fruits containing seeds with a high quality oil representing 35 to 40% of the seed by weight (Achten, 2008; Kumar and Sharma, 2008). It produces high quality oil whose chemical characteristics have allowed it to partially substitute for jet fuels in

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recent test flights (Lilley, 2008). Although, this plant is proving an excellent feed stock for large scale plantation, the full potential of *J. curcas* has not yet been realized due to several reasons such as technological, economic and lack of quality plant material.

The limitations in available germplasm include lack of knowledge of the genetic base, poor yields and vulnerability to a wide array of insects and diseases. Assessment of genetic diversity using molecular markers disclosed low inter-accessional variability in *J. curcas* germplasm (Basha and Sujatha, 2007; Popluechai et al., 2009; Tanya et al., 2011). Also, inter-specific hybridization in *Jatropha* species for transferring useful traits such as yield, high oil content, maximum number of seeds, more femaleness, and hard stems has limited success due to pollen incompatibility (Kumar et al., 2009; Dhillon et al., 2009). The sound breeding program depends upon the availability of genetic variability for desired trait. Collection, characterization and evaluation of germplasm for oil and yield and agro-morphological trait are in nascent stage. The major activity of genetic improvement is selection and breeding. As *J. curcas* is often a cross-pollinated crop and exploitation of genetic variation may be carried out through mass selection, recurrent selection, mutation breeding, heterosis breeding and inter-specific hybridization (Divakara et al., 2010). Mutation breeding in tree crop is preferred due to demerits of conventional breeding such as time consuming, unpredictable results, long juvenile phase, high heterozygosity and fear of loss of unique genotype. Mutation breeding which is an efficient and much cheaper method than others can play an important role in crop improvement either directly or by supplementing the conventional breeding (Khawale et al., 2007).

When no gene or genes, for resistance to a particular disease or for tolerance to stress are found in the available gene pool, plant breeders have no obvious alternative but to attempt mutation induction. Treatment with mutagens alters genes or breaks chromosomes. Mutagenic agents, such as radiation and certain chemicals, can be used to induce mutations and generate genetic variation. Mutation breeding work in *J. curcas* carried out in Thailand using fast neutrons and isolated dwarf or early flowering mutants from the M_3 generation, but the potential productivity of these variants under intensive cultivation conditions was not proved (Sakaguchi and Samabhi, 1987). Dwimahyani and Ishak (2004) used induced mutations in *J. curcas* for improvement of agronomic characters with irradiation dose of 10 Gy and identified mutant plants with early maturity, 100 seeds weight (30% over control) and better branch growth. In India, mutation breeding using chemical and physical mutagens has been initiated to create genetic variation for various traits and developed mutants are being characterized using DNA markers (Punia, 2007). Mutation studies undertaken at National Botanical Research Institute (NBRI), Lucknow, India has led to induction of cotyledonary variabilities in *J. curcas* (Pandey and Datta, 1995). The mutants themselves may not be suitable for direct release, but they do provide the necessary alleles for developing superior cultivars with desirable traits.

In this context, induced mutagenesis is an important breeding tool to improve the desirable characters among the existing commercial varieties. Although, the desired variation is often lacking, radiation can be used to induce mutations and thereby generate genetic variation from which desired mutants may be selected. Different methods are available to investigate the effect of mutagens on plants (Chopra, 2005). However, molecular markers allow a direct comparison of the effects of genotypes at the DNA level. The explorations of Random Amplified Polymorphic DNA (RAPD) as genetic markers have improved the effectiveness of recombinant DNA techniques. RAPD analysis hence can be used for the detection of DNA alterations after the influence of mutagenic agents. Irradiation by gamma rays leads to the increasing level of DNA break formation. These different types of DNA damages must be detected by changes in RAPD profiles (Selvi et al., 2007). Therefore, in the present investigation, mutation induction in *J. curcas* was undertaken to identify the DNA polymorphisms induced by gamma rays.

MATERIALS AND METHODS

Plant source, irradiation and morphology study

In the present study, the seeds of MP-022 accession from Sidhi and MP-031 from Shahdol, Madhya Pradesh, India which showed >95% similarity as investigated in our previous study (Dhillon et al., 2012), were used as basic material. Seeds of MP-031 were irradiated with different doses of gamma rays namely, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 kR, whereas the untreated seeds of MP-022 accession were used as control. Prior to finalization of different treatments of gamma rays, medial lethal dose that causes 50% mortality of the seeds (LD50) was standardized. The gamma rays treated as well as untreated seeds were sown at 1 cm depth in root trainers (250 cc m^3) having vermiculite, perlite and coco-pit in the ratio of 3:2:1. These blocks of root trainers were kept in the poly house of Department of Forestry, CCS Haryana Agricultural University, Hisar. Two seeds were sown in each cell of root trainer and for each treatment 300 seeds were used. Customary care was undertaken after seed sowing. Observations on seed germination and survival of germinated seedlings were recorded regularly.

At the age of four months, 18 plants from each treatment were transplanted in experimental field following randomized block design with three replications and with six plants in each replication. Observations were made for growth, reproductive and yield parameters. The data were analyzed using the statistical model suggested by Panse and Sukhatme (1978).

DNA extraction

DNA extraction was carried out from young leaves of putative mutants (gamma-rays treated) and control plants (untreated) following the cetyl trimethyl ammonium bromide (CTAB) extraction method (Murry and Thompson, 1980) with some modifications. DNA

extraction buffer was comprised of 1.5% CTAB, I00 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% PVP and 2% β-mercaptoethanol. The homogenized mixture (CTAB buffer and ground tissue) was incubated at 60°C for 90 min.

After incubation, chloroform:isoamylalcohol (24:1) extraction was carried out twice and DNA was precipitated with chilled isopropanol. DNA was washed with wash 1 (76% ethanol with 0.2 M sodium acetate) for 20 min and then subsequently washed with wash 2 (76% ethanol with 10 mM ammonium acetate) for 2 min. Afterwards, the DNA was left for air drying. Dried DNA was dissolved in appropriate TE buffer. RNase A (50 µg/ml) treatment was given to remove RNA at 37°C for 2 to 3 h. After incubation, DNA was extracted with chloroform:isoamylalcohol and precipitated with ice-cold absolute alcohol. The quality and quantity of DNA were tested by submerged horizontal agarose gel (0.8%) electrophoresis (Sambrook et al., 1989) using λ DNA (25 and 50 ng) as standards.

RAPD analysis

A total of 47 RAPD primers with high GC content (synthesized from Life Technologies, India) were used for molecular characterization of gamma-rays induced mutants in *J. curcas* (Table 1). PCR amplifications were carried out in 10 µl reaction mixture containing 12.5 ng template DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 µM primer (Life Technologies), 200 µM dNTP mix (MBI Fermentas) and 1.0 U of Taq DNA polymerase (Life Technologies, India). PCR amplifications were performed using MJ Research, Inc. PTC-150 minicycler with initial denaturation at 94°C for 4 min, followed by 40 cycles of amplification with denaturation at 94°C for 1 min, annealing at 35°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min. The amplified products were electrophoresed on 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) using 0.5 × TBE buffer at 80 V for 4 h. A 500 bp DNA ladder was used as a molecular weight standard. The DNA fragments were visualized under UV light and photographed using the Vilber Lourmat gel documentation system.

Data analysis

The data were scored for presence (1) or absence (0) of bands and set in a binary matrix. Jaccard's coefficient of similarity was measured and a dendrogram based on similarity coefficients was generated by using unweighted pair group method with arithmetic mean (UPGMA). Principal coordinate analysis was done using the package NTSYS-PC (Rohlf, 1990).

RESULTS AND DISCUSSION

Seed germination, survival and yield attributing parameters

Table 2 shows that seed germination in the untreated seeds started after five days of sowing; however, germination was delayed in irradiated seeds which commenced after nine days of sowing. Significant variation was observed in seed germination and survival percent of both irradiated and untreated seeds. In case of percent seed germination, the data shows that 5 and 10 kR doses have a stimulatory effect on seed germination (84.9 and 73.1%, respectively); whereas, when the dose was increased to 20 kR and above, the seed germination percentage was reduced up to 16.6% (50 kR). The percent survival was highest (98%) in control plants followed by plants obtained from the seeds treated with 10 kR dose of gamma rays (92.3%). It was observed that the percent survival decreased radically with increase in the irradiation level of gamma rays. The higher exposures to irradiation are usually inhibitor on seed germination of both agricultural crops and tree species (Akhaury et al., 1993; Thapa et al., 1999); whereas, lower exposures stimulated percent seed germination (Taylor, 1968; Chauhan, 1978).

The reasons of these stimulations are acceleration in cell division rates (Zaka, 2004) as well as activation of auxins (Gunckel and Sparrow, 1991). Percent reduction/stimulation in seed germination might have been due to the effect of mutagens on meristematic tissues of the seed. The decrease in germination and survival at higher doses of the mutagens may be attributed to disturbances at cellular level (caused either at physiological or physical level) including chromosomal damages. Kumar and Mishra (2004) reported that in okra (*Abelmoschus esculentus*), germination percentage decreased with increase in gamma rays dose. Reduced germination and survival percentage with increasing doses of gamma radiation was also reported in *Pinus kesiya* and *Pinus wallichiana* (Thapa, 2004) and *J. curcas* (Songsri et al., 2011). Similar pattern was observed for plant morphological (plant height and basal diameter) characters. Stunted plant growth was recorded under 45 and 50 kR treatments. The higher doses of gamma irradiation lead to reduction in plant growth parameters in the present study, which may be partly due to the fact that the cells which have relatively more chromosomal damage at high irradiation exposures are at a disadvantage due to diplontic section, which cannot complete well with the normal cells and are thus prevented from making any further contribution. Days to first flowering ranged from 256 to 327 days (Table 2). A maximum decrease in days to first flowering (256 days) was recorded in 25 kR followed by 50 kR dose (273 days). Gamma radiation induced earliness in flowering and the plants set flowers earlier than that of control, which took longer duration of 327 days for flowering. Total number of flowers per inflorescence had a high degree of variability as 20 kR dose had 292 flowers (male to female flower ratio 23:1) which were more than two times as compared to the 45 kR dose of gamma irradiation which had only 136 flowers (male to female flower ratio 31:1). However, the maximum femaleness (19:1) was observed in the plants obtained from 25 kR treatment.

The days taken for fruiting to maturity ranged from 57 to 65. The least time taken was 57 days by 25 and 40 kR doses and the highest time of 65 days was taken by control plants. Seed yield per plant (g) varied from 53.9 to 213.7 g. The highest seed yield per plant was obtained from the plants obtained from the 25 kR treatment and the lowest seed yield per plant was recorded at 50 kR

Table 1. List of primers and DNA fingerprint profile of γ-rays induced mutants in *J. curcas.*

Primer	Sequence(5'-3')	% GC content	*TNB	**PP	Band size range	PIC value
UP-1	CAGGCCCTTC	70	13	46	250-1900	0.23
UP-2	TGCCGAGCTG	70	10	80	550-2500	0.49
UP-3	AGTCAGCCAC	60	14	71.4	475-1750	0.24
UP-5	AGGGGTCTTG	60	14	57.1	200-1975	0.14
UP-6	GGTCCCTGAC	70	3	33.3	650-950	0.09
UP-7	GAAACGGGTG	60	13	38.5	300-2500	0.19
UP-13	CAGCACCCAC	70	14	35.7	530-1750	0.14
UP-14	TCTGTGCTGG	60	13	53.8	520-2350	0.21
UP-15	TTCCGAACCC	60	5	60	600-2500	0.11
UP-16	AGCCAGCGAA	60	14	57.1	380-2200	0.26
UP-17	GACCGCTTGT	60	5	40	400-1600	0.09
UP-18	AGGTGACCGT	60	13	38.5	270-2100	0.14
UP-19	CAAACGTCGG	60	6	50	650-1800	0.23
UP-20	GTTGCGATCC	60	10	80	400-2000	0.26
UP-24	CCGAACACGG	70	16	56.2	230-2100	0.16
UP-26	TCGTTCCGCA	60	7	57.1	250-2000	0.29
UP-27	CCTCTCGACA	60	8	62.5	380-2400	0.22
UP-29	TGAGCCTCAC	60	13	69.2	250-1950	0.36
UP-30	AAGCCCGAGG	70	9	77.7	280-1200	0.64
UP-32	GTCCCGTGGT	70	6	50	550-2250	0.18
UP-35	TGTAGCAGGG	60	9	66.7	300-1400	0.36
UP-36	CTGCTTAGGG	60	8	75	550-2000	0.28
UP-39	GGACACCACT	60	9	66.7	100-820	0.32
UP-40	AAGCGGCCTC	70	7	28.6	475-2000	0.16
UP-41	CCCAAGGTCC	70	7	57.1	550-2000	0.05
UP-44	GTGACATGCC	60	11	63.6	250-2200	0.21
UP-45	TCAGGGAGGT	60	13	92.3	430-2200	0.43
UP-46	AAGACCCCTC	60	16	75	200-1500	0.38
UP-47	AGATGCAGCC	60	18	83.3	350-2300	0.33
UP-49	CTTCACCCGA	60	9	77.7	550-1700	0.39
UP-51	GAGTCTCAGG	60	11	63.6	250-1950	0.08
UP-54	TGCGGCTGAG	70	11	36.4	200-1900	0.15
UP-55	ACGCACAACC	60	11	72.7	450-1300	0.28
UP-56	GGTGACTGTG	60	8	75	500-2400	0.25
UP-58	GGACTGCAGA	60	14	71.4	300-1850	0.33
UP-60	AACGGTGACC	60	11	63.6	390-1900	0.30
UP-63	CTGTTGCTAC	50	9	88.9	300-1300	0.36
UP-65	CCCAGTCACT	60	8	37.5	600-1900	0.06
UP-67	CAGCACTGAC	60	11	72.7	150-1400	0.25
UP-82	GGGAACGTGT	60	11	63.6	200-2000	0.30
UP-85	GTCGCCGTCA	70	15	80	350-1800	0.27
UP-86	TCTGGTGAGG	60	10	90	475-1750	0.41
UP-87	GGTCTACACC	60	6	83.3	780-2000	0.38
UP-88	CACCGTATCC	60	11	72.7	300-1500	0.32
UP-96	GGGACGATGG	70	13	69.2	475-1475	0.22
OPN-12	CACAGACACC	60	11	54.5	700-2000	0.30
OPO-03	CTGTTGCTAC	50	17	100	300-2500	0.42

*TNB- Total number of bands, **PP- percentage polymorphism.

Table 2. Effect of gamma radiation on percent germination, growth and reproductive parameters of *J. curcas.*

Treatment (^{60}Co)	Germination (%)	Survival (%)	Days taken to first flowering	Flowering plants/ population (%)	Plant morphological characters at reproductive age		Number of flowers/ inflorescence	Male to female flower ratio	Days taken from flowering to fruit maturity	Seed yield/ plant (g)
					Plant height (cm)	Basal diameter (cm)				
Control	67.3 ± 4.49	98.0 ± 1.97	327 ± 16.04	1.7 ± 0.94	127.8 ± 9.78	4.53 ± 1.18	189 ± 23.86	36:1	65 ± 2.43	96.2 ± 6.45
5 kR	84.9 ± 5.03	84.9 ± 4.21	294 ± 29.43	8.3 ± 3.12	114 ± 14.36	5.34 ± 0.86	263 ± 31.42	39:1	60 ± 1.81	163.8 ± 13.87
10 kR	73.1 ± 5.79	92.3 ± 3.37	304 ± 11.68	13.8 ± 1.49	122 ± 5.50	4.84 ± 0.53	251 ± 17.97	33:1	59 ± 0.75	183.3 ± 5.03
15 kR	69.5 ± 2.98	80.8 ± 5.04	281 ± 18.37	32.4 ± 4.76	104 ± 19.42	4.39 ± 0.97	176 ± 24.20	32:1	63 ± 1.50	155.5 ± 9.27
20 kR	50.9 ± 4.23	76.1 ± 2.53	290 ± 8.91	65.4 ± 1.88	97 ± 19.03	4.44 ± 1.05	292 ±22.53	23:1	58 ± 1.08	176.1 ± 7.91
25 kR	44.3 ± 2.52	61.3 ± 6.12	256 ± 19.47	69.7 ± 6.03	94.5 ± 11.27	4.29 ± 1.49	194 ± 16.68	19:1	57 ± 1.67	213.7 ± 12.38
30 kR	36.2 ± 6.40	43.9 ± 4.45	299 ± 28.03	61.5 ± 2.97	100.08 ± .95	4.60 ± 0.70	263 ± 26.34	23:1	59 ± 1.32	189.6 ± 3.66
35 kR	27.4 ± 5.33	33.4 ± 5.10	285 ± 25.32	57.6 ± 2.53	94.3 ± 13.74	4.45 ± 0.37	249 ± 30.72	31:1	59 ± 2.69	147.8 ± 15.62
40 kR	20.5 ± 3.58	28.1 ± 2.09	302 ± 10.93	54.3 ± 5.35	91.4 ± 10.58	5.10 ± 0.66	188 ± 29.16	26:1	57 ± 4.36	101.6 ± 5.84
45 kR	20.1 ± 6.14	23.6 ± 5.71	294 ± 15.89	36.8 ± 2.09	63.8 ± 8.43	4.95 ± 0.28	136 ± 17.53	31:1	61 ± 2.28	98.3 ± 17. 41
50 kR	16.6 ± 5.51	20.1 ± 3.93	273 ± 26.76	8.0 ± 3.63	62.13 ± 5.51	5.13 ± 0.73	162 ± 23.49	22:1	58 ± 5.82	53.9 ± 10.28
CD (P≤0.05)	5.81	7.39	11.96	3.07	4.22	0.91	13.69	-	3.35	16.79

followed by control.

Similar findings were also reported earlier in *Vitis vinifera* (Charbaji and Nabulsi, 1999), *Pisum sativum* (Zaka et al., 2004), *Triticum durum* (Melki and Marouani, 2009) and *J. curcas* (Dhakshanamoorthy et al., 2011). The variation observed in reproductive characters such as days to first flowering, flowering population, number of flowers per inflorescence, male to female flower ratio and days taken for flowering to fruit maturity in the present investigation can be useful for plant cultivation with high seed yield as the primary objective.

The findings on plant morphological and reproductive parameters showed that gamma rays treated plants can change the flowering and its maturity in either a positive or negative direction which result in sufficient variability in the treated population that can be utilized for selection of early or late flowering plants for further improvement of this versatile crop.

Days to flowering and maturity in the case of irradiated populations were consistently shifted towards earliness. It is valuable in obtaining varieties associated with escape from pests, drought and other stress injuries that occur in late growing winter season.

DNA polymorphism

A total of 47 RAPD primers were used which resulted in the generation of a total of 501 reproducible RAPD bands. Out of these, 174 were monomorphic and 327 were polymorphic. The overall polymorphism percentage was 65.27%. The number of bands (DNA fragments) per primer ranged from 3 (UP-6) to 18 (UP-47), the average number of bands per primer being 10.66. The size of amplified products ranged from 100 to 2500 bp (Table 1). The selection of primers with high GC content (mostly 60 to 70%) was useful in terms of reproducibility of results and production of higher frequencies of RAPD because of increase in total frequency of amplified fragments (Fritsch et al., 1993). The PIC values ranged from 0.05 (UP-41) to 0.64 (UP-30) (Table 1).

The highest PIC (0.64) was exhibited by primer UP-30 followed by UP-2 (0.49), UP-45 (0.43), OPO-03 (0.42) and UP-86 (0.41) primers. These primers revealed more informativeness and can be used for further studies. The banding profile of mutants and control with different RAPD primers is depicted in Figure 1. The main changes observed in RAPD profiles were both in the presence or absence of different bands with variations of their intensities as well.

As evident from Figure 1, some of the RAPD bands appeared as new bands in specific γ-ray induced mutants while some of the RAPD bands which were present in the control disappeared in some specific mutants. These effects of γ-rays may be correlated with structural re-arrangements

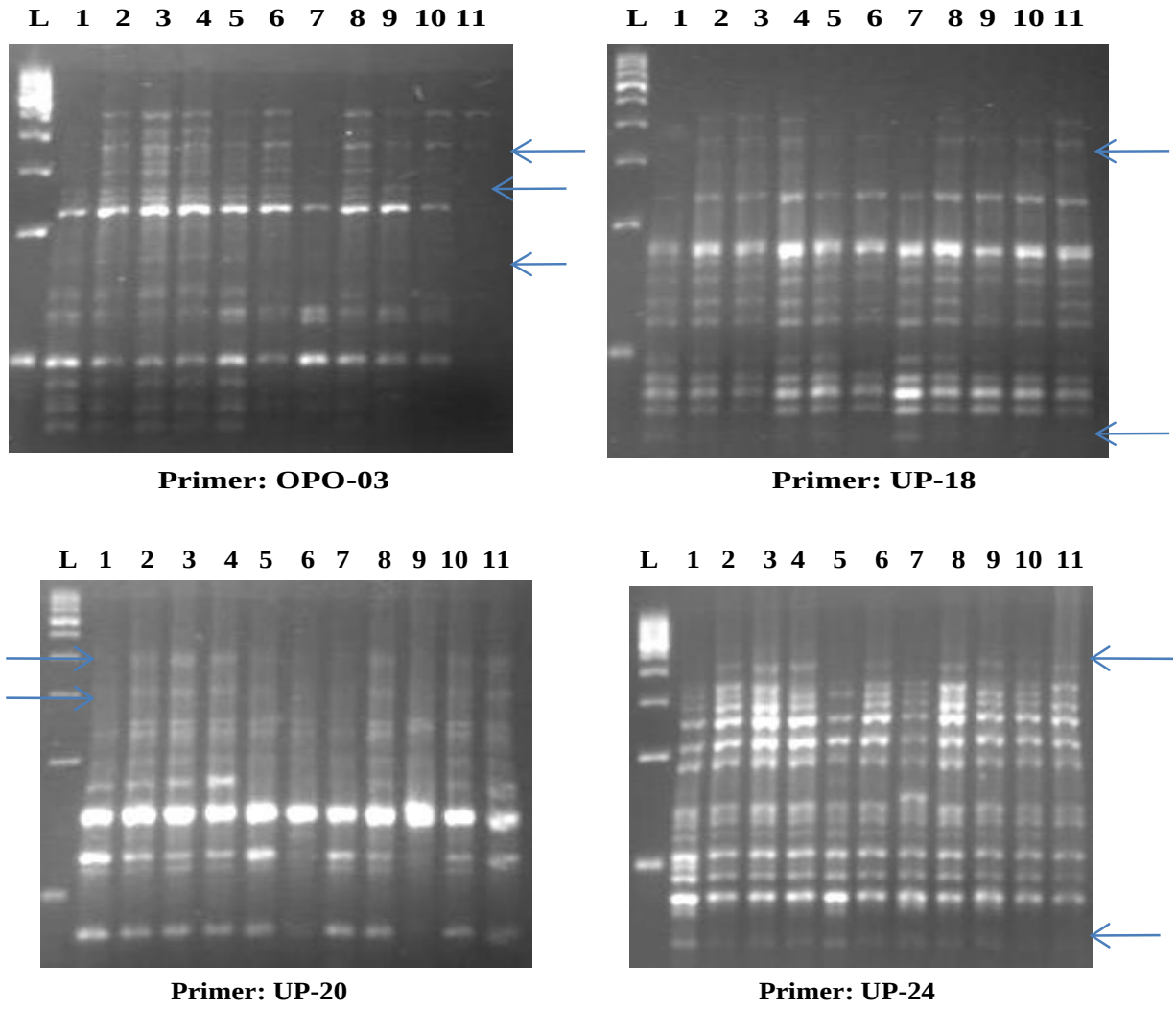

Figure 1. RAPD pattern obtained in γ-rays induced mutants in *J. curcas* with different primers. L, 500 bp DNA ladder; lanes 1 to 11, control, 5 kR, 10 kR, 15 kR, 20 kR, 25 kR, 30 kR, 35 kR, 40 kR, 45 kR and 50kR, respectively.

in DNA (breaks, transpositions, deletions, etc.) caused by different types of DNA damages (Selvi et al., 2007).

Bhagwat et al. (1997) screened radiation induced mutants of groundnut (*Arachis hypogaea*) for RAPD variability and observed 57% polymorphism. Similarly, RAPD method was successfully employed to study genetic variability in radio mutants from *Chrysanthemum* (Ruminska et al., 2004; Kumar et al., 2006) and amla (Selvi et al., 2007).

In these studies, genetic variation rate of mutants was markedly increased as evaluated by RAPD markers. Dhakshanamoorthy et al. (2011) used 23 RAPD primers to evaluate the gamma rays induced mutation in *J. curcas* and reported 55.16% polymorphism. Based on Jaccard's coefficient of similarity values, the maximum similarity (0.906) was found between 10 and 15 kR mutants (Table 3). Minimum similarity (0.634) was found between 40 and 50 kR mutants. Control plant had minimum similarity with 50 kR mutant (0.657) followed by 25 kR (0.687), thereby showing that as the dose of mutagen (gamma rays) increases, variability at the DNA level increases proportionately. However, the trend of genetic variability was not exactly proportional to the doses of gamma rays when similarity coefficient values were examined. The reason may be that genetic variation of radio mutants is proportional to the dosage of mutagen within a certain range (Teng et al., 2008). Average similarity was found to be 0.771 and range of diversity/variation was 9 to 28%. Two main clusters were delineated from the dendrogram (Figure 2). Cluster 1 comprised of control, 20, 25, 30 and 40 kR mutants.

Cluster 2 comprised of 5, 10, 15, 35, 45 and 50 kR mutants. Similar findings were reported by Dhakshanamoorthy et al. (2011) in gamma rays induced mutants in *J. curcas* using 23 RAPD primers. The proximity matrix based on RAPD analysis of EMS-induced mutants showed that

Table 3. Similarity matrix of γ-rays induced mutants in *J. curcas* using database generated by RAPD markers.

	Control	5 kR	10 kR	15 kR	20 kR	25 kR	30 kR	35 kR	40 kR	45 kR	50 kR
Control	1.00										
5 kR	0.728	1.00									
10 kR	0.698	0.862	1.00								
15 kR	0.700	0.844	0.906	1.00							
20 kR	0.816	0.740	0.738	0.764	1.00						
25 kR	0.686	0.798	0.724	0.758	0.742	1.00					
30 kR	0.852	0.716	0.674	0.684	0.796	0.734	1.00				
35 kR	0.710	0.822	0.852	0.858	0.754	0.760	0.730	1.00			
40 kR	0.710	0.662	0.664	0.690	0.714	0.768	0.746	0.684	1.00		
45 kR	0.744	0.780	0.786	0.796	0.772	0.774	0.760	0.822	0.738	1.00	
50 kR	0.656	0.788	0.818	0.804	0.688	0.682	0.672	0.782	0.634	0.808	1.00

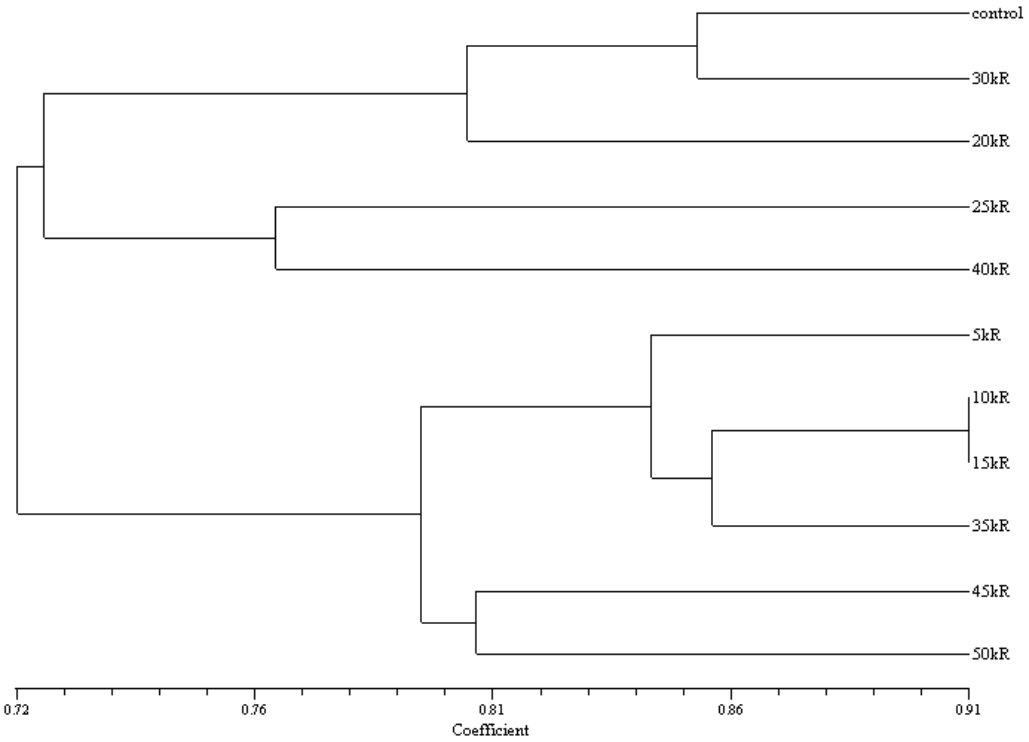

Figure 2. Dendrogram illustrating clustering of γ-rays induced mutants in *J. curcas* based on Jaccard's coefficient of similarity (327 polymorphic bands used).

three mutants were more distinct from the control and other mutants whereas the remaining were quite close to each other and with the control.

In the present study, major differences were observed in RAPD profiles of exposed samples with different doses of γ-rays. The γ-rays exposure changed the patterns of RAPD in comparison with control and hence can be adopted in mutation breeding of *J. curcas.* Further, molecular characterization of mutants might be helpful for the set-up of an efficient mutation induction protocol, for example, identifying proper doses of mutagen by assaying DNA damage. The mutants showing the differences in morphological traits showed DNA polymorphism in PCR profile amplified by RAPD marker. It is concluded that DNA polymorphism detected by RAPD analysis offered a useful molecular marker for the identification of mutants in gamma radiation treated plants.

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A protocol for large scale genomic DNA isolation for cacao genetics analysis

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Advances in DNA technology, such as marker assisted selection, detection of quantitative trait loci and genomic selection also require the isolation of DNA from a large number of samples and the preservation of tissue samples for future use in cacao genome studies. The present study proposes a method for the preservation of sample tissues for DNA extraction and for manual extraction of large number of samples using spheres. The integrity and concentration of the DNA by these methods were assessed and compared with conventional method using mortar. The best parameters in order to obtain a fine powder using spheres was the use of 4 lyophilized leaf disks (50 mg), a single steel ball of 6 mm in diameter, followed by 30 s of manual maceration. The quantity of DNA obtained was four times higher than the conventional method. The purity of the DNA obtained was satisfactory and proved to be amplifiable by PCR using SSR primers. The present approach is a reliable, rapid, simple and consistent DNA isolation method for cacao, compared to the conventional methods. The protocol greatly increases the efficiency of extraction and suggests an inexpensive and practical way of DNA isolation of cacao for large scale.

Key words: DNA extraction, cacao, spheres, lyophilized.

INTRODUCTION

Cacao (*Theobroma cacao* L.), the chocolate tree, is an important tropical species that provides sustainable economic and environmental benefits to some of the poorest and most ecologically sensitive areas of the world. The emergence of molecular marker analyses in genome studies has greatly enhanced the speed and efficacy of crop improvement and breeding programs. In breeding studies, numerous populations are sampled to detect candidate genes or molecular markers associated with economically important traits (Guiltinan, 2007; Maximova et al., 2007; Micheli et al., 2010). Similarly, a large number of accessions are sampled to determine the genetic diversity present in germplasm collections (Lanaud, 1986; Almeida et al., 1995; Pires et al., 1999; Yamada et al., 2001). Advances in DNA technology such as marker assisted selection (MAS), detection of quantitative trait loci (QTL) and genomic selection also require the isolation of DNA from a large number of samples and the preservation of tissue samples for future use. Widespread application of MAS and molecular characterization often require the collection of tissue samples from sites distant from laboratories (such as germplasm collections or fields) or reference populations. This requires proper methods for the conservation of the samples. The most common method used to preserve samples for subsequent DNA extraction is a rapid dehydration of the leaves mainly

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Table 1. Factors tested to optimize the maceration process.

Type of tissue	Number of spheres	Number of leaf disks	Size of the sphere (mm)
Fresh	1	4	6
Lyophilized	1	3	6
Fresh	1	5	4
Fresh	1	4	3
Fresh	1	6	2
Lyophilized	3	4	2
Lyophilized	2	4	5
Lyophilized	3	4	3
Lyophilized	1	4	6

on silica gel (Chase and Hills, 1991). This method is effective for many species with rapid rates of desiccation that prevents DNA degradation. However, improper use of silica gel (too little for a large amount of tissue or insufficiently dry) may result in poor desiccation of leaf tissue and DNA degradation.

In the present study, we report a methodology for DNA extraction based on lyophilized tissue. The use of lyophilized tissue offers several advantages. Dry tissue can be efficiently disrupted while the DNA is unhydrated and thus less susceptible to shear. Since dry tissue can be stored for several years with little loss in DNA quality (Murray and Thompson, 1980), it can be a perfect association with methods for DNA extraction suitable for genotyping and genetic studies. The optimization of this initial stage of collection and storage of plant material depend on the success of subsequent steps of any molecular study. Another major bottleneck encountered in most genomic and molecular markers laboratories, besides the preservation of sample tissue, is the slowness in the maceration of samples. Much of the slowness is due to the way the leaves are crushed. Usually, the samples are macerated individually and transferred to specific tubes, increasing the time for extraction and the risks of contamination and misidentification in the transference from the mortar to tubes. This step is usually performed using mortar and pestle (conventional method) in the presence of large amounts of liquid nitrogen. Equipments specially designed for the maceration of plant tissues in large scale using commercial beads exist in the market (beadbeaters); however, they are usually quite costly, limiting their use in many laboratories, particularly those in third world countries.

Several DNA extraction protocols already available for cocoa have proven unsuitable because of the presence of high levels of mucilage, polysaccharides and polyphenolic compounds within the tissues. Furthermore, the published protocols developed specifically for cocoa are complex, requiring extensive configuration steps (Figueira et al., 1992; Laurent et al., 1993; Lanaud et al., 1995; Faleiro et al., 2002). The present study proposes a method for the preservation of sample tissues for DNA extraction and for manual extraction of large number of samples using spheres (spheres method). The integrity and concentration of the DNA by these methods were assessed and compared with conventional methods.

MATERIALS AND METHODS

Sample preparation and maceration using spheres method

Leaf disks of 15 mm in diameter (approximately 50 mg of lyophilized tissue) per cacao plant were excised from young, green, fleshy leaves, showing no or minimal damage from microorganisms or insects, previously cleaned with a solution of sodium hypochlorite at 1%. Disks were placed inside 2.0 ml Eppendorf tubes. The leaf discs collected were immediately stored in a freezer at -80°C. Prior the extraction, the leaf disks were lyophilized for 24 h in the microtubes, capped and stored in desiccators for conservation free of moisture until use. The leaf disks were then placed in 2 ml microtubes. On these microtubes it was placed stainless steel balls or spheres of different diameters and quantities (Table 1; Figure 1a). The sets of tubes were then immersed in liquid nitrogen (Figure 1b) and then placed inside a plastic box with lid for storage of the microtubes and posterior maceration. In the maceration process, the box with the microtubes was manually stirred for 30 s (Figure 1c), producing a very fine powder (Figure 1d). Aiming to optimize the maceration process using spheres, the effect of various factors were tested (Table 1) in order to obtain a fine powder, required in the next steps of the DNA extraction.

DNA extraction

DNA extraction was performed according to the procedure described in Risterucci et al. (2000) with some modifications to a set of 20 cacao trees from different progenies used for genetics studies. The fine powder (50 mg of tissue) obtained by manual maceration using mortar (conventional method) or the spheres method described earlier (Figure 1d) was mixed with 850 µL of extraction buffer (1.4 M NaCl, 100 mM Tris HCl pH 8.0, 20 mM EDTA, 10 mM Na_2SO_3, 1% PEG 6000, 2% MATAB) pre-heated to 74°C. The extract was then homogenized for 10 s with a vortex and incubated for 30 min at 74°C; after cooling at 20°C (Figure 2a), an equal volume of chloroform-isoamyl alcohol (24:1 v/v) was added, followed by emulsification. The tube was then centrifuged at 7000 g for 30 min (Figure 2b) and the supernatant was precipitated at - 20°C overnight after the addition of an equal volume of isopropanol (Figure 2c). The DNA was removed with a sterile tip (Figure 2d) and re-suspended in 200 µL of TE buffer (50 mM TRIS-HCl, 10 mM EDTA, pH 7.0) with RNAse at 1%. As a control, the proposed

Figure 1. Proposed cacao leaf maceration protocol: a) tube, steel spheres and 15-mm leaf disks; b) freezing the tubes containing the spheres and the leaf discs lyophilized in liquid nitrogen; c) manual agitation of a set of samples; d) fine powder obtained.

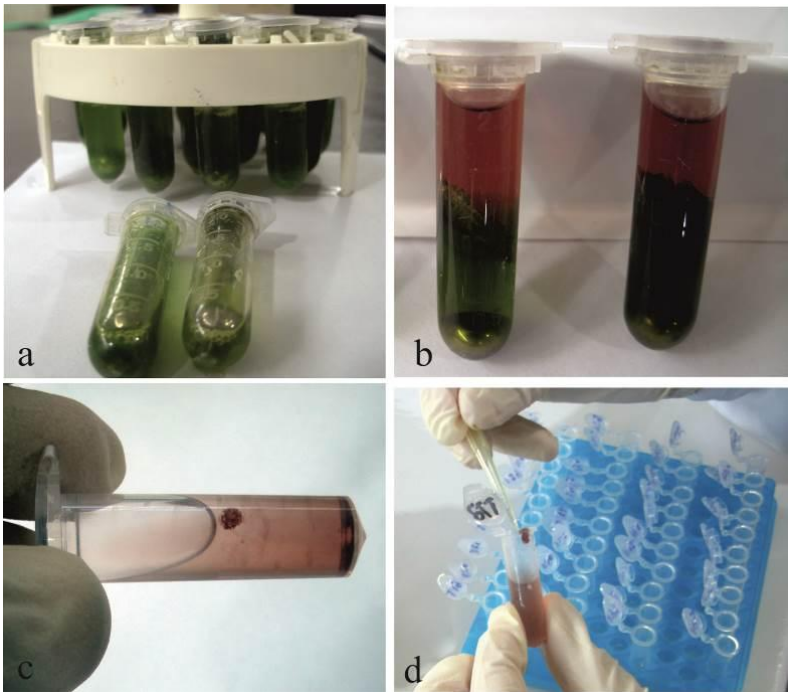

Figure 2. The extraction process: a) leaf disks with the extraction buffer; b) formation of the supernatant after centrifugation; c) formation of the pellet after addition of isopropanol; d) removal of the pellet with sterile tip.

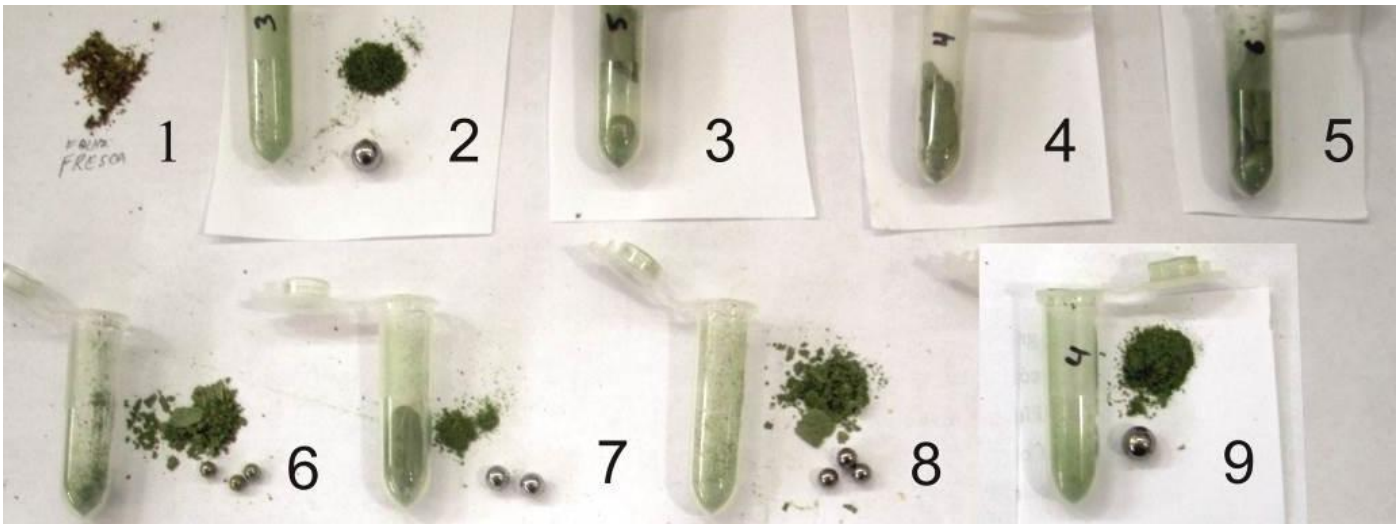

Figure 3. Parameters tested to optimize the maceration process ranging between: type of tissue, quantity of spheres, number of leaf disks and size of sphere (mm), respectively. 1 = fresh, 1, 4 and 6; 2 = lyophilized, 1, 3 and 6; 3 = fresh, 1, 5 and 4; 4 = fresh, 1, 4 and 3; 5 = fresh, 1, 6 and 2; 6 = lyophilized, 3, 4 and 2; 7 = lyophilized, 2, 4 and 5; 8 = lyophilized, 3, 4 and 3; 9 = lyophilized, 1, 4 and 6.

protocol was compared with the standard protocol used for DNA extraction in cacao, using the 20 same samples macerated using mortar, but now using fresh leaf material. After this step the same procedure described earlier was performed.

DNA quantification

Aliquots of 1 µL of extracted genomic DNA were submitted to electrophoresis on 1% agarose gels, stained with GelRed Nucleic Acid Stain (Biotium) and visualized using a transluminator with a system of image capture L-PIX IMAGE 7.1. Images were captured with the software L-PIX IMAGE 1.0.1 (Loccus Biotecnologia, Brazil). Phage lambda DNA (sigma) was used as marker ladder. For each sample, the total DNA extracted was quantified by optical density using a Picodrop spectrophotometer (Picodrop Limited, UK). The ratio A260 /A280 was used to assess the purity of DNA (Sambrook et al., 1989).

PCR amplification using cacao single sequence repeat (SSR) primers

Four samples of isolated DNA were randomly selected and subjected to PCR amplification with two cacao SSR primers [mTcCIR24 and mTcCIR35 (Lanaud et al., 1995)]. The total volume of the PCR reaction was 20 µL, which contained 2 µL of freshly extracted DNA (~ 20 ng), 2 µL of 10x PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl], 1.2 µL of 25 mM $MgCl_2$, 2 µL of each forward and reverse of 2 pmol primer, 1.6 µL of 2.5 mM dNTPs (dATP, dCTP, dGTP and dTTP), 0.5 µL of 5U of Taq polymerase (Fermentas). Amplifications were carried out in a termocycler Mastercycle (Eppendorf). The PCR cycle consisted of initial denaturing at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 51°C annealing for 1 min and 72°C for 1 min. This was followed by further primer extension at 72°C for 7 min. After PCR samples are subjected to electrophoresis on capillary ABI3100 automated sequencer (applied biosystems).

RESULTS

The best parameters in order to obtain a fine powder using spheres for obtaining a suitable material for DNA extraction is the use of 4 lyophilized leaf disks (50 mg), a single steel ball of 6 mm in diameter, followed by 30 s of manual maceration (Figure 3; picture 9). In a dried state, tissue can be ground or pulverized into a fine powder by mechanical means increasing the efficiency of DNA isolation (Figure 3; pictures 2, 6, 7, 8 and 9). For maceration use, mortar was needed more than 4 leaf discs, because of excessive loss of the material in the walls of mortar. The quantity of DNA obtained using the conventional method (mortar and fresh leaves) ranged from 4.5 to 15.7 µg of total DNA, the average being 10.74 µg. On the other hand, the quantity of DNA obtained with the spheres method and lyophilized tissue ranged from 14.6 to 75.5 µg, with an average of 49.17 µg. The A260/A280 ratio was between 1.7 and 2.0, in 40% of the samples using the conventional method and 90% using the spheres method, indicating low contamination with polyphenols, proteins and polysaccharides according to the A260/A280 ratio (Table 2). The purity of the DNA obtained was satisfactory and visible on agarose gel (Figure 4) compared with the conventional method and proved to be amplifiable by PCR using SSR primers (Figure 5).

DISCUSSION

In this study, a manual method of large scale DNA extraction was standardized to isolate DNA from cacao leaf samples. The protocol was then used to extract DNA from several cacao accessions under study in our laboratory. The sampling of the cacao leaves used in this study allowed for efficient storing and normalization of the plant material obtained from lyophilized cacao tissues. This approach opens the possibility for the creation of leaf banks of lyophilized material for cacao, in order to preserve important genotypes, by storing the leaves

Table 2. DNA quantity total (in µg) and ratio A260/A280 from the samples using the conventional and spheres methods.

Plant	Conventional method		Spheres method	
	Quantity (µg)	Ratio 260/280	Quantity (µg)	Ratio 260/280
1	8.4	1.44	50	1.70
2	15.6	1.33	49.7	1.83
3	8.4	1.42	55.6	1.81
4	7.4	1.52	58.9	1.91
5	8.3	1.80	50.7	1.70
6	12.6	1.91	63.6	1.81
7	9.5	1.90	14.6	1.56
8	4.5	1.73	40.4	1.78
9	7.8	1.41	45.8	1.81
10	9.5	1.57	53.5	1.80
11	14.6	1.60	65.4	1.92
12	5.5	1.40	75.5	2.00
13	14.6	1.72	63.5	2.00
14	8.9	1.80	64.2	1.41
15	13.6	1.43	33.1	1.86
16	15.7	1.54	40	1.84
17	14.6	1.61	40	1.91
18	5.7	1.40	44.8	2.00
19	15.7	1.70	44.5	1.92
20	14	1.73	29.6	1.72
Mean	10.74	1.61	49.17	1.81

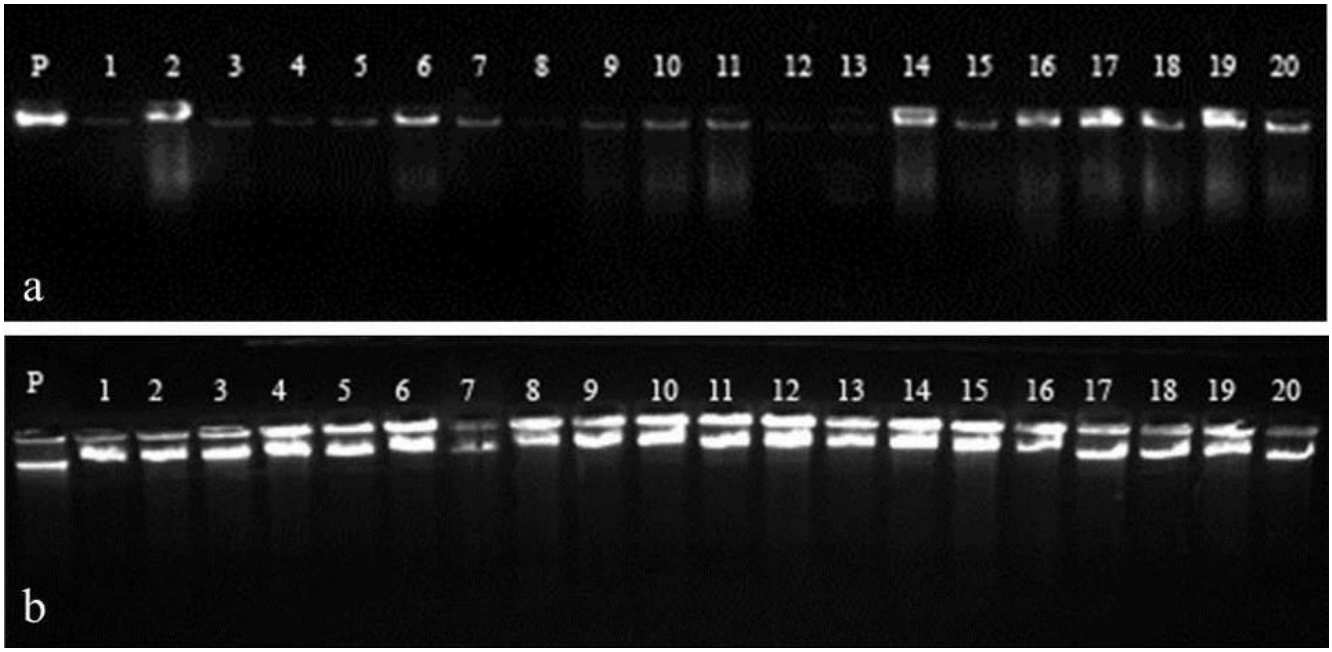

Figure 4. Agarose gel analysis of 20 genomic DNA extracted utilizing conventional method a); 20 same genomic DNA extracted utilizing spheres method b); P, Phage Lambda DNA (100 ng).

before the plant dies. The two major bottlenecks in DNA isolation from large populations of plants are the processing of the tissue to an extractable form and the storage (Tai and Tanksley, 1988). Dry tissue can be stored for

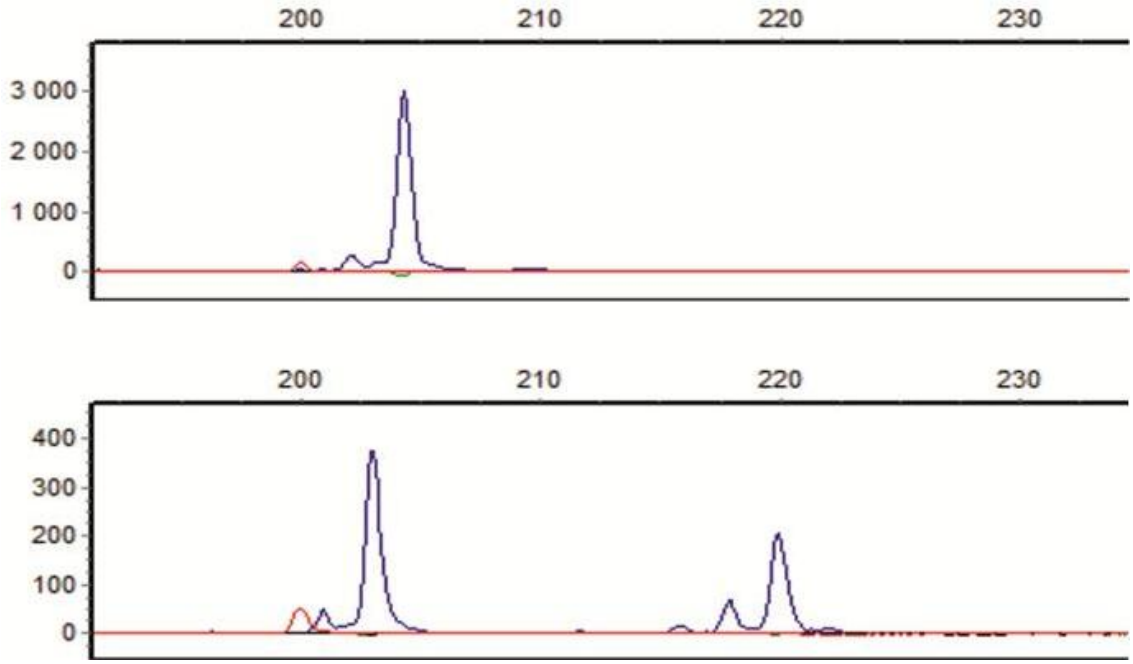

Figure 5. Representative data output for analysis of cacao DNA with marker mTcCIR24. The marker were amplified in a single PCR reaction and separated by capillary electrophoresis. The upper panel depicts the homozygous plants for the alleles, and the lower panel depicts the heterozygous plants. The alleles are represented by the peaks. The sizes in base pairs (bp) are indicated on the top x axis. The values on the y axis indicate fluorescent signal intensity given in relative fluorescent units (RFU) and vary for each sample.

several years with little loss in DNA quality and can be ground or pulverized into a fine powder by mechanical means which increases the efficiency of isolation (Figure 3). Hence, the use of 4 lyophilized leaf disks is sufficient to obtain sufficient amount of DNA in this extraction procedure. Another important point in this procedure also is the fact that it permitted the elimination of the use of liquid nitrogen for maceration, reducing the costs of DNA extraction. According to Tai and Tanksley (1988), the isolation of DNA from plants previously dried proved more efficient than the use of fresh tissue. The major advantage associated with the lyophilization is the decrease in the water content into the tissues and consequently, the reduction of the catabolic process inside the cell, since a less fluid environment slow down the catalytic activity of nucleases and proteases.

Another benefit of the lyophilization is the improvement of the relationship between extraction buffer and dry tissue. With less fluid in the tissue, the dilution of the extraction buffer is smaller and therefore, its activity is better, reflecting a greater amount of DNA extracted (Table 2 and Figure 4b). Unlike other DNA extraction protocols, the modified method presented here was effective to avoid the DNA oxidation in their very beginning. Despite the whole process of lyophilization requires about 24 h for complete dehydration, its applicability is certainly satisfactory, since its inclusion improves the quality and amount of the DNA purified allowing them to be easily removed from the samples before they irreversibly oxidize the DNA molecule. Five minutes was the time required to do the following steps: placing the steel spheres in the 20 tubes, immerging the tubes in liquid nitrogen, placing in the box the tubes, performing the manual agitation, adding the extraction buffer and carrying out the homogenization. In the conventional method, using mortar and pestle, it would take approximately 30 min to perform the same operations. The cost of the beads is extremely low; moreover, they can be reused many times, as long as previous DNA residues are adequately removed between extractions. Decontamination can be done by using the same procedures used to decontaminate the mortar and pestles.

In this study, we preferred the extraction protocol containing MATAB (Risterucci et al., 2000) because it contains fewer steps than the conventional protocol being less laborious and faster. MATAB combined with the use of spheres method for maceration probably reduce the amount of polyphenols and polysaccharides resulting in a viscous substance typically associated with DNA extractions from cacao (Faleiro et al., 2002). These complex molecules interfere with DNA quality, leading to low yields (Tel-Zur et al., 1999), and inhibiting the action of the Taq polymerase (Fang et al., 1992). Hence, their removal is crucial for successful use of DNA in molecular techniques. The quantity of DNA obtained was four times higher than the conventional method utilizing the same quantity of tissue, since it shows greater uniformity, no degradation visible in agarose gels compared with the

conventional method (Figure 4a and 4b). The quality of genomic DNA is evident from high-molecular-weight bands and the absence of RNA along with other polysaccharides that usually affect migration during electrophoresis (Figure 4b) and seen frequently in plants (91%) with ratio A260/A280 between 1.7 and 2.0 (Table 2). The quantity of DNA obtained was high, sufficient for large number of PCR reactions. Amplification products were obtained for all the DNA samples tested with different cacao SSR primers (Figure 5). All samples under investigation amplified the PCR product expected for the SSR primers. Thus, we conclude that the present approach is a reliable, rapid, simple and consistent DNA isolation method for cacao, compared to the conventional methods.

The fact that DNA was successfully extracted indicates that this technique should work on a wide range of plant tissues. This method appears to be very flexible and may be scaled up or down depending upon the need. The spheres method processing of tissue in conjunction with the Risterucci et al. (2000) protocol greatly increases the efficiency of extraction and suggests an inexpensive and practical way of DNA isolation of cacao for large scale.

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Full Length Research Paper

Sensibility of the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin to *Pelargonium sidoides* extract (EPs 7630®) assessed by conidia germination speed parameter

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***Pelargonium sidoides*, known as umckaloabo, is a plant originally from South Africa and its extract is used in popular medicine. The fungus *Metarhizium anisopliae*, an important entomopathogen used in biological control of pests, is also employed as model for the evaluation of toxicity and compatibility of different products, considering, among other parameters, conidia germination speed. Therefore, this study aimed to evaluate the sensibility of *M. anisopliae* var. *anisopliae* in the presence of the extract of *P. sidoides*, at a concentration of 20.625 and 2.0625 mg/ml. During incubation at 28°C, samples were collected at 0, 6, 8, 10, 12 and 24 h and analyzed by light microscopy, with observation of 300 conidia, in triplicate, for both treatments and negative controls (in a completely randomized design). This study shows that the two concentrations of *P. sidoides* extract tested are capable of delaying the *M. anisopliae* conidia germination speed, in comparison to the controls, although the germination frequency has been restored after about 12 h of conidia incubations, with did not lack conidia viability, indicating no toxicity.**

Key words: Model fungus, toxicity, vegetative development, viability.

INTRODUCTION

The plant *Pelargonium sidoides* DC (Geraniaceae family) is native to the coastal regions of South Africa (Van der Walt and Vorster, 1988) and its root extracts has been widely used for the treatment of diarrhea and dysentery (Loureiro et al., 2005). Its ethanolic root extract, named EPs 7630® (Umckaloabo®), had full marketing authorization by the German drug regulatory agency (Conrad et al., 2007). It has antimicrobial and immunoregulatory activities (Franceschini et al., 2001) and it has been reported as efficient to treat herpes virus, respiratory infections, respiratory viruses (Rangel et al., 2004; Schnitzler et al., 2008; Michaelis et al., 2011). Its chemical composition is primarily phenolic and polyphenolic compounds, proteins, minerals, coumarin derivatives and a number of miscellaneous uncommon metabolites (Schötz et al., 2008). The early studies with the asexual filamentous fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin were conducted by the Russian researcher Elie Metschnikoff,

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in 1879, for the control of a species of beetle (Faria, 2001), and its great potential for the biological control of a variety of insect pests was already demonstrated (Makaka, 2008; Allsopp, 2010; Mochi et al., 2010; Niassy et al., 2011). Moreover, formulations based on it are commercialized by companies of Australia, Brazil, Germany, South Africa and USA (Khetan, 2001; Scholte et al., 2004; St. Leger and Wang, 2010). This fungus has been employed as model for evaluating its tolerance / compatibility / toxicity to different factors and substances (as UV-B radiation, fungicides, insecticides, insect grown regulators, pesticides, medicament) using parameters such as morphology, radial and vegetative growth, conidia germination speed (Rangel et al., 2004, 2005; Alves et al., 2011; Akbar et al., 2012; Tonussi et al., 2012; Fabrice et al., 2013; Bulla et al., 2013). Conidia germination speed can be explored as parameter to determine whether physical and/or chemical variables in the substrate on conidia are produced, having influence on fungal development and conidiogenesis (Rangel et al., 2004).

Considering that the *P. sidoides* extracts are used with medicinal application and *M. anisopliae* cells can be used as a referential of toxicity level of chemical products, as for example insecticides and medicaments, this study aimed to verify the sensibility of two concentrations of EPs 7630® extract on the conidia germination speed of *M. anisopliae*, expecting to detect the influence of this plant extract in an eukaryotic cell with economic importance, as an alternative citotoxic analysis of medicinal compounds, complementing another studies, such as mutagenicity tests with *Salmonella typhimurium*.

MATERIALS AND METHODS

Fungal strain, plant extract and culture media

The strain of *M. anisopliae* var. *anisopliae*, isolated from the insect host *Deois* sp., was obtained from the fungal culture collection of Laboratório de Biotecnologia Microbiana from Universidade Estadual de Maringá, Paraná, Brazil. The EPs 7630® (ethanolic root extract of *P. sidoides*) was employed. The culture media Potato Dextrose Agar (PDA) (Smith and Onions, 1983), complete medium (CM) and liquid complete medium (LCM) (Pontecorvo et al., 1953 modified by Azevedo and Costa, 1973) were employed.

Conidia germination speed in the presence of EPs 7630®

The *M. anisopliae* conidia solution was prepared according to Alves et al. (2011), except that the concentration 5.35×10^7 conidia/ml was obtained and inoculated into four flasks containing LCM. One of them was used as the negative control (C1); other received ethanol P.A. that was also used as negative control (C2). The two treatments received EPs 7630® in concentrations of 20.625 mg/ml (T1) and 2.0625 mg/ml (T2). All flasks were incubated in BOD at 28°C for 24 h. Samples from controls and treatments were analyzed in triplicate (three samples from each flask were collected) at 0, 6, 8, 10, 12 and 24 h of incubation. Germinated conidia were counted using Neubauer hemocytometer with light microscopy and the percentage of dormant, embedded, bud and germinated conidia was assessed by randomly observation of 300 conidia.

Viability of *M. anisopliae* conidia treated with EPs 7630®

The process described earlier was repeated, except that the conidia solution was obtained in a concentration of 6.20×10^7 conidia/ml. Samples from controls and treatments were collected at 8 h of incubation, diluted in concentrations of 10^2, 10^3, 10^4 and incubated in triplicate in Petri dishes (9 cm) containing CM (20 ml). Dishes were incubated in BOD at 28°C for 72 h and the number of macroscopic colonies was counted.

Statistical analysis

To verify the possible differences in the number of germinated conidia among treatments, incubation times and their interactions data were analyzed using statistical package BRugs for software R (2008) and the Poisson distribution was assumed, implemented in Bayesian methodology. Significant differences were considered at the level of 5% between the treatments if the zero value was not included in the credibility interval of the desired contrast. The Markov chain was composed of 10,000 samples, with a burn-in period of 1,000 initial values and thin interval equal to 10.

The estimates were based on posterior means of the remaining 900 samples. Non informative priors were used for the parameters. For germination percentage over incubation time, a binomial distribution was assumed. Therefore, logistic regressions were implemented in Bayesian methodology. Data were also analyzed using statistical package BRugs for software R (2008) according to the model:

$\log it(\theta ij) = \beta_0 + \beta_1 time + \beta_2 time^2$, for control 2
$\log it(\theta ij) = \beta_0 + \beta_1 time$, for control 1 and treatments 1 and 2,

Where log *it* is the logistic link function, θij is the germination percentage, β_0 is the intercept, β_1 is the linear logistic regression coefficient, β_2 is the quadratic logistic regression coefficient and, *time* is the number of hours elapsed since the beginning of incubation. The goodness of fit was checked by the coefficient of determination (r^2).

For this analysis, the Markov chain was composed of 10,000 samples, with a burn-in period of 1,000 initial values and thin interval equal to 10. The estimates were based on posterior means of the remaining 900 samples. A credibility interval (CI) of 95% was considered. Non-informative priors were used for the parameters. When a logistic link function is considered, the percentage of conidia germination can be obtained as:

$$\theta_{ij} = \frac{\exp(\beta_0 + \beta_1 time)}{1 + \exp(\beta_0 + \beta_1 time)}$$, for linear regressions, or

$$\theta_{ij} = \frac{\exp(\beta_0 + \beta_1 time + \beta_2 time^2)}{1 + \exp(\beta_0 + \beta_1 time + \beta_2 time^2)},$$

for quadratic regressions.

To verify the possible differences in the viability of *M. anisopliae* conidia in controls and treatments with EPs 7630®, data were also analyzed using statistical package BRugs for software R (2008) and the Poisson distribution was assumed, implemented in Bayesian methodology, as explained earlier.

Table 1. Bayesian estimates for the counting of germinated *Metarhizium anisopliae* conidia in the presence of *P. sidoides* extracts.

Treatment	Mean	Standard error	95% ICr	
			2.50%	97.50%
C1 (culture medium)	186[a]	0.03082	179.7	192.3
C2 (ethanol)	168.5[b]	0.02786	162.6	174.5
T1 (20.625 mg/ml)	93.2[d]	0.02455	88.77	97.73
T2 (2.0625 mg/ml)	100.5[c]	0.0239	95.84	105.2

[abcd] Different letters indicate that the means differ.

Table 2. Means and credibility intervals for counting of germinated *Metarhizium anisopliae* conidia throughout the incubation time.

Time (h)	Mean	Standard error	95% ICr	
			2.50%	97.50%
0	0.009[f]	2.933E-4	5.966E-18	0.081
6	29.770[e]	0.01677	26.840	32.940
8	100.500[d]	0.03386	94.860	106.300
10	154.400[c]	0.03572	147.400	161.400
12	233.300[b]	0.04492	224.800	242.300
24	297.600[a]	0.05318	288.000	307.200

[abcd] Different letters indicate that the means differ.

Table 3. Bayesian estimates for the logistic regression coefficients for control and treatments.

Treatment	b0	b1	b2	R^2
C1 (culture medium)	-6.846	0.916	-	0.991008
C2 (ethanol)	-4.931	0.7123	-0.0117	0.9681967
T1 (20.625 mg/ml)	-1.418e + 01	1.249e + 00	-	0.9949418
T2 (2.0625 mg/ml)	-9.716e + 00	8.697e - 01	-	0.9955921

b0 is the intercept, b1 is the linear coefficient, b2 is the quadratic coefficient and r^2 is the determination coefficient of regressions.

RESULTS AND DISCUSSION

Conidia germination speed in the presence of EPs 7630®

The entomophatogen important fungal *M. anisopliae* was chosen as model system to evaluate the effects of EPs 7630®, since fungi are considered model systems for studies about fundamental cell biological questions because basic principles of many cellular processes are conserved between fungi and animals (Steinberg and Perez-Martin, 2008; Bulla et al., 2013). The means and ICr for counting of germinated conidia are shown in Table 1. A bayesian ICr of 95% is the interval in which 95% of the samples are contained, and smaller is the interval, less dispersed is the parameter. The means of germinated conidia in 24 h were 186 (C1), 168.5 (C2), 93.2 (T1) and 100.5 (T2), with credibility interval formed by 2.5 and 97.5%. Bayesian analysis showed that there was a significant difference between treatments 1 and 2, being the conidia germination in the concentration of 20.625 mg/ml (T1) lower than germination with 2.0625 mg/ml (T2) of *P. sidoides* extracts. The two treatments significantly decreased the conidia germination speed, when compared with controls 1 and 2. According to Alves et al. (2011), the Bayesian statistical method is an approach that can work on datasets considering the true distribution, being reliable for small groups of data. The means of conidia germination in each incubation period sampled throughout 24 h and the credibility intervals are shown in Table 2. According to these results, the incubation periods of 0, 6 and 8 showed a low germination percentage of conidia incubated with *P. sidoides* extracts. Alves et al. (2011) observed that the physiological evolution of conidia germination started to be apparent from 8 h of incubation. In agreement with it, in the present study was observed that the conidia germination increased among 8 and 24 h of incubation. The logistic regression adjusted efficiently the conidia germination percentage over time and the coefficients of determination (r^2) are shown in Table 3. The behaviors of C1, T1 and T2

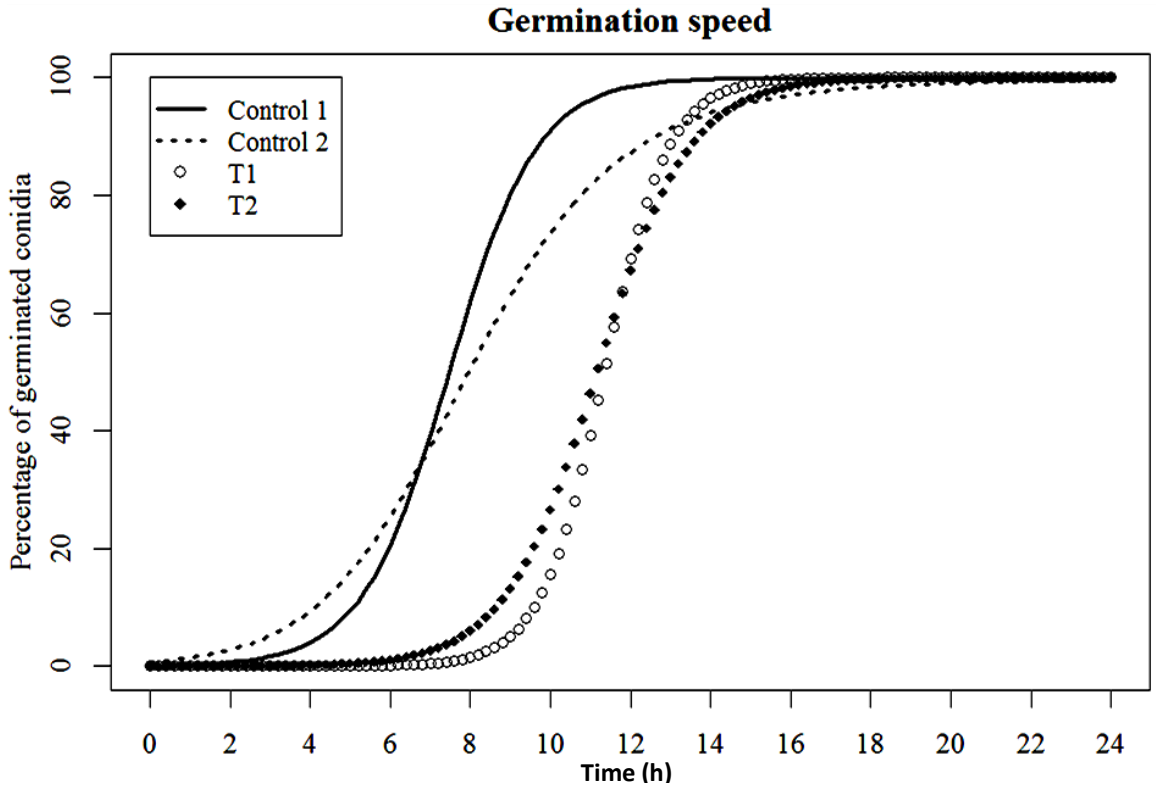

Figure 1. The curve of germination speed of *Metarhizium anisopliae* conidia in the control and treatments.

Table 4. Viability of *M. anisopliae* conidia in controls and treatments with EPs 7630® verified by the number of macroscopic colonies observed after 8 h of incubation. Means and credibility intervals for number of colonies were determined by Bayesian methodology.

Treatment	Mean	Standard error	95% ICr	
			2.50%	97.50%
C1 (culture medium)	2667.0[d]	0.9531	2606.0	2721.0
C2 (ethanol)	123330.0[a]	2.291	12210.0	12460.0
T1 (20.625 mg/ml)	5664.0[c]	1.214	5568.0	5750.0
T2 (2.0625 mg/ml)	6666.0[b]	1.344	6573.0	6758.0

[abcd] Different letters indicate that the means differ.

were similar, where the germination has a linear behavior, whereas in T2 and it was quadratic. The curve of germination speed (Figure 1) showed that the conidia germination started between 0 and 2 h of incubation for C1 and between 2 and 4 h of incubation for C2. The faster initial velocity was observed for C2, but the next 6 h of incubation, conidia in C1 has increased the speed of germination, passing C2. For T2, the conidia germination started near to 6 h of incubation, and it started near to 8 h of incubation for T1. Approximately after 16 h of incubation the germination speed of conidia remained stable for all controls and treatments.

The curve of *M. anisopliae* conidia germination speed obtained by Fabrice et al. (2013) showed that until 14 h of incubation, the germination speed increased for the control and all treatments of thiophanate-methyl (200, 20, 2 and 0.2 µg/ml); however, through incubation time this speed decreased and only it remained stable for controls and treatment with 0.2 µg/ml. Bulla et al. (2013) evaluating toxicity of the anti-hipertensive agent perindopril on the entomathogenic fungus *M. anisopliae* assessed by conidia germination speed parameter observed that the medicament concentrations of 200 and 20 µg/ml increased the germination speed of fungus conidia, indicating no toxicity. The results presented herein indicate that the two concentrations of *P. sidoides* extract tested are capable of delaying the *M. anisopliae* conidia germination, in comparison to the controls. All the germinated conidia has viability preserved (Table 4), that is, the capability of germinated conidia developing in a fungal colony. The process of spore germination can be defined as a sequence of events that activates the resting spore

(d'Enfert, 1997), what involves water uptake and wall growth (Griffin, 1994). The resting spore is converted into a rapidly growing germ-tube from which the mycelium will be formed by elongation and branching (d'Enfert, 1997). This process is directly influenced by the incubation period (Alves et al., 2011) and by environmental factors. Water, oxygen, and carbon dioxide are universally required to activate the spore germination (d'Enfert, 1997). More-over, optimum conditions such as temperature, humidity, pH and nutrient sources are essential for the conidia germination.

Germination speed of *M. anisopliae* conidia was similarly employed by Alves et al. (2011) as parameter to evaluate the toxicity of the insect grown regulator lufenuron. Bayesian analysis showed that conidia germination was not inhibited by the regulator in the lowest concentrations tested (700 µg/ml and 1 mg/ml). These authors suggested the possibility of a future use of a biological-chemical combination with *M. anisopliae* and lufenuron, with a low environmental impact to combat insect-pests.

Akbar et al. (2012) evaluated the toxicity of insecticides and fungicides on spore production and mycelial growth of *M. anisopliae*, showing that the chemicals composed by chlorpyrifos, phosphorus, metalaxyl + mancozeb and profenofos were the most toxic to mycelial growth and conidial germination. Meanwhile, the chemicals that contain acetameprid, cypermethrin, emamectin, imidacloprid and sinophos were less toxic to mycelial growth and spore production. Spinosad and indoxacarb were considered safe and compatible with *M. anisopliae*. The same methodology employed here was used by Tonussi et al. (2012) to evaluate the toxicity of deltamethrin on *M. anisopliae*. The authors observed that the concentration of 50 µg/ml reduced and delayed conidia germination and it was 100% inhibited by high concentrations. Ultra diluted treatments were not inhibitory with concentration of 31.25 ρg/ml and also with the concentration of 31.25 ηg/ml increased the germination of conidia, indicating a possibly occurrence of hormesis effect.

A recent study conducted by Fabrice et al. (2013) showed the efficiency of germination speed parameter to evaluate the compatibility of *M. anisopliae* Sorokin with fungicide thiophanate-methyl.

Data demonstrated that this compound inhibited or delayed the conidia germination, but not interfered in it when used at lower concentrations. The combined use of the entomopathogen and fungicide against *Diatraea saccharalis* larvae showed to be not high compatibly, since there was a 26.84% reduction of larvae mortality when compared with the use of *M. anisopliae* conidia only. This study shows that the two concentrations of *P. sidoides* extract tested are capable of delaying the *M. anisopliae* conidia germination speed, in comparison to the controls, although the germination frequency has been restored after about 12 h of conidia incubations, with did not lack conidia viability, indicating no toxicity.

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Full Length Research Paper

Prospects of increasing the presence of *Helianthemum kahiricum* Dell. pastoral North African plant by means of micropropagation

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Helianthemum kahiricum **has great potential in forage, in traditional medicine, for halting desert encroachment and stabilizing sand dunes through their excellent root systems development, and in the improvement of soil organic matter content. *H. kahiricum* is threatened with extinction because of overgrazing abuse and increasing human disturbance. For these reasons, *in vitro* propagation is essential for developing efficient conservation program. Several cytokinins: indole-3-acetic acid (IAA) ratios and a range of zeatin concentrations were evaluated for their effect on shoot multiplication from apical shoots and nodal segments. The type of cytokinin and the origin of the explants were the most important factors affecting shoot multiplication. The highest shoot multiplication rate was obtained from single-nodal explants on medium hormone free. Increasing zeatin concentrations promotes decreased shoot multiplication independently of explants type, although this effect tends to decrease with higher zeatin concentration. Shoot growth was higher in apical shoots and it was not stimulated by the presence of auxin. A number of experiments were conducted to identify suitable procedures for rooting of *in vitro* produced shoots. Although, rooting frequency rose to 98% by *in vitro* culture on an auxin-free medium, the survival of the plants after 6 months of acclimatization was good (90%).**

Key words: Biotechnology conservation, pastoral species, *ex vitro* rooting, *Helianthemum kahiricum*, *in vitro* culture.

INTRODUCTION

Helianthemum kahiricum Dell. (*H. kahiricum*) is a perennial herb plant [15 cm tall, covered in its various parts by glandular hairs, the leaves are whorled, sessile flowers with five sepals, five yellow petals (Ozenda, 1977), numerous stamens and 3 to 5 carpels defining a unilocular ovary, the fruit is a hairy capsule, photo-synthesis of the plant is C3 belonging to the Cistaceae] (Aïdoud et al., 2006). It is distributed in arid and semi-arid Mediterranean areas. It presents an important ecological, economical and pastoral interest (Radice and Caso, 1990; Hsia and Korban, 1998), besides it has a medicinal interest. Despite its ecological and economic interests, this plant is a rare endemic flora of the western basin of the Mediterranean (Escudero, 2007), as a result of overgrazing. The size of the population is restricted or is greatly diminished; the data indicate that the situation

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Abbreviations: BA, 6-Benzyladenine (cytokinine); **IAA,** indole-3-acetic acid (auxin); **IBA,** indole-3-butyric acid (auxin); **kin,** kinetin (cytokinine); **MS,** Murashige and Skoog medium; **NAA,** 2-naphthalene acetic acid (auxin); **2iP,** 2 isopentenyladenine (cytokinine).

will worsen irreversibly if nothing is done to address this vulnerability. In other words, if the observed situation continues, it is anticipated the disappearance of the specie. The factors responsible include degradation of habitat, exploitation of the species, exposure to pollutants, parasitism, diseases, interspecific competition, climate change, overexploitation; other cause result in the regression in number, but the population level reaches a critical threshold. They have a very important role in the fight against desertification and stabilization of vulnerable areas (Diez et al., 2002). In addition, they are involved in the production of desert truffles (Al-Rahmah, 2001).

Desert truffles, known locally as the "terfess" are edible and wild seasonal mushrooms hypogeal (Mandeed et al., 2007). *H. kahiricum* is considered threatened and is in an extremely precarious situation. The size of their population or their range, or both, is restricted or is greatly diminished. Thus, to maintain the genetic integrity of clones and conservation of this species (Lankova et al., 2001), *in vitro* germination, cultivation microcuttings and stimulation of axillary buds are the most applied in plant micropropagation method (Walali, 1993). For our species, *in vitro* culture seems to be a very interesting alternative for preserving *H. kahiricum* against the scourge of extinction. This work has as purpose the multiplication of the species by micropropagation highlighting the effect of the composition of the medium on the multiplication of plant material.

MATERIALS AND METHODS

Plant material and surface sterilization

Young shoots, 8 to 10 cm length, were collected from 4 to 5-years-old trees of *H. kahiricum* growing in the garden of Arid Lands Institute of Medenine (IRA) (Medenine: latitude 32° 7' 09" N, longitude 11° 38' 26" E, with arid climate characterized by a mean rainfall of 150 mm/year). The types of explants that were used were the nodal and apical explants, defoliated and surface-sterilized according to Romano et al. (1992). Buds were grown under controlled conditions (glasshouse), and developed shoots, 1 to 2 cm in length, were collected for *in vitro* culture according to Romano et al. (1992). These explants were treated with 70% ethanol for 3 min and 15% Clorox (containing 5% sodium hypochlorite) with 0.2% detergent for at least 10 to 20 min for surface sterilization, and then rinsed six times with sterilized distilled water. Explants were individually placed in test tubes (25 to 160 mm) containing 10 ml of MS (Murashige and Skoog, 1962), supplemented with 0.5 mg L^{-1} 6-benzyladenine (BA). Sucrose (2%, w/v) was used as a carbon source and media were solidified with 0.8% (w/v) agar. Media pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. Cultures were incubated at 25 ± 2°C under a 16 h photoperiod at 60 µmol m^{-2} s^{-1} provided by cool-white fluorescent lights for 10 weeks (Iriondi, 1995).

Shoot multiplication

Explants were individually placed in test tubes containing 10 ml of M S (Murashige and Skoog, 1962), supplemented with hormones. During the multiplication stage, many culture media were used: MS with cytokinin, MS with auxin and MS with combination (auxin and cytokinin). Several cytokinins concentrations [zeatin (0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg L^{-1}), 2iP–2-isopentenyladenine (0.5, 1.0 mg L^{-1}), Kin–kinetin and BA (0.5, 1.0 mg L^{-1}) (0.5, 1, 1.5, 2 and 3 mg L^{-1}), and auxins: IAA ratios (0.5, 1, 1.5 mg L^{-1})] were assayed. Twenty (20) cultures per treatment were tested. After 10 weeks, shoot multiplication rate defined as the total number of shoots produced from each initial explants and the length of the longest shoot per culture were evaluated.

Root induction

Shoots were rooted using *in vitro* and *ex vitro* methods, and a combination of both. Individual shoots, 4.0 cm in length, harvested at the end of the multiplication stage after being cultured for 2 weeks on ½ MS growth-regulator-free medium, were used in all rooting assays.

In vitro rooting

For *in vitro* rooting studies, two methods were tested: inclusion of auxins in the rooting medium or dipping the base of the shoots in a concentrated auxin solution. In the first one, shoots were cultured on full or half-strength MS (½ MS), containing IBA or NAA at 1.0 or 2.0 mg L^{-1}. In the second, the basal ends of the shoots were dipped in 1.0 g L^{-1} IBA or NAA for 2 min, followed by culture on MS or ½ MS auxin-free medium. For both methods, shoots were grown in test tubes (450 to 200 mm) containing 20 ml of medium. After 8 weeks of induction rooting frequency, root number and the longest root length per plantlet were evaluated.

Ex vitro rooting

For *ex vitro* rooting, the basal ends of the shoots were dipped in 1.0 g L^{-1} of IBA or NAA for 2 min, potted in peat and perlite (2:1, v/v) and immediately transferred to acclimatization conditions. In another experiment, *in vitro* rooting induction followed by *ex vitro* development of the roots was tried. The shoots were dipped in 1.0 g L^{-1} of IBA or NAA for 2 min, cultured on ½ MS auxin-free medium for 1 week in the dark and then transferred to acclimatization conditions. Rooting was evaluated 8 weeks after induction and was expressed in terms of rooting frequency and the substrate colonization. For all rooting assays (*in vitro* or *ex vitro*), 20 shoots were tested per treatment and the experiment was repeated three times.

Acclimatization

Plantlets with at least five well-developed roots were transferred to plastic pots (150 ml) containing a mixture of peat and perlite (2:1, v/v). Potted plantlets were placed in a growth chamber set at 100% relative humidity, 25 ± 2°C, with a 16 h photoperiod (100 µmol m^{-2} s^{-1}), for three months, then transferred to a glasshouse under natural daylight conditions at 25°C temperature. Plantlets were watered twice a week for 3 months with a diluted solution of ½ MS.

Statistical analysis

The results of analysis of variance (ANOVA) of different parameters were obtained by the software SPSS v.11.5. Multiple comparisons

Table 1. Effects of explants type and cytokinin/IAA balances (mg L^{-1}) on mean number and length of shoots.

Concentration (mg L^{-1})	Number of shoots/culture	Length shoot (cm)
Apical shoots		
0.5 zeatin + 0.5 IAA	3.0^{b}	16.9^{ab}
1.0 zeatin + 1.0 IAA	4.7^{a}	17.1^{ab}
0.5 2iP + 0.5 IAA	2.31^{c}	18.7^{a}
1.0 2iP + 1.0 IAA	2.1^{c}	18.6^{a}
0.5 Kin + 0.5 IAA	2.1^{c}	14.7^{c}
0.5 BA + 0.5 IAA	2.0^{c}	15.09^{bc}
1.0 BA + 1.0 IAA	2.0^{c}	15.6^{bc}
Nodal segments		
0.5 zeatin + 0.5 IAA	3.7^{b}	13.2^{a}
1.0 zeatin + 1.0 IAA	4.5^{a}	13.8^{a}
0.5 2iP + 0.5 IAA	2.7^{cd}	13.0^{a}
1.0 2iP + 1.0 IAA	3.1^{c}	13.5^{a}
0.5 Kin + 0.5 IAA	2.32^{d}	11.07^{b}
0.5 BA + 0.5 IAA	2.4^{d}	15.6^{a}
1.0 BA + 1.0 IAA	2.9^{cd}	7.2^{c}

Means with different letters are significantly different at threshold $p < 0.05$ (Duncan test).

of means and the setting command classes were made by Duncan's test.

RESULTS

The initiation of cultures from axillary and apical segments of *H. kahiricum* taken directly from field material was not possible due to high contaminations (~95%). It decreased after the pre-conditioning treatment of the cuttings. Shoot tip and axillary shoot development from single node explants was observed within 10 to 15 days of culture and all the explants showed growth viability.

Shoot multiplication

The mean number of shoots per explants was significantly affected by cytokines and the origin of explants (Table 1). Nodal shoots allowed a higher proli-feration rate (Table 1) and among the four cytokinins tested in combination with IAA, zeatin gave the best results in terms of mean number of shoots (Table 1). For both apical shoots and nodal segments, best results were observed with 2 mg L^{-1} zeatin with 1 mg l^{-1} IAA. Shoot length was significantly affected by cytokinins, the origin of explants, and their interaction (Table 1). When testing several zeatin concentrations and zeatin: IAA combinations for both explants (apical and nodal shoots), we observed that the mean number and length of shoots were significantly affected by the medium, explants origin and by their interaction (Table 2, Figure 1a and 1b); increasing zeatin concentration promotes shoot multiplication. However, this effect tends to decrease with higher zeatin concentration (2, 2.5 and 3 mg L^{-1}). Shoot growth was higher in nodal shoots than apical shoots and it was not stimulated by the presence of IAA (Table 2). Zeatin and 2iP have been successfully used during shoot proliferation of *H. kahiricum* species (Hsia et al., 1998).

Rooting

The effect of IBA and NAA (1 and 2 mg L^{-1}) added to the culture medium was tested in two macronutrient formulations; full MS and ½ MS. Best results in terms of rooting frequency, mean number of roots and root length were obtained in ½ MS (Table 3). For the two auxins (IBA and NAA) tested, the root length was significantly higher with IBA than NAA. With regard to auxin concentration, 1 mg L^{-1} induced significant root length (Table 3). Basal immersion of shoots in a 1g l^{-1} IBA or NAA solution for 2 min followed by *in vitro* culture on auxin-free medium (Table 3) increased the rate of rooting. Mean root number was significantly affected by the basal medium, auxin, and their interaction. IBA induced a higher number of roots and ½ MS was the most adequate medium. In all the *in vitro* rooting assays, roots developed at the base of the shoots but, most of them grew outside the medium,

Table 2. Effects of explants type and zeatin concentration (mg L^{-1}) combined with IAA on mean number and length of shoots.

Concentration (mg L^{-1})	Number of shoot/explant	Length shoot (mm)
Apical shoot		
0.25 zeatin	1.0^{e}	14.3^{d}
0.5 zeatin	1.51^{de}	15.91^{d}
1.0 zeatin	3.7^{a}	19.02^{a}
1.5 zeatin	2.2^{cd}	18.9^{a}
1.5 zeatin + 0.5 IAA	2.16^{d}	16.3^{b}
2.0 zeatin + 1.0 IAA	2.5^{bc}	15.8^{cd}
2.5 zeatin + 1.0 IAA	3.7^{a}	13.1^{bc}
3.0 zeatin + 1.5 IAA	3.1^{ab}	13.7^{cd}
Nodal segment		
0.25 zeatin	2.7^{d}	11.4^{c}
0.5 zeatin	3.4^{bc}	12.9^{ab}
1.0 zeatin	3.5^{bc}	13.7^{a}
1.5 zeatin	3.6^{c}	13.62^{ab}
1.5 zeatin + 0.5 IAA	2.7^{d}	12.9^{a}
2.0 zeatin + 1.0 IAA	4.18^{a}	11.7^{a}
2.5 zeatin + 1.0 IAA	3.27^{bc}	11.5^{a}
3.0 zeatin + 1.5 IAA	3.9^{b}	9.8^{b}

Means with different letters are significantly different at threshold $p < 0.05$ (Duncan test).

possibly due to poor aeration on the agarified medium. Many shoots rooted were observed either in direct *ex vitro* rooting or *in vitro* rooting induction followed by *ex vitro* development of the roots (Table 4). In both assays, IBA was found to be the auxin determining best substrate colonization. Shoots developed high number of thin roots that spread all over the substrate (Figure 1c). These results are in agreement with previous results with other plants *ex vitro* rooting of micropropagated shoots has been successfully used (Briggs et al., 1994; McCulloch and Briggs, 1994). Furthermore, in commercial practice, micropropagated shoots of plants are rooted *ex vitro* since it is effective.

Acclimatization

The *in vitro* regenerated plantlets were acclimatized in a growth chamber at high relative humidity (90 to 95%) for two months, and then they were transferred to the glasshouse. Results showed that after 6 months of acclimatization, the percentage survival was 60%. These results were improved when shoots were induced to root *ex vitro* with a surviving frequency of 100%. This increase could be due to a more developed and efficient rooting system. Micropropagated plants showed good growth and uniformity *ex vitro* and exhibited normal development. When reintroduced into their natural habitat during eight months, these plants showed 85% of survival. *Ex vitro* rooting was found beneficial both in terms of rooting results (100% rooted shoots) and acclimatization success (100% surviving plants) (Figure 1d and e). The results show the utility of combining *ex vitro* rooting and acclimatization in one step in order to reduce costs of multiplying plants via micropropagation. This micropropagation protocol could be used to clone some of this threatened native plant, and therefore it may represent an important tool for the preservation of biodiversity.

DISCUSSION

Shoot length was significantly affected by cytokinins; the origin of explants and their interaction, present a higher proliferation rate among the four cytokinins tested (zeatin, 2iP–2-isopentenyladenin, Kin-kinetin and BA-6 benzyladenine). The work of Armstrong et al. (2001) on *Ceratopetalum gummiferum* showed that the number of shoots per explants increased with increasing the concentration of BAP, Kinetin, Zeatin and 2iP. However, the use of excessive concentrations cytokinin causes a decrease in the number of shoots per explants, the shoot

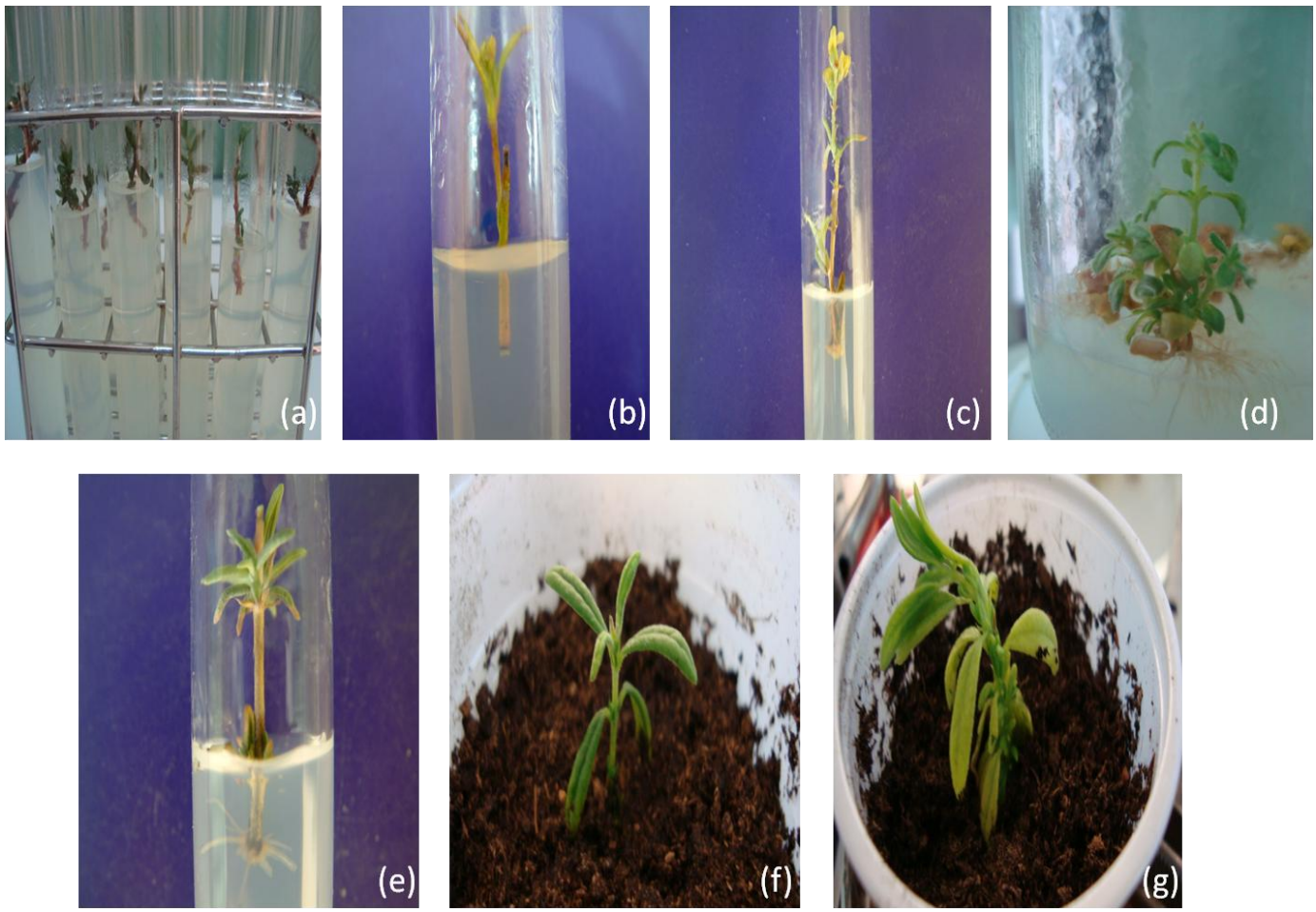

Figure 1. (a) Shoots at the beginning of multiplication phase in medium containing 1.0 mg L^{-1} zeatin. **(b)** Proliferation of axillary shoots from nodal segment on MS medium supplemented with 1.0 mg L^{-1} zeatin. **(c)** Proliferation of apical shoot on MS medium supplemented with 1.0 mg L^{-1} zeatin, (d) plant regenerated from nodal segment with 3 shoots. **(e)** Root regenerated from shoot cultured on MS medium containing 1.0 mg L^{-1} ANA, (f) *in vitro* regenerated planted of *Helianthemum kahiricum* transferred into plastic cup. **(g)** Acclimatized plant transplanted soil in the glasshouse conditions.

length and shoots weight. Shoot growth was higher in nodal segments that the apical shoots and it was not stimulated by the presence of IAA. The addition of auxin has a negative effect on the induction of shoots. Figueiredo et al. (2001) have shown that increasing the concentration of NAA decreases the proliferation of axillary shoots *Rollina mucosa*. The effect of auxin-cytokinin combination was also studied by Fracaro et al. (2001) for *Cunila galioides*. These studies show that the addition of different auxins: NAA, IAA or IBA to a multiplication medium (MS + BAP) significantly reduced the number of shoots per explants. However, Souayah et al. (2003) showed that in *A. halimus*, adding NAA enhances the rate of multiplication but with increased callus. The addition of BAP in *H. lippii*, improves the rate of buds, but the increase in the concentration of BAP induced a decrease in growth of shoots. Best results in terms of rooting frequency, mean number of roots and root length were obtained in ½ MS (Table 3).

Morte and Honrubia (1992) showed that the roots of *Helianthemun almeriense* occur after dilution of the culture medium. Similarly, for *Atriplex halimus* (Souayah et al., 2003), for which rooting is obtained on media without growth regulators indeed increased with diluting the mineral medium.

Conclusion

The shoot growth of *H. kahiricum* was higher in nodal shoots than apical shoots and it was not stimulated by the presence of IAA, shoot length was significantly

Table 3. Effect of media (MS and ½ MS), auxins (NAA and IBA) added to the medium and concentration (1 and 2 mg L^{-1}) on rooting frequency, mean number and length of root.

Medium	Auxin	Concentration (mg L^{-1})	Rooting (%)	Number of roots/explants	Length root (mm)
***In vitro* rooting**					
MS	NAA	1	61	5.4[a]	10.7[b]
		2	64	3.1[b]	8.0[b]
	IBA	1	42	5.2[a]	17.4[a]
		2	23	4.9[a]	14.3[a]
½ MS	NAA	1	69	6.8[a]	17.0[cd]
		2	83	6.9[a]	14[d]
	IBA	1	80	6.4[a]	27.9[a]
		2	82	5.8[a]	19.4[b]
***Ex vitro* rooting[a]**					
MS	NAA	1	94	7.8[b]	6.9[c]
	IBA	1	91	7.6[b]	8.1[b]
½ MS	NAA	1	94	7.6[b]	7.2[c]
	IBA	1	98	11.7[a]	12.6[a]

Means with different letters are significantly different at threshold $p < 0.05$ (Duncan test). [a]The basal ends of the shoots were dipped in 1 g l^{-1} auxin for 2 min, followed by culturing on auxin-free medium.

Table 4. Influence of auxins (IBA and NAA, 1 g L^{-1}) applied by basal immersion of the micropropagated shoots (during 2 min) on rooting frequency and substrate colonization.

Treatment	Rooting (%)	Substrate colonization (%)
***Ex vitro*[a]**		
NAA	100	64[b]
IBA	100	76[a]
***In vitro/ex vitro*[b]**		
NAA	100	62[b]
IBA	100	73[a]

Means with different letters are significantly different at threshold $p < 0.05$ (Duncan test). [a] Basal ends of the shoots were dipped in 1 g L^{-1} auxin for 2 min, potted in peat and perlite (2:1, v/v) and immediately transferred to acclimatization conditions; [b] Shoots after the basal immersion described earlier were cultured on ½ MS auxin-free for 1 week in the dark and then transferred to acclimatization conditions.

affected by cytokinins. The best results in terms of rooting frequency mean number of roots and root length were obtained in ½ MS. For the two auxins (IBA and NAA) tested, the longest root length was significantly higher with IBA than NAA. These results were improved when shoots were induced to root *ex vitro.* Results showed that after 6 months of acclimatization, the percentage survival was 60%. Micropropagated plants showed good growth and uniformity and exhibited normal development. When reintroduced into their natural habitat during 8 months, these plants showed 85% of survival.

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Influence of spectral properties on cassava leaf development and metabolism

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Cassava's importance as a food security crop in Sub Saharan Africa will be enhanced by its special traits such as tolerance to drought and high yields under hydrothermal stress. Some of the special traits which include the light reflective and absorptive properties of the leaves that depend on the surface characteristics of the leaves, are variety dependent and may influence the plants' reaction to light; hence, its photosynthetic capacity. We investigated the differences in the leaf spectral properties in different cassava varieties and related them to leaf biochemical properties using 20 cassava varieties established in a randomized complete block design in Kasese, western Uganda. Time dependent changes in leaf spectral characteristics were studied using Digimizer software and related to changes in sugar and pigment properties. Changes in the amount of reflected light were observed for the three main wavelengths used by plants (blue, green and red) with the blue being the most preferred. Total soluble free sugars exhibited a diurnal pattern from lower values (0.07 mg/g) after the dark period to higher values (0.313 mg/g) as the day progressed and was different from those of translocatable sugars such as sucrose. Chlorophyll a exhibited a curved pattern in all varieties increasing with increase in light intensity from 09:00 h (0.18 ug/g), peaking at 15:00 h (0.22 ug/g) and dropping down in concentration by 18:00 h (0.16 ug/g). Significant differences were observed in cassava varieties for the concentration of chlorophylls and carotenes. The results were obtained at a time of optimal growth conditions (four months after planting) and were used to classify these varieties into three broad groups showing that studies on spectral properties of leaves can still give a lot of insights in selection for stress tolerance under less optimal stress. The significant changes observed in the phenotype especially the foliar portion of the plant with the stay green and early recovering mechanisms of tolerance identified also tarried well with observed spectral differences. The results show that studies on plant spectral properties can be important in making inferences on the plants physiological and growth status.

Key words: Spectral properties, tolerance mechanisms, physiology, reflectance.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is the third most important source of calories in the tropics (FAO, 2010) and depicts various tolerance mechanisms to abiotic stresses. For cassava, a number of studies have been carried out to understand plant response to abiotic stresses such as moisture stress (Turyagyenda et al., 2013; Alves and

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Setter, 2004), soil salinity and related stresses (Okgbenin et al., 2010; Fregene and Setter, 2007), but still, the development of drought tolerant varieties remains a challenge. One way of solving challenges related to moisture and salinity stress susceptibility is the selec-tion of varieties that exhibit tolerance characteristics. Most of these characteristics are phenotypic in nature (Turyagyenda et al., 2013) and are related to leaf morphology and leaf response to light (Okgbenin et al., 2010). Light mediates a number of responses including leaf growth and development which are important factors in the deployment of stress tolerance mechanisms and manifestations such as the stay green trait. Therefore, the type and amount of light used during plant growth and development determines the way plants respond to stresses like drought. In a study carried out by Samuoliene et al. (2010), it was reported that the presence of red and blue light was important in plant morphogenesis whereby absorbed light intensity influence morphogenesis through support of processes such as photosynthesis and plant development which ultimately affect total plant yield. In fact light quality, quantity and photoperiod have been found to affect whole plant growth and developmental transitions (Folta et al., 2005).

Basic plant research has shown that specific light wavelengths such as the blue light are important in plant processes such as germination and stem growth, biomass accumulation (Kim et al., 2004) and transition to flowering (Valverde et al., 2004). Red light is important in stem/shoot elongation, phytochrome responses and changes in plant anatomy (Schuerger et al., 1997). The red light also contributes to plant photosynthesis by allowing the opening of stomata at appropriate times for the plant to take up enough carbondioxide and hence metabolic influence of the red light cannot be ignored (Okamoto et al., 1996). On the other hand, blue light is important in chlorophyll biosynthesis and chloroplast maturation, stomatal opening, enzyme synthesis and the whole process of photosynthesis (Samuoliene et al., 2010). The importance of blue light in sustainable morphological development cannot, therefore be ignored. The contributions of the two spectral wavelengths during photosynthesis have been found to be efficient in advancing the developmental characteristics associated with autotrophic growth habits. Photosynthetically, inefficient light wave lengths also impart important environmental effects to a developing plant. In particular, the green light affects plant respiration and growth via cryptochrome-dependent and cryptochrome-independent means hence affecting plant development by either slowing down or increasing the rate at which respiration and plant growth occurs (Greiger, 1994). This is through the balance between used radiation where the green light opposes the effects of blue and red wave bands (Folta and Mahrunich, 2007). Therefore, because plants have become adapted to gradually changing daily irradiance, it is advantageous to study regulation of photosynthesis and carbon metabolism in the context of the diurnal light cycle and how this affects the way plants respond to stress in relation to its effects on photosynthesis and hence plant yield (Geiger, 1994).

Plants are exposed to the same amount of light, but the amount of reflected light intensity off the plant leaf surface may depend on a number of factors including the leaf surface properties (Barnes and Cardosa-Vilhena, 1996), its orientation and its physiological state (Blatzer and Thomas, 2005). However, it is a fact that the more the reflected intensity observed, the less efficient the plant is at conversion of received light into useful photosynthetically active radiation (PAR) that drives photosynthetic process (Geiger, 1994). The under-standing of this pattern would thus be important in easing the selection process based on the use of PARby plants. It would also allow us to understand the basis of tolerance mechanisms which are mainly based on changes in plant morphology, biomass acquisition and relatedly low productivity. Although, lot of work has been done in the area of plant spectral response (Folta and Mahrich, 2007); much has not been done for cassava and the results from such studies have not been exploited fully in this important crop.

In the present study, results from reflective spectra of the three light wavelengths of red, blue and green were studied and compared to observed biochemical and phenotypic differences in cassava varieties and the comparisons were used to select varieties showing varied responses to plant abiotic stresses.

MATERIALS AND METHODS

Field design and sampling plan

Twenty (20) varieties of cassava were selected based on known parameters of dry matter content, resistance to Cassava Mosaic Disease (CMD) and farmer preference. They were established in a randomized complete block design (RCBD) in Mubuku Irrigation Scheme, Kasese Western Uganda. The trial consisted of two experimental and two control blocks in plot sizes of 9 × 9 m and up to 81 plants per plot. The different cassava varieties were observed on a daily basis for two weeks at an interval of one week in between and changes in main metabolites and plant spectral response were recorded. Results were taken at 4 months after planting (MAP) representing a period of optimal growth in cassava (Alves and Setter, 2004). Each day, tagged leaf surfaces (5th leaf on individual plants) of three plants per plot were photographed at 09:00, 12:00, 15:00 and 18:00 h at a distance of 30 cm away from the leaf surface. At the same time, leaf samples were picked on the same plant at every sampling period. To maintain the metabolic state of the leaf samples, leaves were preserved in liquid nitrogen by freeze drying. The samples were used to study the relationship between metabolites, physiological state of the plant and time. The metabolites considered were sugars, chlorophylls and carotenoids within particular leaf samples.

Cassava leaf spectral properties

Using online computer software –Digmizer (digmizer.com), leaf surface photos taken at different times were analyzed for emitted

Table 1. Average weather conditions at the time different photographs were taken for analysis.

Time (h)	Temperature	Humidity	Dew point	Heat index
09:00	20.53[a]	78.57[a]	16.65[a]	21.15[a]
12:00	25.08[b]	65.14[a]	17.92[a]	25.83[b]
15:00	29.5[c]	44.86[b]	16.0[a]	29.95[c]
18:00	28.26[c]	53.22[ab]	17.37[a]	29.06[c]

Values followed by the same letter in a column are not significantly different at P = 0.05.

intensity at the different time periods. The leaf surfaces which differed in area and size were harmonized by considering an approximate leaf surface of 25 cm^2 on the surface directly exposed to sunlight at right angles. In all cases, the leaf midrib, big leaf veins near the bottom of the leaf and near apex surfaces were avoided. The selected portion was then analyzed for specific emitted intensities for the blue, red and green light intensities using a computer. The average emitted intensity and the hue were calculated using the software. The generated data was then converted into excel sheets and the data obtained averaged for each time period for the 10 day intervals and used to make inferences on plant response to light at different times. From analysis of spectral properties, average intensities were obtained for the different reflected wavelengths of blue, red and green. These were given in unit ratios of reflected light. The data collected was subjected to analysis of variance using the Genstat Software (Genstat, discovery edition, 2011).

Mean values for the different parameters at different times recorded for the 10 day period were used to study observed variations in leaf reflectance.

Chlorophyll and sugar extraction

After leaf optical properties were measured, chlorophyll and sugar concentrations of the same leaves were determined. Six circular disks, each 5 mm in diameter, were punched from the leaf portion for which optical properties were measured. The disks were placed immediately into 8 ml of 100% ethanol, and pigments were allowed to extract in the dark at 30°C for 48 h. During this period, free sugars in the leaf were extracted. Pigments were determined directly from the extracted solution with the organic solvent. Reducing sugars were extracted too after the solution was exposed to light at 50°C for up to 24 h until when the solution was completely decolorized.

Quantification of leaf sugar and pigment contents

Leaf samples collected were analysed for their individual pigment concentration at different times of the day for the 10 day sampling periods. Pigments of interest included chlorophylls a and b (Chla and Chlb) and carotenoids. The total amount of reducing sugars included both free and bound sugars excluding high molecular weight free carbohydrate components. Quantitative measurements for chla, chlb and carotenoids were carried out using a spectrophotometer by taking absorbencies at 662, 644 and 445 nm, respectively according to Wettshstein (1957). Free reducing sugars were taken to be the water extractable sugars at 30°C while bound reducing sugars were taken to be the hot ethanol extractable sugars at 70°C for 5 min. The quantities of these sugars were determined using phenol sulfuric method by Dubois et al. (1956).

Data analysis

For the analysis of spectral properties, average intensities were obtained for the different reflected wavelengths of blue, red and green. These were given in unit ratios of reflected light. The data collected was converted into an excel file and the resultant data obtained was subjected to analysis of variance using the Genstat Software (Genstat, discovery edition, 2011). Mean values for the different parameters at different times recorded for the 10 day period were used to study observed variations in leaf reflectance. Carbohydrate and pigment related data was obtained from the different assays for samples obtained at different days and for the different times. Mean values for each variety were recorded and relationships between different parameters were studied. Microsoft excel software was used to study the trends in the different properties of the plant studied.

RESULTS AND DISCUSSION

Prevailing weather conditions

Results showing the weather conditions during the experimental time are shown in Table 1. These are conditions that have direct effects on leaf properties and its ability to intercept incoming radiation and include the relative humidity, the dew point and the wind speeds. During the experimental time, the relative humidity was considerably high and varied widely during the day (80 to 88%). Day and night temperatures ranged from 19.6°C at 09:00 h to about 31.1°C at 15:00 h. This represents the normal temperature range for the growth of cassava which is between 19 to 32°C (Alves et al., 2000); although, the variations in this case had a significant effect on the thermal and solar characteristics of the cassava leaves. The curve linear trend for daily temperature determined the type of photosynthetic metabolism carried out by the cassava leaves in relation to total metabolism (Yin and Kropff, 1996). Average daily humidity decreased with the time of the day and ranged from 87% at 09:00 h and 45% later in the day at 15:00 h representing a wide range of air water saturation which affects leaf temperature, stomatal opening and carbondioxide intake as suggested by Katahata et al. (2007). Low average humidity (39 to 48%) at 15:00 h was due to increased incoming light and low rates of air water saturation as a result of high wind speeds during the day sweeping off any existing water near plant surfaces.

Table 2. Variations in leaf reflectance for the different light intensities at different times of the day for the different cassava varieties. DU = digmizer units).

Intensity	09:00 (h)	12:00 (h)	15:00 (h)	%CV
Red (DU)	0.494[a]	0.471[a]	0.475[a]	8.80
%Reflected	30.95	30.04	30.00	
Green (DU)	0.681[b]	0.671[b]	0.676[b]	6.47
%Reflected	42.67	42.79	42.70	
Blue (DU)	0.421[c]	0.426[a]	0.432[c]	12.54
%Reflected	26.37	27.17	27.29	
Hue	105.14±7.08	111.6±9.87	111.31±7.02	7.53

Values for DU followed by the same letter in a column are not significantly different at P<0.05. Values for Hue are followed by the standard deviation across the row.

The dew point was high earlier (16.7%) at 09:00 h and later during the day (21.4%) at 18:00 h and reduced with increase in in-solation as the day progressed being at its lowest at 12:00 h (Table 1).

Phenotypic basis of grouping varieties

Based on observed phenotypic characteristics such as leaf retention, biomass accumulation and general plant growth characteristics, varieties were ranked into three broad groups according to their response to moisture and heat stress. The groupings included varieties that maintained a moderately high leaf area index (LAI) during periods of high temperature and low soil moisture or stay green varieties (SGV) such as NASE 2, NASE 4, I/92/0067 and MH/96/0068. The other grouping included those that lost all their leaves immediately after onset of stress only to recover immediately with increase in relative humidity and they were named as early recovering varieties (ERV) and some varieties had both mechanisms but were not very pronounced in each case. A few varieties completely lost their leaves as stress progressed and the ones which were retained were dechloropyllated and rendered incapable to photosynthesize. The same varieties showed little or no capacity to recover easily after stress and they were named susceptible varieties (SV).

Variations light intensity

Since the amount of absorbed light is directly proportional to the ability of the plant to utilize photosynthetically active radiation, the less the reflected light intensity, the more photosynthetically efficient the plant or the particular plant variety. Thus, studies on the variations in the reflected light intensity were carried out to allow for selection of photosynthetically efficient and high biomass accumulating plants which as a result would have a higher degree of tolerance to hydrothermal stress. The results for this total reflected intensity are shown in Table 2. In all cases, the percentage reflected light was high for the green wavelength (~43%) compared to the red (31%) and blue (26%) wavelengths. High reflected green wavelengths were expected due to the high chlorophyll concentration in the leaves of the cassava plant. It represented almost twice of the total reflected intensity at all times though it changed considerably with the time of the day. Daily changes in reflected light for the blue, red and green wavelengths were observed where for the green light reflected intensity was low at beginning and end of the day while it was high between 12:00 and 15:00 h. This can be attributed to the amount of light intensity received by the plant at different times of the day (Folta et al., 2005). Like the green intensity, slight increments were observed for the blue wavelength reflected intensity as the day progressed while slight reductions were observed for the red wavelength. The plants had selective usage of three different main wavelength intensities at different times of the day, although, the blue wavelength intensity was the most preferred.

The selective usage of particular wavelengths by plants has been observed among other plants (Bjorn, 2008). This is important in allowing the plants to capture and convert all energy to chemical energy hence reducing the risk of photo-oxidation (Chalker-Scott and Cahill, 2001). However, selective use of either wavelengths at any time may be due to changes in plant morphology where if plants develop thicker leaves with cuticle meant to protect the plant against irrelevant irradiation may limit the use of particular light wavelengths and in instances were plants accumulate certain pigments that are also important in protection of membranes against oxidative damage may result into increased reflected intensity corresponding to a particular colored pigment (Spalding and Folta, 2005). Such scenarios happen during stress and may lead to changes in plant leaf spectral patterns. It also shows that

Table 3. Variations in leaf reflectance among variety groups and total average reflectance for the different main spectral groups.

Variety group	Intensity	09:00 (h)	12:00 (h)	15:00 (h)	Average
Stay green	Red (%)	30.99[a]	29.84[a]	29.57[a]	30.13
	Green (%)	43.89[c]	42.04[b]	42.83[b]	42.92
	Blue (%)	25.31[b]	28.12[a]	27.59[a]	27.01
	Average intensity	0.523	0.538	0.537	0.533
Early recovery	Red (%)	31.81[a]	30.19[a]	30.69[a]	30.90
	Green (%)	42.81[c]	44.54[c]	43.05[c]	43.47
	Blue (%)	25.36[b]	25.27[b]	26.26[b]	25.63
	Average intensity	0.531	0.501	0.520	0.517
Susceptible	Red (%)	31.07[a]	30.30[a]	29.38[a]	30.25
	Green (%)	42.43[c]	42.63[c]	42.73[c]	42.59
	Blue (%)	26.51[b]	27.08[a]	27.89[a]	27.16
	Average intensity	0.548	0.495	0.559	0.534

Values followed by the same letter in a column within a variety group are not significantly different at P = 0.05. Note the high green and low blue reflectance for early recovering varieties compared to the stay green varieties.

the highly absorbed blue intensity mediated most of the physiological related reactions during the day for cassava plants. Cassava leaves selectively absorb the blue intensity to mediate phytochrome dependent processes such as photo-dormancy and for chemical energy while avoiding photo-oxidation which is mainly as a result of higher light intensities such as near ultraviolet wavelengths (Chalker-Scott and Cahill, 2001).

Importantly, it was observed that diurnal changes in irradiance follow a particular pattern beginning slowly during the day and generally matching the time constants of processes related to photosynthesis later in the day (Table 3). These results into differences in the rate of photosynthesis in the time course of the day which is mediated by the ability of the plantto absorb light of a particular wave length (Weller and Kendrick, 2008). Thus, the selective use of blue intensity in cassava is also important since the blue intensity has been found to support photosynthesis and increase the total plant biomass in form of the products. In addition, the red intensity is mainly important in flowering and physiological activities related to seed formation and seed development. The combination of the two lights is important for plants to achieve specific leaf area good enough for the plants physiological activities (Yorio et al., 2001). Plants use these different intensities selectively providing for the different physiological requirements. The use of particular wavelengths allows the plant to efficiently use light balancing different processes carried out by the plant (Spalding and Folta, 2005). Thus, cassava plants at mid growth cycle tend to selectively use the blue light to increase their vegetative growth and biomass accumulation. The selective use of different intensities at different times of the day also has an impact on plant photosynthesis and morphogenesis (Chalker-Scott, 2010). In particular, chlorophyll synthesis is mediated by light perception and it was found out that in cassava, chlorophyll concentration patterns increased with increase in the amount of absorbed photo-synthetically active radiation in the blue and red range of the spectrum (Table 4). The changes in pigment concen-trations result into the observed shift in biochemical and physiological alteration within the plants metabolic profiles. For example high chlorophyll concen-trations result into high photosynthetic rates and hence high sugar production (Rolland et al., 2006).

The sugars and metabolites are used in biomass accumulation in the plant which directly influences the development of stress tolerance traits such as the stay green trait (Xu et al., 2000). In this respect, plants that can withstand high temperature and low soil moisture stress are those that have accumulated reserves of metabolites before stress. These metabolites (especially the free sugars) arise from photosynthesis and hence are directly proportional to the amount of active radiation used by the plant. The use of most of the photosynthetically active radiation in stay green and early recovering varieties thus makes them better suited for biomass accumulation in preparation for adverse conditions later in the growing cycle. The differences point to the fact that in selection from a number of populations, the amount and type of radiation used is important as a selection tool for stress tolerant varieties. According to Geiger (1994), the light available to leaves for photosynthesis depends both on the time course of diurnal irradiance and on factors such as leaf orientation, that affect the interception of the incident light.

The evolution of photosynthesizing organisms under cyclic diurnal irradiance has equipped plants to regulate the photosynthetic process in ways that allow carbon to

be assimilated efficiently over the wide range of diurnal cycle irradiance.

Variations in reflected intensity in variety groups

To understand the differences between different varieties or groups of varieties, the amount of light reflected off the leaf surfaces of these varieties was determined and recorded as the reflected intensity (Table 3). Over all, the average intensity varied within the different variety groups and at different times of the day. A lot of variation ($P<0.05$) was observed for the early recovering (0.501 to 0.531) and susceptible varieties (0.495 to 0.559) compared to the stay green varieties whose average intensity ranged from (0.523 to 0.538). In both the early recovering and susceptible varieties, average reflectance was low at 12:00 h unlike the stay green varieties where average reflectance was low at 09:00 h. High average reflectance for early recovering varieties was observed at 09:00 h while for susceptible varieties it was at 15:00 h. The reflectance also varied with the time of the day for the different variety groups. At 09:00 h, the amount of reflected light was high for susceptible varieties and low for stay green varieties while at 12:00 h, total reflected light was high for stay green varieties and low for susceptible varieties. At 15:00 h, overall average intensity was high for susceptible varieties and low for early recovering varieties (Table 3). This meant that stay green cassava varieties absorbed most of the in-coming radiation early in the day while susceptible varieties absorbed most of the radiation later in the day. Early recovering varieties absorbed most of the radiation throughout the day.

Total changes in reflected light during the day were thus low for the early recovering varieties compared to both stay green and susceptible varieties implying that at optimal conditions, the early recovering varieties had high optimal absorption of photosynthetically active radiation compared to other varieties. This means that these varieties had high optimal photosynthetic rates and hence converted more of the absorbed carbon dioxide into sugars. The results thus show that the use of different wavelengths of light by different varieties may be due to differences in pigment concentration in photosystem I and photosystem II (Hogewoning et al., 2012) which results into wavelength dependent excitation of the photosynthetic apparatus hence requirement for different wavelengths at different times of the day. This timing (the use of certain PAR at particular times of the day) is important because the effectiveness of light in the regulation of plant processes varies with the phase of the diurnal cycle as determined by the plant's time-measuring mechanisms (Weller and Kendrick, 2008). This explains the differences observed in different varieties in relation to selective use of certain light intensities at different times of the day. In addition, the differences observed may reflect the differences in the photosystem used by cassava for main photosynthetic processes where each photosystem has different components and hence differences in light energy use capabilities (Samuoliene et al., 2010). In addition, not only does this light drive photosynthesis but it also affects plant morphology, physiology, leaf display and chloroplast orientation (Spalding and Folta, 2005; Weller and Kendrick, 2008). Thus, the variation observed in different cassava varieties point to different morphological and physiological responses of cassava towards available light. The percentage reflected light also varied among variety groups but followed the same pattern with the green light wavelengths being the most reflected as expected and the blue light being the least. After 09:00 h and as the day progressed, green light was reflected highly for early recovering varieties compared to stay green varieties and susceptible varieties while the blue and red light was reflected the least in these varieties. This explains the differences in the use of light with high spectral quality among different varieties.

It also shows the adjustments carried out by the cassava plants' light capturing apparatus in order to capture light of the best spectral quality. It also highlights the ability of the light harvesting complexes in both PSI and PSII to harness light of particular wavelengths as the pigment concentration in each of these photosystems changes with time (Hegonowing et al., 2012). The pigment concentration change is accompanied by changes in the ratio of chlorophyll a and chlorophyll b; hence, a shift towards absorption of particular light wavelengths. According to Geiger (1994), spectral quality, the wavelength composition of light, is important because photons differ in their probability of being absorbed by the light harvesting complex and hence their ability to drive carbon assimilation. The various light receptors for light-mediated regulation of plant form and physiology have characteristic absorption spectra and hence photons differ in their effectiveness for eliciting these responses (Spalding and Folta, 2005). The high amount of reflected green intensity for all varieties but especially the early recovering varieties points to the ability of plants to avoid incoming green radiation which from the action spectra of most crop plants is not highly used for photosynthetic processes (Folta and Mahrunich, 2007; Nishio, 2000). There seemed to be a balance in the type and amount of PAR used in each of the varieties with the ratios of the different light intensities being the same all through. This balance is necessary since imbalance in the amount and type of light available for plant metabolism results into imbalances in plant development (Weller and Kendrick, 2008).

In relation to this, poor plant development results into poor yields and susceptibility to both abiotic and biotic stresses (Chalker-Scott, 2010). Thus, the way varieties use available irradiance or the percentage of light absorbed determines the way the plant is adapted to

Table 4. Diurnal changes in reducing sugar (RS) contents for the different varieties.

Variety group/RS (ug)	09:00 (h)	12:00 (h)	15:00 (h)	18:00 (h)	Daily average
Susceptible varieties	0.222[a]	0.060[a]	0.149[a]	0.330[a]	0.190
Stay green varieties	0.359[b]	0.072[b]	0.143[b]	0.316[ab]	0.223
Early recovering varieties	0.291[a]	0.078[c]	0.152[a]	0.293[b]	0.204
P -value	0.005	0.008	0.038	0.042	

Values followed by the same letter within a column are not significantly different at P<0.05. Note that the quantified include total free sugars (monosaccharides).

different environments. From the results, the differences observed point to the fact that different cassava varieties vary genetically in their light harvesting complexes and related pigments and hence perform differently in terms of morphogenetic and photosynthetic processes. The efficiency in utilization of incoming photons results into increased output of photosynthetic products due to increased carbon dioxide fixation which in turn impacts on biomass accumulation in these plants. It was also clear that the use of different forms of light depends on the accumulation of different pigments within the light absorption apparatus of the plant at different times of the day. This also explains the time dependent differences in the way different plants respond to light. High reflected light intensities at different times of the day point to less absorbed light; hence, low rates of photosynthesis and ultimately yield (Tables 3 and 4). Properties of light that affect growth and morphology of the plant, in turn, can affect photosynthesis.

Photosynthesis generates a positive feedback system in which photo-assimilation of carbon contributes to the growth of leaves that absorb photons and assimilate carbon to contribute to further growth. The compounding aspect of the production and growth of leaves obviously is affected by plant properties such as leaf area and thickness, which are regulated by light.

Relationship between light intensity and production of reducing sugars

The results for changes in sugar contents with the time of the day are shown in Table 4. Reducing sugars showed a diurnal rhythm with high amounts observed during the early and late hours of the day (Table 4). By 12:00 h, the reducing sugar content was four times lower than the amount at 09.00 h or later in the day at 18:00 h. However, an increment was observed from 12:00 to 18:00 h and beyond with significant differences observed during the light and the dark period. This was the time when the amount of PAR used in form of the blue and the red intensities increased showing the importance of active PAR in reducing sugar production. Variety differences were observed at 09:00 h with the stay green varieties having higher values compared to other varieties while the susceptible varieties had lower values. The reducing sugar values were not significantly ($p < 0.05$) different at 12:00 and 15:00 h for all varieties although differences were observed at 18:00 h, the stage at which sugar accumulation was also observed. The pattern observed was so different from the pattern observed for translocatable sugars such as sucrose (Rapacz and Hura, 2002; Usuda et al., 1987). A similar pattern for sugar changes has been reported in tree species and explains the importance of sugars in the plants metabolism especially their conversion into storable carbohydrate forms (Dickson, 1987).

In this study, it was observed that export of sugars from the source leaf depends on the leaf carbohydrate metabolism at particular times of the day (Table 4). Their increase during the day shows that plants prepare for production of sucrose during the day by increasing the daily concentration of reducing sugars as photosynthesis progresses reaching the peak as the day closes. It also suggests that the process of converting manufactured glucose and fructose into sucrose and starch does not occur concurrently with simple sugar production during photosynthesis. This is the suggested model for carbohydrate metabolism in C3 to C4 plants such as cassava and other C4 plants such as maize (Usuda et al., 1987). Particularly, it is known that increments in primary products of photosynthesis result into increments in sucrose accumulation which follows a net photosynthetic rate that follows increments with irradiance as the day progresses. The sugars especially glucose 6-phosphate and fructose 6-phosphate which are monomers for sucrose change in a different pattern compared to sucrose being low at the beginning of the day only to increase as the dark hours are approached (Usuda et al., 1987). Thus, the observed increase in reducing sugars at 18:00 h led to increase sucrose accumulation. Some of the reducing sugars are changed into storable carbohydrates while others are maintained to help in the providing energy for leaf metabolism at night and during the early hours of the day (Blasing et al., 2005).

Carbon partitioning thus depends on the amount and type of organic compounds required for leaf metabolism; hence, a different pattern is observed compared to that of sucrose which is mainly destined to storage organs where it is converted into starch as was observed by Thum et al. (2004) in *Arabidopsis*. Carbon partitioning also depends on the leaf age where in highly metabolizing

Table 5. Daily changes in the concentrations of the different plant main pigments among the broad phenotypic classes of cassava after stress.

Variety	09:00 (h)			12:00 (h)			15:00 (h)			18:00 (h)		
	Chla	Chlb	Cart	Chla	Chlb	Cart	Chla	Chlb	Cart	Chla	Chlb	Cart
Susceptible	0.21[a]	0.12[a]	0.42[a]	0.20[a]	0.11[a]	0.42[a]	0.22[a]	0.11[a]	0.45[a]	0.15[a]	0.07[a]	0.35[a]
Stay green	0.18[b]	0.10[b]	0.35[b]	0.19[b]	0.10[b]	0.39[b]	0.14[b]	0.07[b]	0.30[b]	0.18[b]	0.09[b]	0.40[b]
Early recovering	0.17[b]	0.10[b]	0.34[b]	0.19[b]	0.09[c]	0.38[b]	0.26[c]	0.13[c]	0.55[c]	0.16[a]	0.08[c]	0.37[c]
P- value	0.0021	0.002	0.004	0.002	0.0005	0.007	0.004	0.0002	0.01	0.001	0.0003	0.002

Chla = Chlorophyll a; Chlb = chlorophyll b; Cart = carotenoids. Values followed by the same letter in a column are not significantly different at P<0.05.

and expanding leaves, most reducing sugars (fructose and glucose) are retained overnight in the leaf to allow for leaf development in terms of night respiration, leaf construction and construction of other support organs in the plant (Givnish, 1998). If the plant does not maintain the relevant sugar reserve, the costs for net carbon gain will be high since the plant cannot form relevant support structures (Geiger and Serveittes, 1994). The reducing sugar pattern followed the *chla* pattern hence showing the importance of *chla* in cassava plant photosynthesis and production of carbohydrates (Chalker-Scott, 2010). As the *chla* content increased, reducing sugar increased in turn showing that increases in *chla* increases photosynthetic rate.

Relationship between light intensity and pigment properties

Because of the role of pigments in leaf growth and metabolism, variations in pigment content may provide information concerning the physiological state of leaves. Chlorophyll and carotenoids concentrations tend to decline more rapidly when plants are under stress or during leaf senescence but under optimal conditions differences observed allude to plant behavior. Generally, levels of *chla* increased with time reaching the peak at 15:00 h after which they dropped significantly to twice as low the value by 18:00 h (Table 5). *Chlb* followed the same pattern although there were no significant increments between 09:00 and 12:00 h for this pigment. The patterns observed appear to be linked to increments in active irradiation as the day progresses. This is likely because it has been earlier demonstrated that increase in light intensity influences the production of the chlorophyll and carotenoids (Weller and Kendrick, 2008; Chalker-Scott and Cahill, 2001). Notable too were variety differences which were observed for the two pigments (P<0.05). For *chla*, values were higher for susceptible and early recovering varieties between 09:00 and 15:00 h compared to the stay green varieties. However, at 18:00 h, the value for *chla* did not show any significant differences (P<0.05) inbetween the susceptible and early recovering varieties; although, the early recovering varieties seemed to maintain a higher value at this time. Notable were the high values observed for *chla* and *chlb* for early recovering varieties later in the day (Table 4).

Thus, the concentrations of different chlorophylls in the leaf varied with the time of the day but were generally at the peak by 15:00 h. They followed the same pattern with *chla* being higher in concentration compared to *chlb* in all instances.The high concentrations of chloropyhlls coincided with the higher absorbed light intensities for the blue and red wavelengths. This shows the efficiency of these pigments in light capture and utilization and the role of light in morphogenesis; hence, an increase in chlorophyll concentration. Chlorophyll pigments also varied within varieties and were found to be higher for stay green and susceptible varieties during the 9 h cycle. Their concentrations also showed a varied pattern when the different varieties were compared. Changes in irradiance mediate the changes observed for pigment concentration especially chlorophylls.

It has been observed that increase in irradiance results into increase in pigment composition until the saturation point resulting into photo-inhibition (Gordillo et al., 2001). It was also observed by Weller and Kendrick (2008) that high irradiance resulted into reductions in *chla* concentrations. This explains the differences observed among different varieties of cassava in terms of pigment concentrations pointing to different saturation points for different variety groups and even individual varieties within these groups. Much as the differences observed especially for chlorophyll concentrations were not significant, the downstream effects of the differences were detectable in the reducing sugars patterns observed (Table 4).

Carter and Knapp (2001) observed differences in tree species for chlorophyll content which also resulted into differences in reflective intensity patterns in these different plants. Accordingly, the observed differences in chlorophyll concentrations serve to explain the differences observed in reflectance especially between the early recovering varieties and stay green varieties. This was also observed by Thomas and Blatzer (2005) in temperate trees grown under different intensities.

Carotenoids also showed a similar diurnal pattern as the chlorophylls. Much earlier during the day, susceptible and early recovering varieties showed higher concentrations while later in the day early recovering varieties showed higher concentrations. Changes in leaf pigment concentrations are dependent on a number of factors but mainly the physiological state of the plant (Carter and Knapp, 2001). Apart from changes in irradiance during the day as observed earlier, diurnal changes observed in different plants are also a result of changes in the photochemical efficiency of the leaves during the day depending on the amount of irradiance available. According to Farghali (1997), diurnal changes in leaf pigment content were observed in old leaves of *Senna* and coincided with increase in dry matter content during the day. This study showed that these changes are related with the accumulation of dry matter (in form of carbohydrates and carbohydrate derivatives) in plants which is a function of photosynthesis; hence, variations in the photosynthetic efficiency of different crops.According to Pollet et al. (2009), response of chlorophyll is light dependent and affects the quantum efficiency of Photo System II. The increases observed with increase in light intensity in this study allude to this. Such increments also allow increments in the concentration of sugars produced during the day (Table 4) which progresses with increase in photosynthetic efficiency.

Spectral property based selections for stress resilience in cassava

A measure of successful diurnal regulation of photo-synthesis is the ability of the plant to lessen the impact of environmental stresses such as high temperature stress and use what light is available efficiently. The result is a general correspondence between carbon flux through the assimilatory segment of the cycle and the course of diurnal irradiance. According to Torres and Huber (1987), the accumulation of sugars in leaves of maize showed a diurnal pattern where they accumulated progressively during the day reaching its peak by 15:00 h. In particular, this was affected by the amount of enzymes involved in sucrose metabolism. In our case, it was observed that, the amount of sugar in the leaves increased during the day as observed earlier. The high *chla/chlb* ratios are notable of plants highly adapted to sunny conditions (Givnish, 1988). These ratios determine the amount of absorbed light and also are elated to total reflectance.

Alterations in reflectance are mediated by the differences in chlorophyll content within the leaves (Carter and Knapp, 2001). Effect of stress on chlorophyll thus leads to significant changes in the reaction of the plant to the different levels of moisture and heat stress. The importance of light in plant growth and metabolism determines its role in influencing the ability of the plant to accumulate relevant biomass. Light based biomass accumulation is very relevant in plant growth but importantly in plant physiological response to the environment. Thus, the differences observed in the use of light in form of PAR are important in providing a biomass based selection tool for cassava varieties that are resilient to abiotic stress but importantly hydrothermal stress.

In particular, light based production of primary metabolites such as sugars and the differences observed among varieties for production of these metabolites is important in pointing out varieties that can tolerate stress. Such varieties have a strong source and in addition deposit a lot of stored metabolites in the sink resulting into biomass accumulation and an important trait described as the stay green trait. This trait allows cassava to tolerate various environmental stresses in field conditions but importantly hydrothermal stress. For varieties that have the ability to recover after stress, the root based biomass plays a critical role in providing metabolic resources for recovery and as such, these resources are mobilized and accumulated during growth under optimal conditions. Biomass accumulation is also as a result of photomorphogenic processes that occur during cassava growth and development. These processes are driven by particular wavelengths but mainly the red and blue based wavelengths which allow development of biomass based structure for driving physiological processes and for attaining a specific leaf area index that can support physiological processes. In addition, light is key to production of relevant pigments that drive physiological processes. Thus, in this study, the importance of cassava leaves in mediating the morphological and physiological processes of the plant was elucidated. Such processes are related to the means by which cassava plants manage stress and attain various levels of tolerance to abiotic stress. The responses of leaf/leaves to available light and how it affects the physiology of the plant have also been explained in cassava.

Deductions from the results show that much as spectral characteristics of the plant are mediated by the quality of light available to the plant, there are effects of quality of light on pigment concentrations although the main effects are observed in downstream processes such as sugar metabolism and carbohydrate production by the plant which leads to growth and development. Thus, the differences in the use of available quality light have an impact on the type of response by the plant to the environment but especially under abiotic stress. In addition, leaf related traits such as chlorophyll content and average absorption of light at different times of the day if well manipulated can be important in producing stress tolerant varieties.

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Full Length Research Paper

AtNEA1-identification and characterization of a novel plant nuclear envelope associated protein

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In animal and yeast cells, a cross nuclear envelope structure linker of nucleoskeleton and cytoskeleton (LINC) is formed by outer nuclear membrane SUN proteins and inner nuclear membrane KASH proteins. However, little information was acquired about plant SUN-KASH structure until they were found in plant SUN proteins in 2010 and KASH proteins in 2012. The SUN-KASH complex alongside with actin in the microfilament cytoskeleton and nucleaoskeleton together shape the main cell skeleton structure and involve in many important biological functions including cell structure stability, cell movement and cell division. In searching of other plant nuclear envelope associated proteins, arabidopsis nuclear envelop associated (AtNEA1) protein 1, a plant nucleoplasmic protein, was identified from biological studies. AtNEA1 was predicted to have a nuclear localisation signal (NLS), two coiled coil domains and one transmembrane (TM) domain. The mutants with the deletion of respective putative domains were observed under confocal microscopy. The subcellular localisation of mutants implied that putative NLS is not essential for AtNEA1 to diffuse through NPC but can strongly increase the efficiency, both coiled coil domains participate in the interaction of AtNEA1 with its unknown INM intrinsic interaction partner, and putative TM appeared to be non-functional. The function of AtNEA1 in plant was studied through observing tDNA lines.

Key words: Arabidopsis nuclear envelope associated protein 1 (AtNEA1), bioinformatics search, confocal microscopy, nucleoplasmic protein, domain deletions, truncation.

INTRODUCTION

The Nuclear Envelope (NE) consists of an inner nuclear membrane (INM) and an outer nuclear membrane (ONM). The pore membrane accommodates the nuclear pore complex (NPC) and connects INM and ONM (Evans et al., 2004, 2011; Fiserova et al., 2009). The ONM is also connected with the endoplasmic reticulum (ER), and hence; allows the NE to function with the endomembrane network (Crisp and Burke, 2008; Razafsky and Hodzic, 2009). The INM and ONM contain a series of proteins and these proteins execute important cellular functions. A number of proteins of the INM are associated with the nuclear lamina - a protein meshwork composed of lamins. The nuclear lamina attaches chromatin to the NE and stabiles the structure of nucleus. The ONM proteins together with cytoskeletal components function in cell movement and nuclear positioning. Anchored to the nuclear pore membrane is the nuclear pore complex (NPC), which has a framework of a central gated channel with cytoplasmic and nuclear filaments. The NPC facilitates the exchange of macromolecules (RNA, proteins, carbohydrates and lipids) between cytoplasm and nucleus (Doye and Hurt, 1997; D'Angelo and Hetzer, 2006; Stewart et al., 2007; Meier and Brkljacic, 2009a).

The INM can connect to chromatin directly and through the lamina. The nuclear lamina is a meshwork of about 10 to20 nm thick and is formed by lamins and

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lamin-associated proteins (Gruenbaum et al., 2005; Stewart et al., 2007; Prokocimer et al., 2009). The nuclear lamina helps to support the structure of the nucleus in terms of providing a framework at the INM and is involved in various cellular processes including mitosis and nuclear activities (Broers et al., 1999; Gruenbaum et al., 2005; D'Angelo and Hetzer, 2006; Prokocimer et al., 2009). The nuclear lamins are type V intermediate filaments. The IF has a tripartite structure, which is shaped by a helical rod domain in the centre with a short head domain and a long tail (Prokocimer et al., 2009). Four coiled coils form the centre rod domain and they are linked by a linker structure (Stuurman et al., 1998; Herrmann and Aebi, 2004). The linkage from cytoskeletal elements to INM proteins, from INM proteins to lamins, and from lamins to nucleoskeleton and chromatin forms a bridge across the nuclear envelope to participate in cellular processes like mitotic spindle assembly, nuclear positioning and cell movement. In addition, lamins participate in gene transcription activities through interaction with BAF, RNA and other transcriptional regulators (Goldberg et al., 1999). The lamins interact with proliferating cell nuclear antigen (PCNA) protein, chromatin and DNA matrix attachment region (MAR) to support the scaffold for DNA replication.

MAR are specific DNA sequences functioning in maintaining and mediating chromatin structure in the regulation of gene expression (Moir and Spann, 2001; Vlcek et al., 2001; Gruenbaum et al., 2005; D'Angelo and Hetzer, 2006). An important INM protein family is the SUN (Sad1/Unc-84) domain protein family. SUN domain proteins consist of an N-terminal nucleoplasmic portion and at least one transmembrane domain embeded in the INM. The nucleoplasmic portion of SUN domain proteins interact with nucleoskeletal components like type-A and type-B lamins (Hodzic et al., 2004; Haque et al., 2006; Tzur et al., 2006; Wang et al., 2006). The regulation of interactions between lamins and SUN domain proteins is largely unknown (Razafsky and Hodzic, 2009). The C-terminal portion of SUN domain proteins is in the lumen and interacts with KASH domain proteins. KASH domain proteins are a major family of ONM-intrinsic proteins in animal and yeast cells (Wilhelmsen et al., 2006). The interaction of SUN domain proteins and KASH domain proteins forms the linker of nucleoskeleton and cytoskeleton (LINC) complex, which provides a physical connection across the NE (Crisp et al., 2006). It is crucial for the duplication of centrosomes and spindle pole bodies (SPB) at NE, and formation anchorage of the chromosome bouquet in mitosis. The LINC complex is also important for spanning the width of the nuclear periplasm (approximately 30 to 50 nm) (Chikashige et al., 2006; Crisp et al., 2006; Tomita and Cooper, 2006; Tzur et al., 2006; Chi et al., 2007; Kemp et al., 2007; Starr, 2009; Graumann et al., 2010). Some INM proteins take part in biochemical signaling in the nucleus. MAN1 contains a LEM domain and the C-terminus of MAN1 binds to Smad proteins, which are receptor-regulated (Massague et al., 2005).

The Smad proteins then mediate downstream signaling of TGF-β superfamily including bone morphogenetic protein (BMP) signaling (Osada et al., 2003; ten Dijke and Hill, 2004; Lin et al., 2005). Human MAN1 binds to Smad2 and Smad3 and antagonizes signaling by transforming growth factor-β (TGF-β) (Gruenbaum et al., 2005; Lin et al., 2005). In yeasts, INM proteins amino acid sensor independent (Asi)1, Asi2 and Asi3 function as negative regulators of Ssy1-Ptr3-Ssy5 (SPS) sensor signaling. The SPS sensor regulates the activity of transcriptional factors Stp1 and Stp2, which are synthesized as latent cytoplasmic factors (Forsberg and Ljungdahl, 2001; Andreasson and Ljungdahl, 2002). Emerin is associated with the death promoting factor Btf, the splicing associated factor YT-521B and the transcriptional repressor germ cell less (GCL) (Holaska et al., 2003; Wilkinson et al., 2003; Haraguchi et al., 2004). GCL and BAF compete *in vitro* to bind with emerin (Holaska et al., 2003). BAF recruits emerin to chromatin in nuclear assembly in animal cells. The recruitment is crucial for emerin to be localized at the reformed NE (Haraguchi et al., 2001; Holaska et al., 2003). In animal cells, SUN1/matefin is the pro-apoptotic Ced4 receptor and is required for programmed cell death (Tzur et al., 2006). LBR is another vital INM proteins which was first identified in 1988 (Worman et al., 1988). It contains an N-terminal nucleoplasmic portion of 210 amino acids forming two globular domains, a C-terminal hydrophilic domain and 8 transmembrane domains (Worman et al., 1988; Chu et al., 1998). The independent hinge region of LBR binds to chromatin (Ye et al., 1997). The binding is mediated by heterochromatin protein (HP1). The second globular domain of LBR binds to HP1 and the interaction between LBR and HP1 is involved in vesicle targeting to chromosomes at the end of mitosis (Pyrpasopoulou et al., 1996; Buendia and Courvalin, 1997; Ye et al., 1997). In human cells, LBR antibody can also detect lamin B, implying interaction between LBR and lamina (Lassoued et al., 1991; Lin et al., 1996). The interaction of LBR and the lamina supports the attachment of the lamina to the INM and stabiles the NE structure. Figure 1 illustrates the overview of the nucleus and INM proteins. Mutations in emerin and LBR cause diseases (Morris, 2001; Hoffmann et al., 2002; Waterham et al., 2003).

Other typical plant nuclear proteins include MAR-binding filament like protein 1 (MFP1), MAR-binding filament like protein 1 associated factor 1 (MAF1), filament-like plant proteins (FPP), nuclear intermediate filament (NIF) and nuclear matrix protein 1 (NMP1) (Meier et al., 1996; Gindullis et al., 1999, 2002; Rose et al., 2003; Blumenthal et al., 2004). MFP1 functions to connect chromatin to the nuclear lamina and is considered as the homologue of MAR-binding proteins in animal cells (Harder et al., 2000). MAF1 is the MFP1 binding partner in plants. It consists of a WPP (tryptophan-

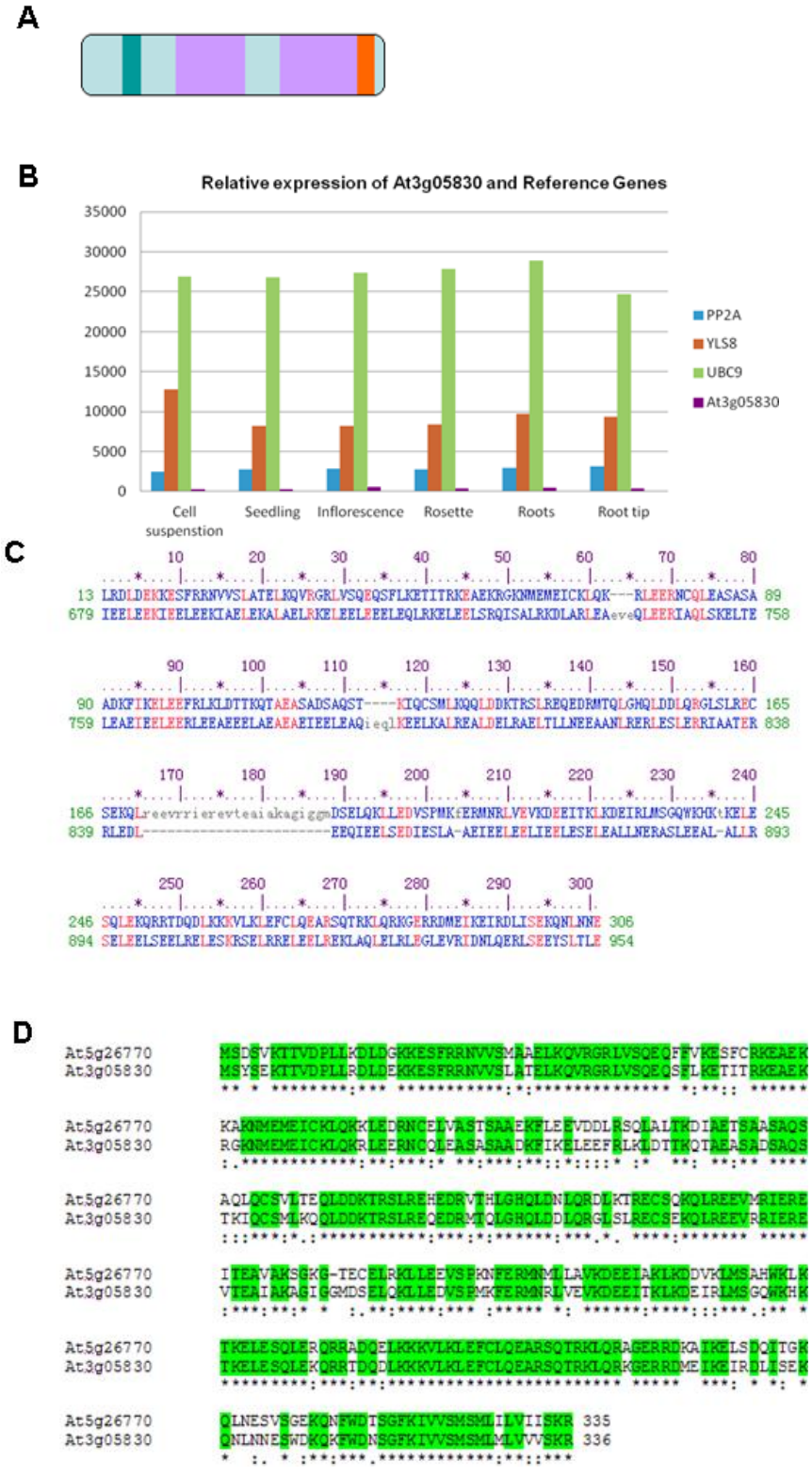

Figure 1. Bioinformatics information of At3g05830 (AtNEA1). Bioinformatics searches were conducted and the relevant information is shown as: (A) Domain structure prediction of At3g05830, cyan: bipartite nuclear localization signal (NLS), purple: coiled coil domains, orange: putative transmembrane domain, location and size of domains were determined by SMART and MotifScan facilities. (B) The relative expression pattern of At3g05830 in *A. thaliana* from Genevestigator. Reference genes: blue: Protein phosphatase 2A subunit-PP2A (At1g13320); red: Yellow leaf-specific protein 8-YLS8 (At4g27960); green: Ubiquitin conjugating enzyme-UBC9 (At4g27960): mauve: At3g05830. (C) The structure alignment of At3g05830 with a typical SMC protein SMC_prok_B (TIGR02168) from NCBI CDD facility. (D) The DNA alignment of At5g26770 with At3g05830 from NCBI BLAST facility.

proline-proline) domain and is unique to plants (Gindullis et al., 1999; Patel et al., 2004; Rose et al., 2004). FPP is also exclusive to plants and interacts with MAF1. NIF is a sequence homologue to lamins in animal cells. NMCP1 is conserved in plants and is located at nucleus (Gindullis et al., 2002; Rose et al., 2003; Blumenthal et al., 2004). LITTLE NUCLEI 1 (LINC1) and LINC2 proteins are two important plant nuclear proteins. LINC1 is localized to the nuclear periphery and LINC2 is localized to the nucleoplasm (Dittmer et al., 2007). LINC1 and LINC2 are NMCP1 homology proteins in arabidopsis. The deletion of either LINC1 or LINC2 causes nucleus size reduction and nuclear morphology change. The deletion of both genes causes not only nuclear shape alteration but also chromocenter number reduction and whole-plant morphology dwarfing (Dittmer et al., 2007). In addition to LINC1 and LINC2, LINC 3 was identified as a nucleolar protein and LINC4 was identified as a plastid protein (Kleffmann et al., 2006; Pendle et al., 2005).

One of the potential plant NE specific proteins is DMI1 (does not make infection 1). DMI1 responds to Nod factor signaling which is released by nitrogen-fixed bacteria to participate in generating perinuclear calcium oscillations (Peiter et al., 2007; Riely et al., 2007). Ran GTPase activating protein (RanGAP) has also been found in the plant ONM (Meier et al., 2010).

The anchoring of plant microtubules to the NE is through the interaction of γ-tubulin ring complexes (γ-TuRC) with plant gamma-tublin complex proteins (GCP) AtGCP2 and AtGCP3. The NE localization domains of AtGCP2 and AtGCP3 can interact with ONM intrinsic proteins, anchoring γ-TuRC to the plant NE (Seltzer et al., 2007). The nuclear calcium level is regulated by calcium channels and pumps located in both INM and ONM (Xiong et al., 2006). A lycopersicon Ca^{2+} ATPase (LCA) in tomato cells is a homologue of the SERCA calcium pump found at the NE in plants (Downie et al., 1998). DMI1 is essential for calcium spiking through the conductance of a K+ (RCK) domain at its C-terminus (Peiter et al., 2007; Riely et al., 2007). Both DMI1 and LCA were shown at the NE by confocal and electron microscopy using immunofluoresence labeling or immunogold labeling (Riely et al., 2007). RanGAP is anchored to the ONM and is crucial for NPC transport, SPB assembly and NE reformation after cell mitosis (Xu and Meier, 2008; Zhao et al., 2008). Plant RanGAP has a unique WPP domain, which is important for RanGAP localization at ONM (Meier, 2007). Although, similarities do exist, significant differences have been found between plant cell nuclei and those of animals or yeast cells.

For example, no lamin sequence homologues have been identified in the plant cell nucleus (Graumann and Evans, 2010; Goldberg et al., 1999; Meier, 2001). However, a lamin-like protein nuclear matrix constituent protein 1 (NMCP1) can be detected using anti-lamin antibodies (Li and Roux, 1992; McNulty and Saunders, 1992; Masuda et al., 1997; Meier, 2007). NMCP1 has a structure of a coiled coil domain, α-helix and a nuclear localization signal (NLS). NMCP1 contains phosphorylation sites for kinases as well. These characteristics imply NMCP1 is a lamin substitute in plant (Masuda et al., 1997).

The aim of the project was to identify potential novel NE or NE-associated proteins in plants and to characterise them. Arabidopsis nuclear envelope protein 1 (AtNEA1), was probed as a plant nuclear envelope associated protein, which is anchored to the INM by interacting with an unknown interacting partner. The subcellular localization of AtNEA1 was identified by approaching *in vivo* fluorescence transient expression as well as BY-2 cells stable expression observed by confocal microscopy. The function of AtNEA1 was probed by applying tDNA mutant lines study and BY-2 cell stable expression study. The novel discovery of AtNEA1 has boarded the view of plant nuclear envelope (NE) structure and NE associated proteins.

MATERIALS AND METHODS

Bioinformatics

The accesses Pfam, Sanger, Aramemnon and Interpro predictive protein and pattern database (http://pfam.sanger.ac.uk/search?tab=searchSequenceBlock; http://smart.embl-heiderberg.de; http://aramemnon.botanik.uni-koeln.de/index.ep; http://www.ebi.ac.uk/interpro) were used to select At3g05830 (in this study, At3g05830 refers to At3g05830.1 subtype) as putative KASH-like gene in arabidopsis. The amino acid sequence of At3g05830 was compared with amino acid sequences in EXPASY, NCBI BLAST and NCBI CDD accesses (http://expasy.org/; http://blast.ncbi.nlm.nih.gov/Blast.cgi; http://www.ncbi.nlm.nih.gov/cdd) to investigate DNA and structural homologues. Relative expression data for AtNEA1 and reference genes were obtained from the genevestigator database (http://www.genevestigator.ethz.ch/).

Cloning and transient expression of AtNEA1 in *Nicotiana benthamiana*

The gene encoding At3g05830 (AtNEA1) was fused into donor vector pDONR207 (Invitrogen, Paisley, UK) by the Gateway BP reaction. YFP fused expressions vector pCAMBIA-YFP-casA and pCAMBIA-casB-YFP (Invitrogen, Paisley, UK) encoding AtNEA1 were gained by the Gateway LR reaction. Built up expression vectors were transfected into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986). Bacterial cells were resuspended in infiltration buffer [0.5% (w/v) glucose, 50 mM MES, 2 mM trisodium phosphate, 100 µM acetosyringone, pH 5.6]. For transformation, *A. tumefaciens* cells containing YFP- tagged expression vectors were adjusted to a D_{600} = 0.4 ratio and infiltrated in 5 week old *N. benthamiana* leafs. Infiltrated *N. benthamiana* leafs were cropped and observed 72 h after transformation.

Cloning, expression and purification of AtNEA1 in *Escherichia coli*

The gene encoding AtNEA1 was amplified from a cDNA library obtained by RT-PCR from cauline leafs of *A. thaliana* and cloned

into the expression vector pDONR207 (Invitrogen, Paisley, UK). The gained vector was transfected into the *E. coli* strain DH5α and cells were grown in LB [1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, and 1% sodium chloride] medium at 37°C. Cells were harvested 12 h after induction and the vectors were purified. For purification of pDONR207-AtNEA1, cells were pelleted and resuspended in 250 µl resuspension solution (Promega, Southampton, UK). The same amount of cell lysis solution (Promega, Southampton, UK) was added to mix. 10 µl alkaline protease solution (Promega, Southampton, UK) was added to mix. 350 µl neutralization solution was added to mix. After centrifugation of the cell lysate at 140 00 *g*, the supernant was decanted into the spin column. 750 µl wash solution (Promega, Southampton, UK) was added and the collection tube (Promega, Southampton, UK) was centrifuged at 140 00 *g*. The wash was repeated once with 250 µl wash buffer. The DNA was eluted in 40 µl nuclease free water and the sequence was analyzed by gene sequencing.

Confocal microscopy

Confocal microscopy was conducted by a Zeiss LSM 510 Laser Scanning Microscopy (Carl Zeiss GmbH, Hertfordshire, UK) with a Plan-Neoflur 63 × / 1.4 oil immersion objective. The following settings were used throughout the experiments: YFP excitation 514 nm, CFP excitation 458 nm, detection wavelength 530 to 600 nm for YFP and 475 to 525 nm for CFP. Images were processed using Zeiss LSM 510 Software (Carl Zeiss GmgH, Hertfordshire, UK). For fluorescence resonance energy transfer (FRET) interaction studies, YFP- and CFP- tagged expression vectors encoding for AtNEA1 and AtSUN1/AtSUN2 were co-infiltrated in *N. benthamina*. FRET was measured with the acceptor bleaching method. The acceptor YFP was bleached 10 times with 100% laser power of the 514 nm diode laser line in a region of interest (ROI). FRET was quantified by measuring CFP fluorescence intensity in the bleached area before (5 frames) and after (10 frames) bleaching. FRET efficiency ($FRET_{eff}$), which is the percentage fluorescence intensity, was calculated according to the following expression:

$$FRET_{eff} = \{(I\ [CFP_{after\ bleaching}] - I\ [GFP_{before\ bleaching}] / I\ [GFP_{after\ bleaching}])\} \times 100$$

The test of AtNEA1 solubility *in vivo*

Transiently YFP- tagged AtNEA1 transformed leaf tissue was crushed on ice with 50 µl exaction buffer (10 mM Tris HCl, 150 mM NaCl, 0.5 to 1.0% Triton X-114). After centrifugation at 140 00 *g* at 4°C, supernatant was decanted to a fresh eppendorf tube and brought up to 250 µl with extraction buffer. Protein sample was gently overlaid on 300 µl sucrose cushion (6% sucrose, 10 mM Tris HCl, 150 mM NaCl, 0.06% Triton X-114). Samples were centrifuged at 300 *g* at 30°C (Sorvall, Bishop's Stortford, UK) after 3 min incubation at 30°C. Upper aqueous phase was decanted into a fresh eppendorf tube. The lower detergent phase was overlaid on the sucrose cushion for 3 min incubation at 30°C followed by 3 min centrifugation at 30°C. The supernatant was discarded. The aqueous phase was rinsed with 2% concentrated Triton X-114. Both aqueous phase and detergent phase were brought up to the equal volumes as well as concentrations of salt and surfactant. Bovine serum albumin (10 mg/ml) was added to samples in a ratio of 3%. 1.5 m^3 of saturated ammonium sulphate solution was added and mixed. The tubes were incubated for 2 h followed by 10 min full speed centrifugation at 4°C. The supernatant was discarded.

Buffer TE (50 mM Tris, 2 mM EDTA) was added to the pellets in a ratio of 20% and the samples were incubated on ice for 30 min. Samples (both aqueous phase and detergent phase) were analyzed by SDS-PAGE gel followed by Western blot (Bordier, 1981).

Domain deletions and truncation

The bioinformatics study predicted 4 putative functional domains in AtNEA1 (SMART: http://smart.embl-heidelberg.de/ and MotifScan: http://myhits.isb-sib.ch.cgi-bin/motif_scan). They are: putative nuclear localization signal (NLS) - from 60 to 75 amino acids; coiled coil 1 - from 121 to 180 amino acids; coiled coil 2 - from 238 to 306 amino acids; putative transmembrane domain - 321 to 334 amino acids. By adopting splicing by overlapping extension (SOE) PCR protocol (Dahm and Jennewein, 2010), the putative NLS and coiled coil domains were deleted and putative transmembrane domain was truncated (the last two amino acids were removed from the gene as well). The mutants were fused into pCAMBIA- YFP vectors by Gateway protocol and transiently expressed in *N. benthamiana* at a D_{600} = 0.4 ratio. The infiltrated leafs were observed by confocal microscopy 72 h after transformation.

SAIL_SALK T-DNA lines

Six arabidopsis agar medium plates [2.2 g/l M&S media (Sigma M0404), 0.5 g/l MES, pH 5.7, 0.6% agar] were prepared under sterile conditions, half of the plates with kanamycin (10 mg/l) and the other half without. A small quantity of seeds - wild type (provided from this lab), SAIL line-SAIL_846_B07.v1 (N837770) (NASC, Nottingham, UK) and SALK line -SALK_021615.44.45.x (N521615) (NASC, Nottingham, UK) were placed into respective 1.5 ml Eppendorf tube. The bleach solution (10% NaClO; 0.02% Tween-20) was added and the tubes were shaken. The tubes were laid in laminar flow hood for 10 min to let the seeds precipitate to the bottom of tube. The supernatant was decanted and the bleach solution was added again. The wash was repeated 3 times. The seeds were resuspended in 500 µl dH_2O. The resuspended seeds were aliquoted onto a filter paper. The seeds were picked and dropped onto agar plates by using a sterile cocktail wooden stick. The plates were sealed with semi-permeable tape. The plates were wrapped with foil and stored at 4°C for 2 days to stratify, the plates were taken out, the foil was removed and the plates were incubated at 28°C in a culture room.

T-DNA line nucleus size measurements

Seedlings (14 day old) showing the same development stage were placed in 6.252 mg/ml ethidium bromide for 16 min at room temperature. Fluorescence of the nucleus of the leaf tissues were observed by laser scanning confocal microscopy to measure the size of the nucleus. Images were taken in the focal plane where the nucleus diameter was largest and then, the surface of this section was measured using ZEISS LSM image browser software. The settings for confocal microscopy were 514 nm excitation wavelength, ×40 objective lense, beam splitters: HFT 458-514, NFT 515, channel: Ch2, filter: BP560-615. The data was analysed by F-test followed by the student t-test with 5% significance level probability.

T-DNA line genotype examination

Leaf tissue was punched out by closing a microfuge lid on the leaf. The leaf material was ground in the microfuge using a disposable pestle for 15 s without buffer. 400 µl extraction buffer was added at room temperature, the microfuge was vortexed for 5 s. The samples were left at room temperature for 1 h. The sample was centrifuged (Beckman Coulter, High Wycombe, UK) at 13000 rpm for 1 min. Supernatant (300 µl) was added to 300 µl isopropanol at room temperature. The samples were left at room temperature for 2 min. The samples were centrifuged (Beckman Coulter, High Wycombe,

UK) at 13000 rpm for 5 min to pellet the DNA. Isopropanol was removed and the pellets were dried in a speed-vac. The DNA was resuspended in 100 µl sterile water. All the sample DNA was set in 4 PCR reactions with primers of T-DNA insertion - PCR reaction 1: SALK left primer (LP)*: 5' - AAT TTC TGG TCG AAT GCA TTG - 3', SALK right primer (RP)*: 5' - CCT GAA GAC GAC ACA TAC ATG AG - 3'; PCR reaction 2: SAIL left primer (LP)*: 5' - CTC TGC AGC TTT CTT GTC TGG - 3', SAIL right primer (RP)*: 5' - AGC TTG AAG CTT CTG CAT CTG - 3'; PCR reaction 3: LBb1.3*: 5' - TAA AAC GGC TAA AGC CTT G - 3', SALK right primer (RP)*: 5' - CCT GAA GAC GAC ACA TAC ATG AG - 3'; PCR reaction 4: LBb1.3*: 5' - TAA AAC GGC TAA AGC CTT G - 3', SAIL right primer (RP)*: 5' - AGC TTG AAG CTT CTG CAT CTG - 3'. LP (SAIL), RP (SAIL), LP (SALK), RP (SALK), LBb1.3 sequence information was obtained from SIGNAL: http://signal.salk.edu/T-DNAprimers.2.html.

The primers were ordered from Invitrogen (Paisley, UK). The PCR products were examined using DNA agarose gel.

BY-2 cells synchronisation and study

7 ml of 7-day old BY-2 cells (transformed with AtNEA1-YFP and YFP-AtNEA1) were transfered into 50 ml fresh BY-2 medium and 50 µl 5 mg/ml aphidicolin was added. The culture was incubated at 130 rpm, 27°C for 24 h. Cells were washed with 10 × 50 ml fresh BY-2 medium in a scintered funnel. Cells were resuspended in 50 ml fresh BY-2 medium and returned into incubator. Cells were observed with confocal microscopy.

RESULTS

Bioinformatics

To select the potential KASH-like proteins in plants, the conserved domain features (actin-binding domain, coiled coil domain and transmembrane domain) of KASH proteins were used as the query in a protein BLAST search, in which At3g05830 (one NLS, two coiled coils and one putative transmembrane domain) was selected as potential KASH-like protein in plants. At3g05830 is 336 amino acids long, with predicted molecular mass of 39.58 kDa. The predicted NLS (amino acids 60 to 75), coiled coil 1 domain (amino acids 121 to 180), coiled coil 2 domain (amino acids 238 to 306) and transmembrane domain (amino acids 321 to 334) are shown in Figure 1A. The *in vivo* expression pattern for At3g05830 in *A. thalianas* (Genevestigator: http://www.genevestigator.ethz.ch/) shows At3g05830 is universally expressed in a relative very low manner. But the expression level in embryonic system is comparably higher than other systems, suggesting its involvement in development. Figure 1B shows the relative expression pattern of At3g05830 in *A. thaliana*s. The structure study (NCBI CDD: http://www.ncbi.nlm.nih.gov/cdd) showed At3g05830 matches structural features of structural maintenance of chromosomes (SMC) protein family, suggesting its potential function in regulating the structure and organization of chromosomes. Figure 1C shows the alignment of At3g05830 with typical SMC protein: SMC_prok_B (TIGR02168).

The DNA homology search (NCBI BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi) for At3g05830 indicated very low similarity in animal and yeast cells from searching but has one highly matching (81%) homologue in plant (At5g26770: an uncharacterized protein). Figure 1D shows the DNA alignment of At3g05830 with At5g26770. This fact indicates that At3g05830 may be plant conserved.

Subcellular localization of the YFP- tagged AtNEA1

To identify the subcellular localization of AtNEA1, a full-length form of AtNEA1 fused to YFP at the N- and C-terminal were constructed and transiently expressed in *N. benthamiana*. Expression of the fluorescent AtNEA1 fusion vectors were examined by confocal microscopy. As shown in Figure 2, the fluorescence signals emitted concentrate at the nuclear envelope periphery region. The identity of the stained pattern and the subcellular localization was demonstrated by co-expression of the CFP- labeled NE marker AtSUN1. The perfect overlay of both expression patterns shown in Figure 2C demonstrate the co-localization of both proteins and verified localization of AtNEA1 at the NE periphery.

Test of AtNEA1-AtSUN1 and AtNEA1-AtSUN2 interactions via FRET microscopy

FRET was adopted to test the potential interactions of AtNEA1 with AtSUN1 or AtNEA1 with AtSUN2 using the fluorophores YFP as donor and CFP as acceptor. The acceptor fluorophor was fused to the N- and C- terminus of AtSUN1 and AtSUN2, whereas the donor was attached to the N- and C- terminus of AtNEA1. *N. benthamiana* leaf cells were co-transformed with both vector constructs and the leaf samples were fixed by latrunculin B (Caorsi et al., 2011). Upon excitation of doubly transformed leaf cells at 514 and 458 nm, both YFP and CFP showed clear fluorescence. However, the statistic calculation did not indicate interactions between AtNEA1 with either AtSUN1 or AtSUN2 upon bleaching. The FRET statistic data table is shown in Table 1. The CFP fluorescence did not increase upon acceptor bleaching. Control experiments were carried out by CFP- tagged AtSUN1 or AtSUN2. No significant increase ($p<0.05$) of CFP fluorescence after YFP photobleaching is observed. The same information is gained by FRET of AtNEA1-YFP with CFP-tagged AtSUN1 or AtSUN2.

The solubility test of AtNEA1 from *N. benthamiana* transiently expression

Fused YFP-AtNEA1 and AtNEA1-YFP constructs were transiently expressed in *N. benthamiana* leafs (infiltrated at a ratio of $D_{600} = 0.4$). The expression was examined by confocal microscopy observe. The infiltrated leafs were

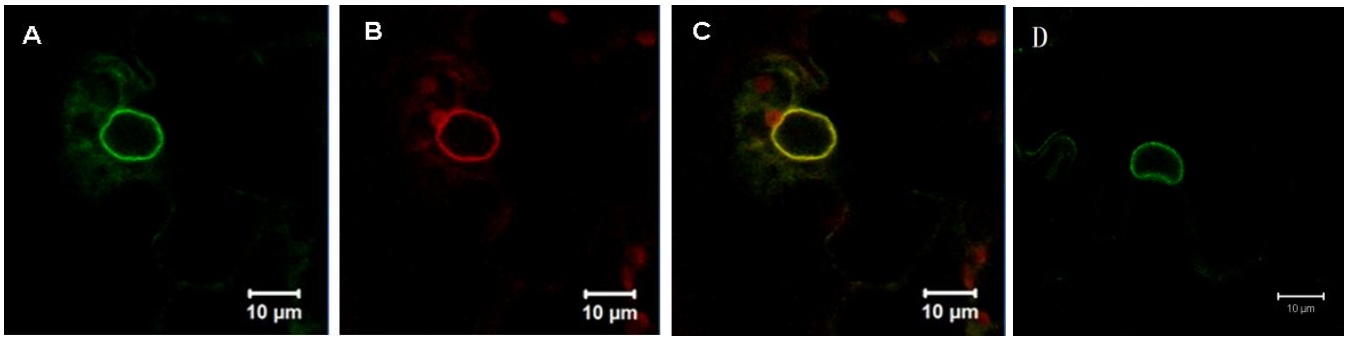

Figure 2. Subcellular localization of AtNEA1 transiently expressed in *N. benthamiana* epidermal leaf cells. Confocal laser scanning microscopy images of representative *N. benthamiana* cells showing fluorescence of (A) AtSUN1-CFP marker protein, (B) AtNEA1-YFP. YFP (530-600 nm) and CFP (475-525 nm) channels were scanned simultaneously. (C) Merged images of (A) and (B). The excitation wavelengths were 514 nm for YFP and 458 nm for CFP. The same information is gained by YFP-AtNEA1 transiently expression. (D) YFP-AtNEA1. YFP (530-600 nm) and CFP (475-525 nm) channels were scanned simultaneously.

Table 1. Fluorescence resonance energy transfer (FRET) efficiencies statistic data.

Co expressed proteins	E_f (%)	Control E_f (%)
CFP-AtSUN1+YFP-AtNEA1	2.62±3.89	2.82±3.89
AtSUN1-CFP+YFP-AtNEA1	3.20±5.12	1.45±3.78
CFP-AtSUN2+YFP-AtNEA1	4.04±7.91	3.81±6.18
AtSUN2-CFP+YFP-AtNEA1	3.24±6.01	3.78±7.84

crushed and the proteins were extracted. The extracted proteins were separated by Triton X-114 bi-phase: aqueous phase and detergent phase. A single band of YFP-AtNEA1 and a single band of AtNEA1-YFP in aqueous lanes on Cy5 stained Western blots indicate AtNEA1 as a soluble protein, rather than a membrane intrinsic protein (insoluble) (Figure 3A and 3B). GFP-CXN (calnexin) (insoluble) and GFP-HDEL (soluble) were used as the markers.

Intracellular localizations of AtNEA1 mutants

By applying SOE PCR methods (Dahm and Jennewein, 2010), putative NLS, coiled coil 1, coiled coil 2 and transmembrane domains were deleted respectively. The mutants were fused into YFP- tagged vectors and transiently expressed in *N. benthamiana* and observed by confocal microscopy at YFP channel settings described earlier. The mutant of NLS deletion (AtNEA1ΔNLS)'s subcellular localization appeared to be both cytoplasmic and nucleoplasmic, indicating NLS is not essential for AtNEA1 to enter nuclei but can strongly increase the transport efficiency, at the same time NLS participates in anchoring AtNEA1 to the INM. The mutants of coiled coil 1 domain deletion (AtNEA1ΔCC1) and coiled coil 2 domain deletion (AtNEA1ΔCC2)'s subcellular localizations both appeared to be nucleoplasmic, indicating both coiled coiled coil 1 domain and coiled coil 2 domain participate in anchoring AtNEA1 to INM. The mutant of putative transmembrane domain truncation (the last 2 amino acids after the putative transmembrane domain were removed)'s subcellular localization appeared to be NE periphery, indicating putative transmembrane domain is not functional. Figure 4A, B, C and D show the subcellular localizations of AtNEA1 mutants.

The sub-cellular localisations of fluorescence tagged AtNEA1 in BY-2 cells

The sub-cellular localisations of both AtNEA1-YFP and YFP-AtNEA1 were observed to be at the nuclear envelope periphery. This observation further confirmed the previous observation data that AtNEA1 is a nuclear envelope periphery localized protein. Also, the BY-2 cell study indicated the coherent information for sub-cellular localisation of AtNEA1-YFP and YFP-AtNEA1 in Arabidopsis thaliana in addition to the sub-cellular locaisation of AtNEA1 in transient transformed *Nicotiana tabacum* (Cv. Petit Havana SR1) (Figure 5).

Characterisation of T-DNA knockouts

The SAIL_SALK T-DNA insertion lines of At3g05830 as well as the wild type (WT) line were grown on agar plates (the SAIL_SALK T-DNA lines grew in agar plates with and without the antibiotic kanamycin) and the morphology of the seedlings was observed. The SAIL line grew on agar plates with and without kanamycin, suggesting the SAIL T-DNA insertion has silenced the kanamycin resistance gene.

The growth and morphology of the shoot apex, cotyledons, hypocotyl and roots did not show any significant differences (Figure 6). The T-DNA SAIL_SALK insertions were screened by genome sequence. In order to determine

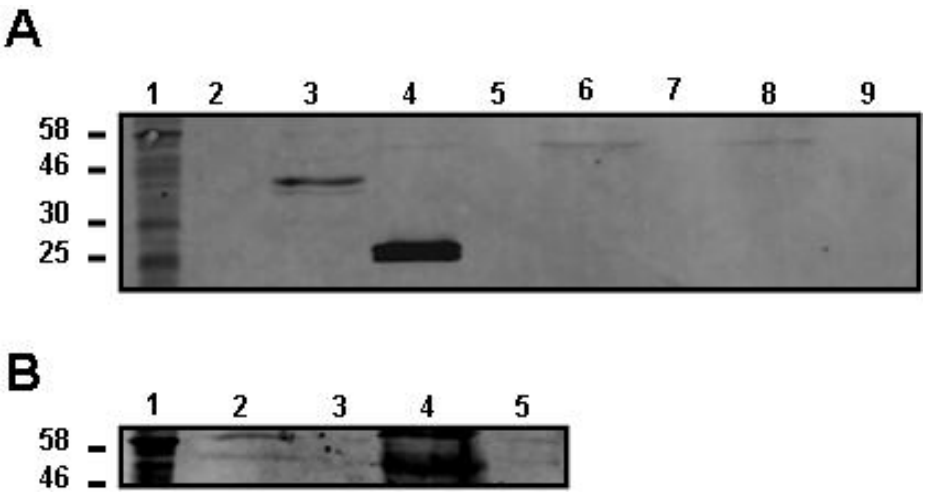

Figure 3. Solubility test of AtNEA1. *A. tumefaciens* (GV3101) was transformed with YFP- tagged AtNEA1, as well as GFP-CXN (calnexin) and GFP-HDEL as the markers. Extracts from the transformed *N. benthamiana* cells were phased by Triton X-114 separation and analyzed by Western blotting. (A) Western blotting of transformed *N. benthamiana* leaf cell extracts using anti-GFP antibodies (abcam, Cambridge, UK). Lane 1, protein marker with molecular masses to the left in kDa; lane 2, cell lysate from GFP-CXN transformed leaf in TX-114 aqueous phase; lane 3, cell lysate from GFP-CXN transformed leaf in TX-114 detergent phase; lane 4, cell lysate from GFP-HDEL transformed leaf in TX-114 aqueous phase; lane 5, cell lysate from GFP-HDEL transformed leaf in TX-114 detergent phase; lane 6, cell lysate from YFP-AtNEA1 transformed leaf in TX-114 aqueous phase; lane 7, cell lysate from YFP-AtNEA1 transformed leaf in TX-114 detergent phase; lane 8, cell lysate from AtNEA1-YFP transformed leaf in TX-114 aqueous phase; lane 9, cell lysate from AtNEA1-YFP transformed leaf in TX-114 detergent phase. (B) Solubility was confirmed by Western blotting using newly raised anti-AtNEA1 antpeptides (Genosphere Biotech, Paris, France). Lane 1, protein marker with molecular masses to the left in kDa; lane 2, cell lysate from YFP-AtNEA1 transformed leaf in TX-114 aqueous phase; lane 3, cell lysate from YFP-AtNEA1 transformed leaf in TX-114 detergent phasse; lane 4, cell lysate from AtNEA1-YFP transformed leaf in TX-114 aqueous phase; lane 5, cell lysate from AtNEA1-YFP transformed leaf in TX-114 detergent phase.

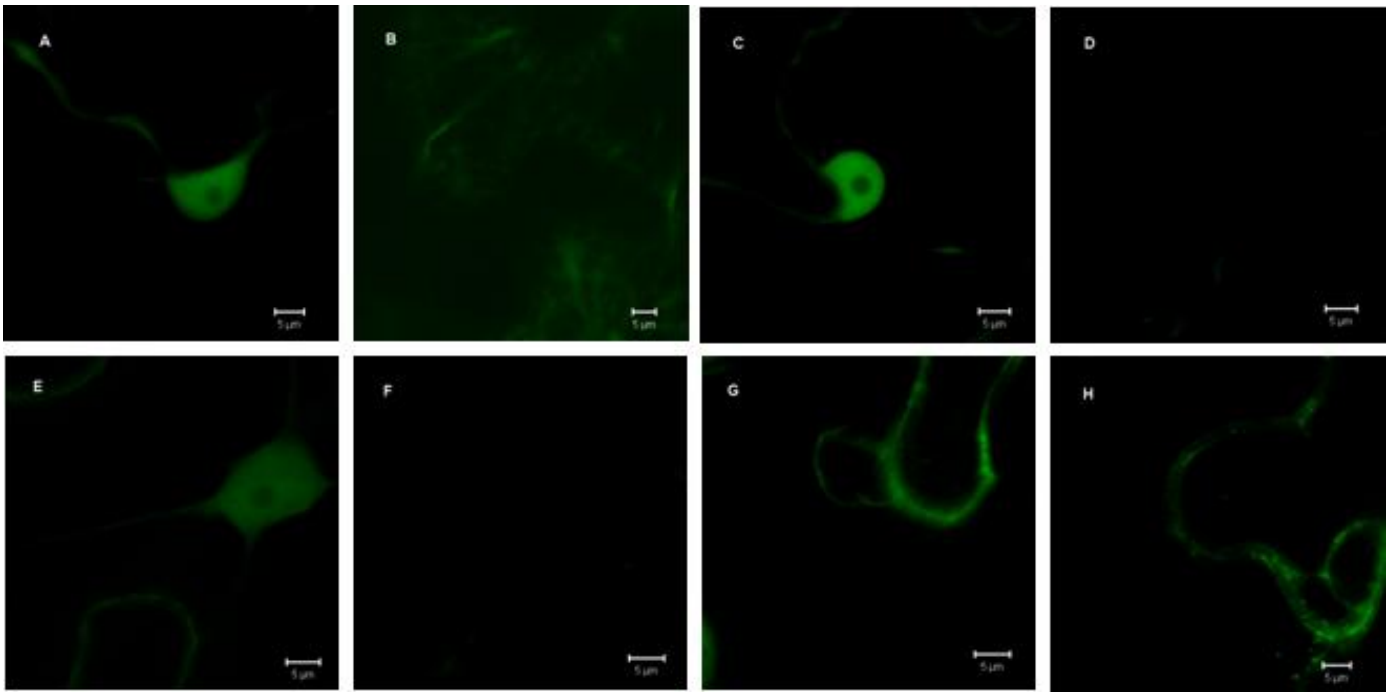

Figure 4. Subcellular localization of AtNEA1 domain mutants transiently expressed in *N. benthamiana* epidermal leaf cells. A) Confocal laser scanning microscopy images of representative *N. benthamiana* cells showing fluorescence of (A) AtNEA1ΔNLS-YFP in nucleoplasmic focus. (B) AtNEA1ΔNLS-YFP in cytoplasmic focus. (C) AtNEA1ΔCC1-YFP in nucleoplasmic focus. (D) AtNEA1ΔCC1-YFP in cytoplasmic focus. (E) AtNEA1ΔCC2-YFP in nucleoplasmic focus. (F) AtNEA1ΔCC2-YFP in cytoplasmic focus. (G) YFP-AtNEA1ΔTM. (H) AtNEA1ΔTM-YFP. YFP (530-600 nm) channels were scanned simultaneously. The excitation wavelengths were 514 nm for YFP. The same information is gained by YFP-AtNEA1ΔNLS, YFP-AtNEA1ΔCC1, YFP-AtNEA1ΔCC2 and YFP-AtNEA1ΔTM transiently expressed.

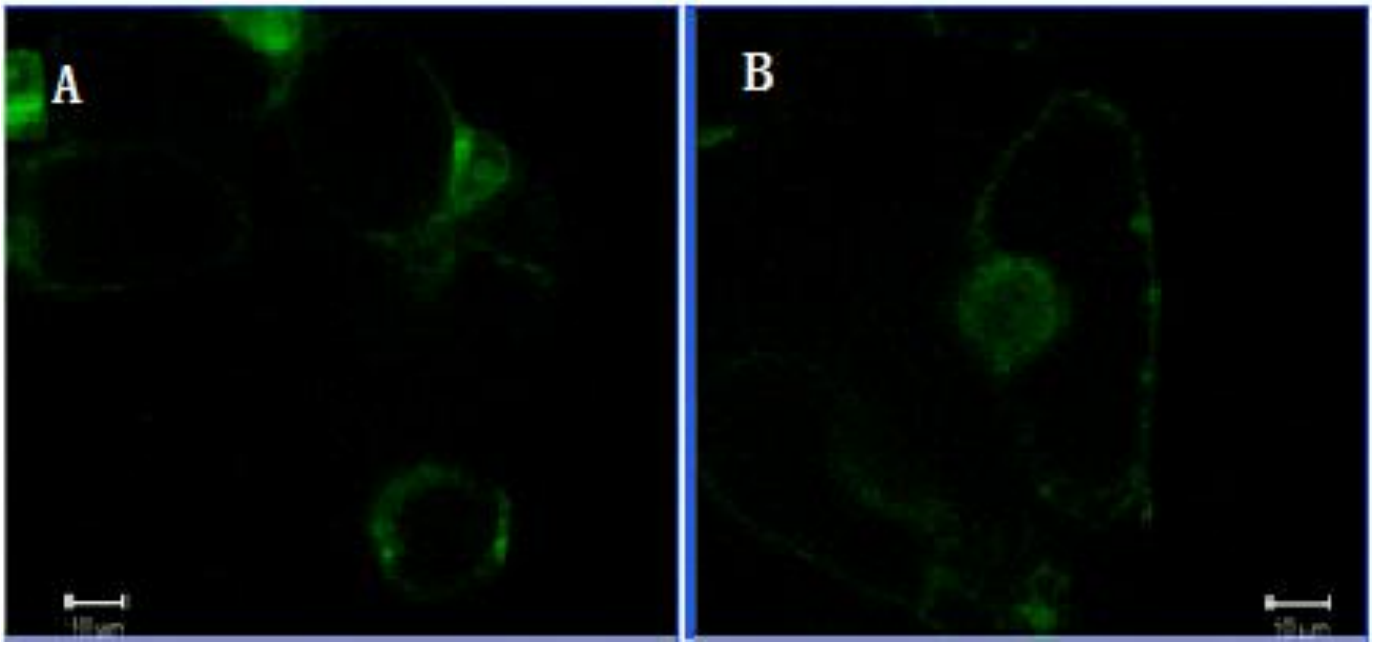

Figure 5. The sub-cellular localisations of YFP-AtNEA1 and AtNEA1-YFP in BY-2 cells. A. Confocal laser scanning microscopy images of representative BY-2 cells showing fluorescence of (A)YFP-AtNEA1 in nuclear envelope periphery focus, (B) AtNEA1-YFP in nuclear envelope periphery focus channels were scanned simultaneously. The excitation wavelengths were 514 nm for YFP.

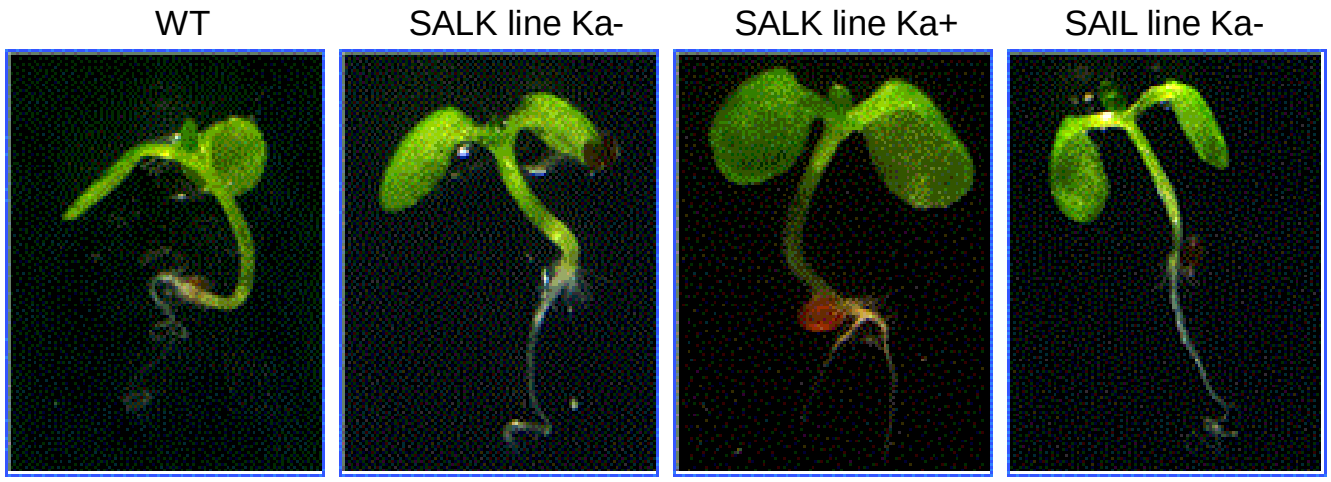

Figure 6. Comparison of the seedlings morphology of *Arabidopsis* wild type, SALK line (without kanamycin), SALK line (with kanamycin) and SAIL line (without kanamycin). SALK (N521615) and SAIL (N837770) lines were provided by NASC, Nottingham, UK.

the size of the nucleus of T-DNA SAIL_SALK lines as well as wild type (WT), leaf pieces were stained with ethidium bromide (EB) and scanned by confocal microscopy. The nucleus size was measured by LSM image browser software.

Samples (30) for each line were measured at the greatest diameter and the data was analyzed by F-test and t-test (P>0.05). The SAIL (N837770) line and the SALK (N521615) line were not significantly different from the wild type (WT). The images of nucleus are shown in Figure 7 and the statistical analysis is shown in Table 2.

DISCUSSION

Bioinformatics of AtNEA1

AtNEA1 (At3g05830) has a predicted NLS sequence at

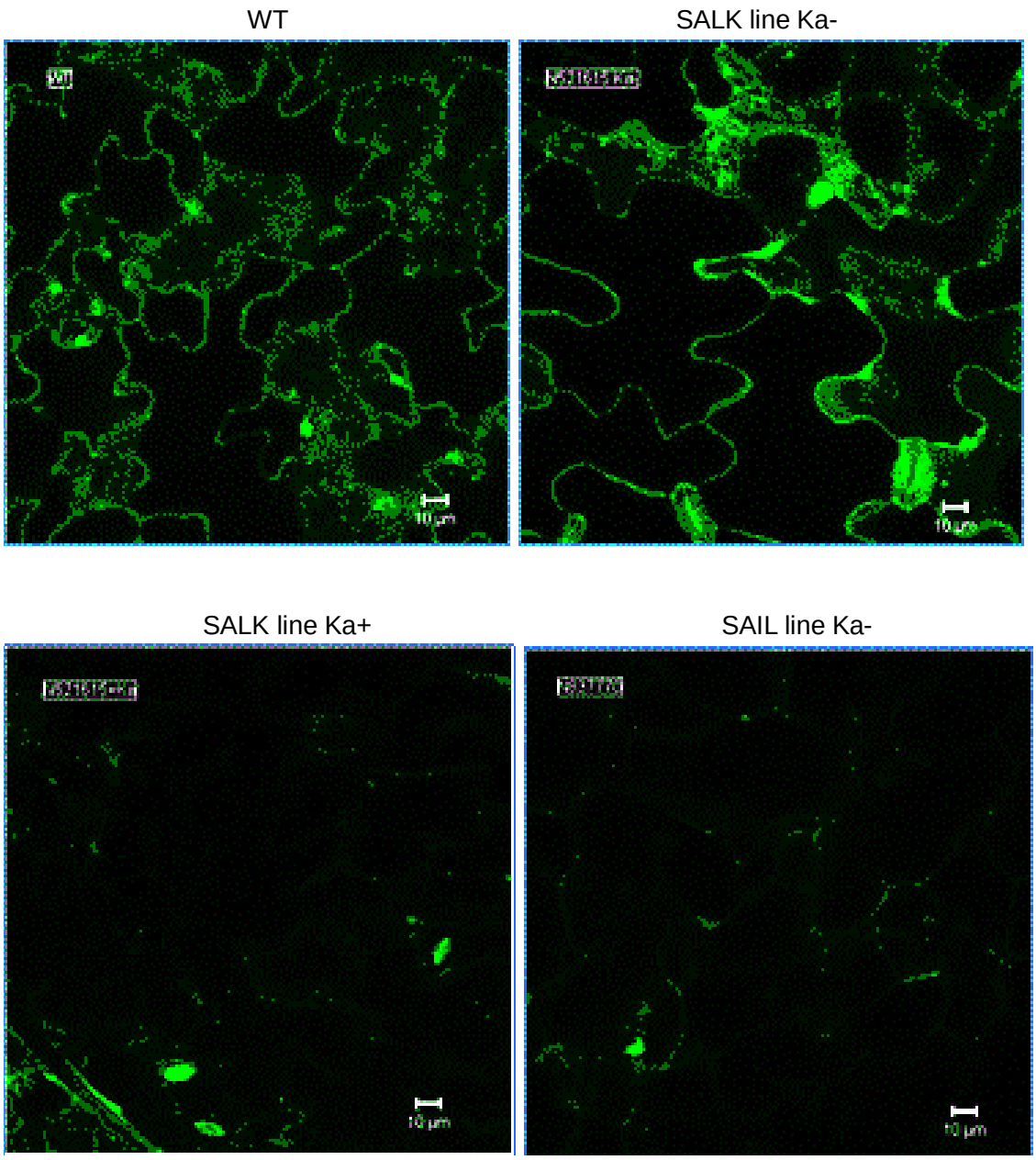

Figure 7. The nucleus images of *Arabidopsis* wild type, T-DNA SAIL (N837770) line (in plate without kanamycin), SALK (N521615) line (in plate without kanamycin), SALK (N521615) line (in plate with kanamycin). SALK (N521615) and SAIL (N837770) lines were provided by NASC, Nottingham, UK. YFP (shown in green) was visualised using an argon laser at 514 nm wavelength. Images were taken with ×63 objective oil immersed lens at various zoom levels. Leaf pieces were stained with ethidium bromide (EB) and scanned by confocal microscopy.

Table 2. The nucleus size measurements of *Arabidopsis* wild type, T-DNA SAIL (N837770) line (in plate without kanamycin), SALK (N521615) line (in plate without kanamycin), SALK (N521615) line (in plate with kanamycin).

Arabidopsis lines	Nucleus size (the greatest diameter of nucelus) unit: μm	Standard deviation
Wild type	7.60	±1.89
SALK line (Ka+)	7.13	±1.06
SALK line (Ka-)	8.27	±1.47
SAIL line (Ka-)	7.19	1.30

SALK (N521615) and SAIL (N837770) lines were provided by NASC, Nottingham, UK.

the N-terminal (from 60 ~ 75 aa) and a predicted transmembrane domain at the C-terminal (from 321 ~ 334 aa) and two coiled coil domains (120 ~ 180 aa and from 238 ~ 306 aa) (SMART: smart.embl-heidelberg.de/; PredictNLS: www.predictprotein.org/cgi/var/nair/resonline.pl). The NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) homology protocol was applied to the sequence of At3g05830 against proteins in the *Arabidopsis thaliana* database (TAIR). At5g26770 was indicated to show 81% homology (E value: $2e^{-47}$) with it. It can be inferred that At5g26770 shares similarities with At3g05830 in terms of function and structure (BP-NLS, coiled coil domain and TM domain are conserved domains for At3g05830 and At5g26770). Therefore, At5g26770 may have close function similarity with At3g05830 or it may be a functional substitute of At3g05830. There are no characterised animal proteins that show homology with At3g05830. This implies At3g05830 is a plant exclusive protein. The expression pattern for *A. thaliana* indicates At3g05830 is relative highly expressed in Arabidopsis embryo, micropylar endosperm, peripheral endosperm, chalazal endosperm, lateral root cap, general seed coat and suspensor. The expression pattern implies a role for At3g05830 in development. The richness of At3g05830 in endosperm implies At3g05830 may function in nutrition of the embryo or release of storage reserves. The stimuli map of At3g05830 (data not shown) shows At3g05830 is stably expressed under various physical or chemical stresses. The data indicates the expression of At3g05830 is ubiquitous in Arabidopsis thaliana and implies At3g05830 has an essential role in *A. thaliana*.

The localization information together indicates: AtNEA1 (At3g05830) is a plant nuclear envelope associated protein; the putative NLS is not essential for At3g05830 to enter nuclei, but can strongly increase the efficiency of transport; the putative NLS also participates in anchoring At3g05830 to nuclear envelope periphery; both the putative coiled coil domain 1 and coiled coil domain 2 participate in anchoring At3g05830 to the nuclear envelope periphery; the putative transmembrane domain appeared to be non-functional. The integrity and stability of the YFP constructs were confirmed by expression followed by western blotting against anti FP antibodies, where proteins of appropriate Mr were detected.

Properties of AtNEA1

At3g05830 was observed to be localized at tobacco leaf cell nuclear envelope periphery by confocal microscopy in transient expression. It was also observed to be co-localized with AtSUN1 and AtSUN2 at the same location in transient expression. The putative NLS, coiled coil domain 1, coiled coil domain 2 and putative transmembrane domain were deleted or truncated respectively. The subcellular localization of AtNEA1ΔNLS appeared to be both nucleoplasmic and cytoplasmic. The subcellular localization of AtNEA1ΔCC1 appeared to be nucleoplasmic. The subcellular localization of AtNEA1ΔCC2 appeared to be nucleoplasmic. The subcellular localization of AtNEA1ΔTM appeared to be nuclear periphery. In the Triton X-114 solubility study, At3g05830 was shown to be in the aqueous phase (soluble phase) of Triton X-114 phase partition.

In combination with the results from domain mutant study, At3g05830 is predicted to be a free-diffuse nucleoplasmic protein, which is anchoring to the INM by interacting with an unknown INM-intrinsic protein. The NLS sequence and two coiled coil domains are all taking part in forming the interaction structure for AtNEA1 with its INM interaction partner. The protein itself can diffuse through the NPC, but the NLS can also be recognized by importins and directed through NPC.

According to the FRET data statistical analysis (Table 1), the efficiencies Ef (Ef is the quantum yield of the energy transfer transition) of CFP did not increase after the bleach of YFP. Therefore, it was not indicated that At3g05830 was interacting with AtSUN1 or AtSUN2; however, FRET does not prove the absence of interaction *in vivo*, as other factors, like the effect of the presence of the fluorescent protein groups, could prevent co-localisation. Study of the phenotype of the Arabidopsis T-DNA lines of AtNEA1 (At3g05830) (SAIL and SALK) did not reveal observable differences in comparison with wild type for any of the parameters measured. Hence, little information can be acquired about the potential function of At3g05830 in terms of affecting Arabidopsis morphology. The nucleus size comparison of Arabidopsis T-DNA lines of At3g05830 (SAIL and SALK) did not show statistically significant differences in comparison with wild type. Hence, little information can be acquired about the potential function of At3g05830 in terms of affecting the size of Arabidopsis cell nucleus. The presence of the T-DNA lines genome insertion was confirmed and the T-DNA lines were proved as homozygous. Absence of identified phenotype therefore may be because another gene, or a homologue, is present which replaces At3g05830 function. One candidate for this could be At5g26770, which shows structural similarity.

AtNEA1 is a novel plant nuclear envelope associated protein and was shown to contain a NLS and two coiled coil domains. Results suggested that a hydophobic domain at the C-terminus was not as predicted a transmembrane domain. Deletion of the coiled coil domains, however, resulted in altered localization and lack of interaction with the NE. The NLS appears to increase localization of the protein to the nucleus. No homologues for At3g05830 were shown in animals or yeasts, though plants appear to have once copy of At3g05830 and one of its putative homologue At5g26770. When At3g05830 function was investigated using SALK and SAIL T-DNA intertion mutants, no phenotype was detected, in morphology, development on size of nucleus.

It is therefore possible to assume AtNEA1 is a functionally substitute of At5g26770 in *A. thalian* but further functions and roles need to be explored.

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Managing physicochemical parameters in compost systems to enhance degradation of petroleum wastes from a sludge dam

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Physical, chemical and biological parameters were optimized during composting to enhance degradation of oil sludge. Mixtures of oil sludge, garden soil, poultry manure and the bulking agents were co-composted in static piles of about 1 m^3 on wooden pallets overlaid with nylon fibre sheets. Temperature, moisture, electrical conductivity (EC), pH, total carbon, total N, heterotrophs and respiration of compost microorganisms were monitored in each pile. Moisture was maintained at between 60 and 70% field capacity. Temperatures reached a mean of 63°C in the compost containing manure and sawdust. The C:N ratio of composts changed significantly during the composting process, reaching 100:41 (sawdust+manure) 100:39 (hay+manure) and 100:31 (woodchips+manure). Respiration of compost organisms rose from 1490 to 3850 CO_2 (µg)/dwt/day in the sawdust+manure compost. Total petroleum hydrocarbons (TPH) decreased by between 52 and 66% in the composts and concentrations of selected polycyclic aromatic hydrocarbons (PAH) by between 78 and 100%. The *Bacillus, Pseudomonas, Arthrobacter* and *Staphylococcus* species were predominant in all the experiments and all temperature regimes.

Key words: Bulking agents, compost bioremediation, crude oil sludge, microorganism, polycyclic aromatic hydrocarbons (PAH), total petroleum hydrocarbons (TPH).

INTRODUCTION

Large amounts of liquid effluents and solid wastes are generated from different stages in the petroleum refining process. These including waste waters from cleaning processes and storage tank sediments (Shie et al., 2004; Wang et al., 2010; Mandal et al., 2012). The resulting waste is a viscous oily sludge containing high amounts of petroleum derived hydrocarbons, including alkanes and paraffins of 1-40 carbon atoms, cycloalkanes, aromatic compounds with different numbers of benzene rings (US EPA, 1997; Marin et al., 2006; Pakpahan et al., 2011), asphaltenes and resins (Diallo et al., 2000). Oil sludge from crude oil storage tanks typically consist of sulphides, phenols and heavy metals along with petroleum based hydrocarbons of the aliphatic and aromatic type in very high concentrations. Irrespective of the source, oil sludge contains large amounts of polycyclic aromatic hydrocarbons (PAHs), polychlorinated hydrocarbons and other organic compounds of environmental interest many

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Abbreviations: PAHs, Polycyclic aromatic hydrocarbons; **PCB,** polychlorinated biphenyls; **TPH,** total petroleum hydrocarbons; **IRS,** infrared spectroscopy; **MSA,** mineral salts agar; **CTAB,** cetyltrimethyl ammonium bromide; **EDTA,** ethylene diamine tetraactic acid buffer; **SDS,** sodium dodecyl sulphate; **PCR**, polymerease chain reaction; **PEG,** polyethylene glycol; **BLAST,** basic local alignment search tool; **EC,** electrical conductivity.

of which are potentially carcinogenic, genotoxic, cytotoxic, mutagenic or of threat to humans (Bojes and Pope, 2007; Kriipsalu et al., 2008; Mandal et al., 2012; Jiang et al., 2013). The environmental impacts due to oil sludge contamination include physical and chemical alteration of natural habitats, lethal and sub-lethal effects on aquatic and terrestrial ecosystems. Thus, oil sludge is a hazardous complex of organic compounds (US EPA 1997; Liu et al., 2010) of environmental concern and should be treated as such.

The treatment and successful disposal of large amounts of oil sludge generated during crude oil refining constitute some of the major challenges faced by oil refineries and petrochemical industries (Srinivasarao et al., 2011). A number of treatment methods have been employed in treating oil sludge including physical, chemical, and biological methods (Mahmoud, 2004, Srinivasarao et al., 2011). These methods, however, have different challenges, which include high cost, production of toxic by-products and lack of available space for landfilling. Landfilling is also faced with the problems of managing leachates, emissions and burrowing animals that periodically expose the wastes (Srinivasarao et al., 2011). Biological methods have been lauded as the most environmentally friendly approaches for treating contaminated sites because they are nature-compatible, reliable, cheaper and easy to adopt compared to physical and chemical methods (Machin-Ramirez et al., 2008). The end products are usually harmless carbon dioxide, water and fatty acids. Oil sludge components, for example, organochlorines and high molecular mass PAHs, however, have continued to be resistant to such treatments, largely due to poor solubility of the sludge matrix in aqueous media. Most biological methods are economically unsound, as they are prone to prolonged periods of treatment, as such may not be suitable for treating some contaminants (Ward et al., 2003). Heavy hydrocarbon components such as high molecular weight PAHs are generally recalcitrant to most bioremediation treatments (Atagana, 2003) due to their low volatility, hydrophobicity and low water solubility which is largely responsible for their low availability to microbial actions hence their persistence in the environment (Kriipsalu et al., 2008; Cameotra and Makker, 2010; Liu et al., 2011).

Compost bioremediation is the application of composting technology in treating contaminants. It is an *ex situ* remediation technology which relies on mixing of organic materials with contaminants (Kriipsalu et al., 2008), a process referred to as co-composting, is becoming popular (Kriipsalu and Nammari, 2010; Srinivasarao et al., 2011). Compost have a rich and diverse microbial population, which could promote degradation of xenobiotic compounds such as polychlorinated biphenyls (PCB) and PAH, high temperatures generated by high metabolic activities of the microbial population, which is conducive for increased solubility of the organic substrate and availability of large amounts of organic substrate to co-metabolize while the population is adapting the contaminant organic substrate. These characteristics have made composting an attractive option to bioremediation practitioners. Successful aerobic degradation depends on adequate temperature, aeration, and appropriate amounts of moisture (Suler and Finstein, 1997; Steger et al., 2007; Kriipsalu et al., 2008; Rasapoor et al 2009). The temperature profile of a composting system determines the type of microorganisms that would prevail in the compost matrix and subsequently the rate of degradation of organic substrates. The composting process tends to reach thermophilic levels easily thus promoting thermo-tolerant organisms. While this may be advantageous to the composting process, the challenges of enzyme denaturing cannot be overlooked. Hence it is essential to manage the temperature with aeration technics during composting to achieve the desired goals. In composting of contaminant organic substrates, the type and amounts of amendments used and the carbon to nitrogen (C:N) ratio is important, as these influences the degradation of the target compounds.

The aim of this study was to employ and manage the improved metabolic activities offered by the large microbial load, abundant nutrients and high temperatures available in a compost mixture and the enhanced aeration and carbon supplement offered by the bulking agents in improving the degradation of oil sludge.

MATERIALS AND METHODS

Oil sludge

Crude oil sludge was obtained from a sludge dam in a petroleum refinery in Durban, South Africa. The sludge contained (mg kg^{-1}), TPH 300 592, total organic carbon 395, phenol 633, ammonia 65, chlorides 1, fluorides 1.8, sulphates 21.5, nitrates 23, lead 123, nickel 175, zinc 1445, cadmium 2.8, chromium 165 and copper 105. The sludge was stored at room temperature until required for the experiments.

Soil

Soil was obtained from an open field, air dried, cleaned and homogenized before use in the experiments. The characteristics are pH 5.45, EC (dS cm^{-1}) 2.85, total N 0.08%, total extractable phosphorus 4.75 mg kg^{-1}, total organic carbon 5.3 g kg^{-1}.

Poultry manure

Poultry manure was obtained from the University of KwaZulu-Natal experimental farm in Pietermaritzburg.

Bulking materials

Wood chips measuring between 3 and 5 cm in length were obtained from the yard of a wood-processing factory in Pietermaritzburg, South Africa. Sawdust was obtained from the same wood-processing factory as the woodchips. Hay was obtained from the University of KwaZulu-Natal experimental farm in Pietermaritzburg.

Table 1. Experimental design for the compost treatments.

Treatment	Oil sludge + soil	Wood chips	Sawdust	Hay	Poultry manure
1	X				
2	X	X			
3	X		X		
4	X			X	
5	X				X
6	X	X			X
7	X		X		X
8	X			X	X

Compost experimental design

Sludge was mixed with soil in a ratio of 3:1 (sludge:soil) (w/w). This mixture was then used to formulate eight treatments labelled 1-8. Treatment 1 contained the sludge/soil mixture only and was designated the control. Treatment 2 contained the sludge/soil mixture and woodchips in a ratio of 1:1 (w/v). Treatment 3 contained the sludge/soil mixture and sawdust in a ratio of 1:1 (w/v). Treatment 4 contained the sludge/soil mixture and sawdust in a ratio of 1:1 (w/v). Treatment 5 contained the sludge/soil mixture and poultry manure in a ratio of 3:1 (w/w). Treatment 6 contained the sludge/soil/woodchips mixture and poultry manure in a ratio of 3:1 (w/w). Treatment 7 contained the sludge/soil/sawdust mixture and poultry manure in a ratio of 3:1 (w/w). Treatment 8 contained the sludge/soil/hay mixture and poultry manure in a ratio of 3:1 (w/w) (Table 1). All experiments were placed on wooden pallets ($1m^2$), which were covered with nylon fibre sheets and incubated for six months. Each experiment was set up in triplicate. Moisture was maintained at between 60 and 70% field capacity. The compost heaps, except the control were turned with a garden fork once a week for aeration.

Analytical procedures

Total petroleum hydrocarbons (TPH) was measured by infrared spectroscopy (IRS) using the EPA method 8440 (USEPA, 1997), PAH concentration by GC/MS and respiration of microorganisms was measured by CO_2 evolution by using the closed jar method. Electrical conductivity, pH, moisture and nutrients were measured by standard methods.

Counts of compost miroorganisms

Counts of culturable compost microorganisms were done by the plate count method for viable cells. 5 g of compost was shaken with 45 ml of 0.28% sodium pyrophosphate for 30 min at 10°C and 150 rpm. Dilutions prepared in 0.9% NaCl were spread onto nutrient agar plates to enumerate aerobic heterotrophs. Counts of heterotrophic microorganisms were done in colony forming units (CFU) monthly for the duration of the experiments.

Isolation and characterisation of microorganisms from compost

The enrichment of organisms was done in mineral salts medium containing per litre KH_2PO_4 5g, MgSO4. $7H_2O$ 5g, NaH_2PO_4. H_2O 5g, NH_4Cl 5g, NaCl 40g. Trace elements solutions containing per litre distilled water; 15 g $FeCl_2.H_2O$, 90 g NaCl, 19.7 g $MnCl_2.4H_2O$, 9 g $CaCl_2$, 23.8 g $CoCl_2.H_2O$, 0.17 g $CuCl_2.H_2O$, 28.7 g $ZnSO_4$, 0.5 g $AlCl_3$, 0.62 g H_3BO_3, 0.24 g $NiCl_2.6H_2O$, 10 ml of 10.18 M HCl (32%). About 15 g of homogenised compost was put in 250 ml Erlenmeyer flasks containing 100 ml sterile MSM spiked with 1 ml crude oil. The flasks were stoppered with cotton wool wrapped in aluminium foil and incubated in the dark at 28°C on a rotary shaker at 150 rpm for 21 days. Sub-cultures were made by aseptically transferring 1 ml of the culture into another set of 250 flasks containing 100 ml sterile MSM spiked with 1 ml crude oil, as the only source of carbon for the bacteria growth and incubated for another 21 days at 28°C in a rotary shaker in the dark. The process was repeated for the third time. Oil degrading bacteria were isolated from serial dilutions (10^{-8}) of the enrichment culture. Aliquots (0.1 ml) of each of the dilutions 10^{-6} to 10^{-8} were inoculated onto mineral salts agar (MSA) plates overlaid with 50 µL of oil filter-sterilised through 0.2 µm Millipore filter membrane and incubated for 21-28 days at 28°C and checked daily for bacteria growth to avoid overcrowding of the plates. The enrichment and isolation procedures were adapted from Atagana (2003) and Mashreghi and Marialigeti (2005). Distinct colonies from the MSA plates were inoculated onto nutrient agar plates and incubated for three days at 28°C. Pure colonies were obtained from the selected colonies further purified by serial dilution and streaking to obtain single colonies.

Genomic DNA extraction of the bacteria isolates

Genomic DNA was extracted using cetyltrimethyl ammonium bromide (CTAB) method. The samples were centrifuged and the pellets were suspended in a solution containing 567 µl tris ethylene diamine tetraactic acid buffer (tris EDTA), 30 µl 10% sodium dodecyl sulphate (SDS), 3 µl of proteinase K (20 mg/ml) and incubated in a digital dry bath incubator (Accu block™) at 65°C for 1 h. Then 180 µl 5 M NaCl and 80 µl 10% CTAB solutions were added and incubated at 65°C for 10 min. Then 400 ml each of phenol and chloroform were added to each tube. The tubes were centrifuged at 14000 rpm for 15 min and 300 µl of the supernatants was transferred into new sterile Eppendorf tubes and the DNA was precipitated by adding 0.6 volume of cold isopropanol to the tubes. The DNA pellets were collected by spinning at 14000 rpm for 15 min and washing with 200 µl 70% ethanol and spinning at 14000 rpm for 10 min. The DNA pellets were then air-dried and 100 µl TE buffer was added and incubated at 37°C for 60 min to dissolve the DNA pellets. Then 1 µl of RNAase was added to remove bacterial RNA and incubated at 37°C for 60 min. The DNA template was separated by electrophoresis with ethidium bromide (0.1 µg/ml)-stained 1% agarose gel running at 80 V for 60 min, using TAE electrophoresis buffer.

Polymerease chain reaction (PCR) and sequencing

The PCR was performed using MJ Mini thermal cycler (Bio-Rad,

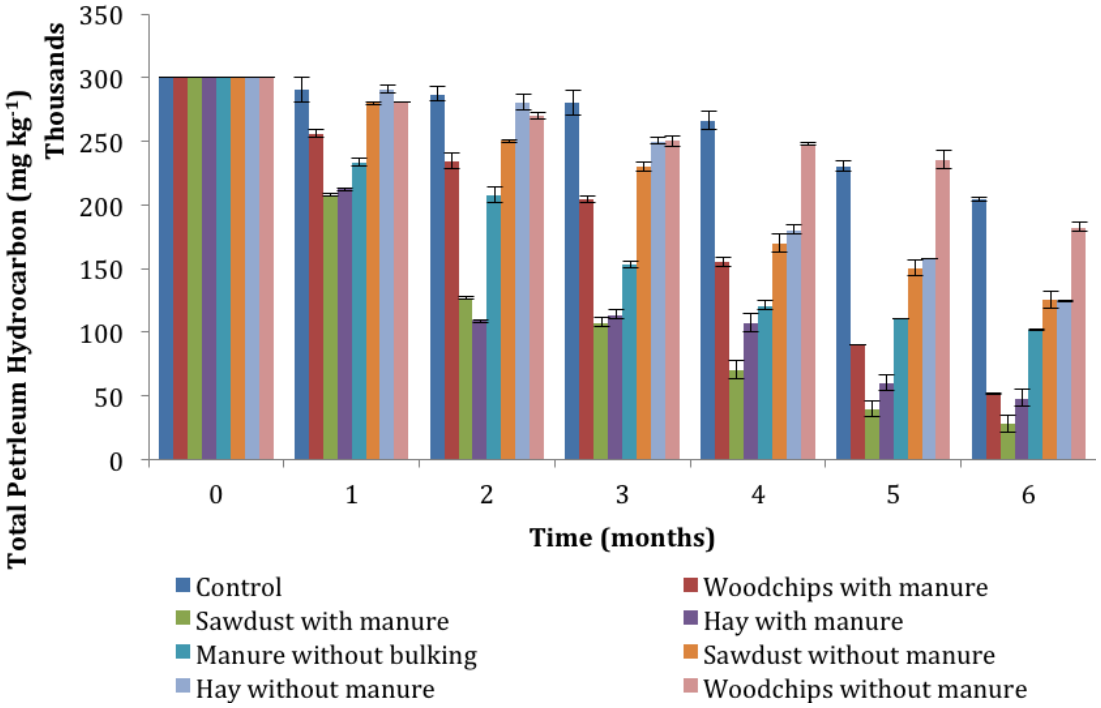

Figure 1. Changes in total petroleum hydrocarbons (TPH) (mg kg^{-1}) during composting. Values are means of three ± standard deviation.

Hercules, CA, USA). The PCR products were separated electrophoretically with ethidium bromide (0.1 µg/ml)-stained 1% agarose gel running at 80 V for 60 min, using TAE electrophoresis buffer. The PCR products (20 µl each) were cleaned by using 160 µl 13% polyethylene glycol (PEG) 8000, 20 µl 5 M NaCl solution and 200 µl 70% ethanol and sequenced.

Basic local alignment search tool (BLASTing) of DNA sequences

The sequences of 16S rDNA region obtained were edited using BioEdit software. The edited sequences were copied in a fasta format. Blasting was done on National Centre for Biotechnology Information (NCBI) website. This was to check and compare the sequences with those on the database (Adeleke et al., 2012).

RESULTS AND DISCUSSION

TPH was reduced in all compost experiments including the control during the six months of incubation. Reduction in TPH in the manure amended sawdust compost progressed more rapidly, reaching 30% reduction in the first month of incubation, as compared to 14 and 29% in the manure amended woodchips and hay compost respectively (Figure 1). Reduction in the non-manure experiments with bulking was not significantly different from the control at p =0.05 at the end of the first month of incubation. After the first month, reduction in the manure amended sawdust and hay composts progressed rapidly and continued to do so for the rest of the incubation period. However, the manure amended sawdust compost continued to show lager reduction in TPH than the hay compost during the last three months of the composting period. The manure amended woodchips compost was slower in TPH reduction compared to sawdust and hay composts, despite the higher aeration probably due to slow rate of breakdown of wood material used for bulking. Poultry manure clearly enhance the reduction in TPH. This is possibly due to the nutrient composition of the manure, particularly the high nitrogen content, which is believed to have offset the initial nitrogen deficiency in the sludge-compost mixture (Table 2). Microorganisms involved in the metabolism of the carbon substrate present in the compost require adequate amounts of nitrogen, which was low in the sludge-compost. Thus the addition of poultry manure provided additional nitrogen, microorganisms and other nutrients to facilitate the early microbial actitiy observed in the compost.

The experiment amended with manure but without bulking showed comparable TPH reduction in the first month but gradually became slower probably due to the lack of bulking, which may have hindered aeration. At the end of the composting period, the experiments amended with manure showed the following TPH reduction: Sawdust 91%, hay 84%, woodchips 82% and the experiment amended with manure but no bulking 66%, as compared to the control, which showed a reduction of 32%. It is evident that inoculation of the compost with

Table 2. Changes in C:N ratio during composting. Values are means of three.

Composts treatment	Time (month)						
	0	1	2	3	4	5	6
Control	100:3	100:10	100:15	100:18	100:21	100:23	100:25
Woodchips without manure	100:3	100:10	100:15	100:16	100:20	100:21	100:24
Sawdust without manure	100:3	100:10	100:15	100:18	100:20	100:21	100:24
Hay without manure	100:3	100:11	100:12	100:16	100:18	100:22	100:25
Manure without bulking	100:5	100:15	100:20	100:20	100:31	100:28	100:28
Woodchips with manure	100:5	100:10	100:18	100:22	100:25	100:27	100:31
Sawdust with manure	100:23	100:27	100:30	100:37	100:35	100:37	100:41
Hay with manure	100:15	100:25	100:29	100:32	100:35	100:43	100:39

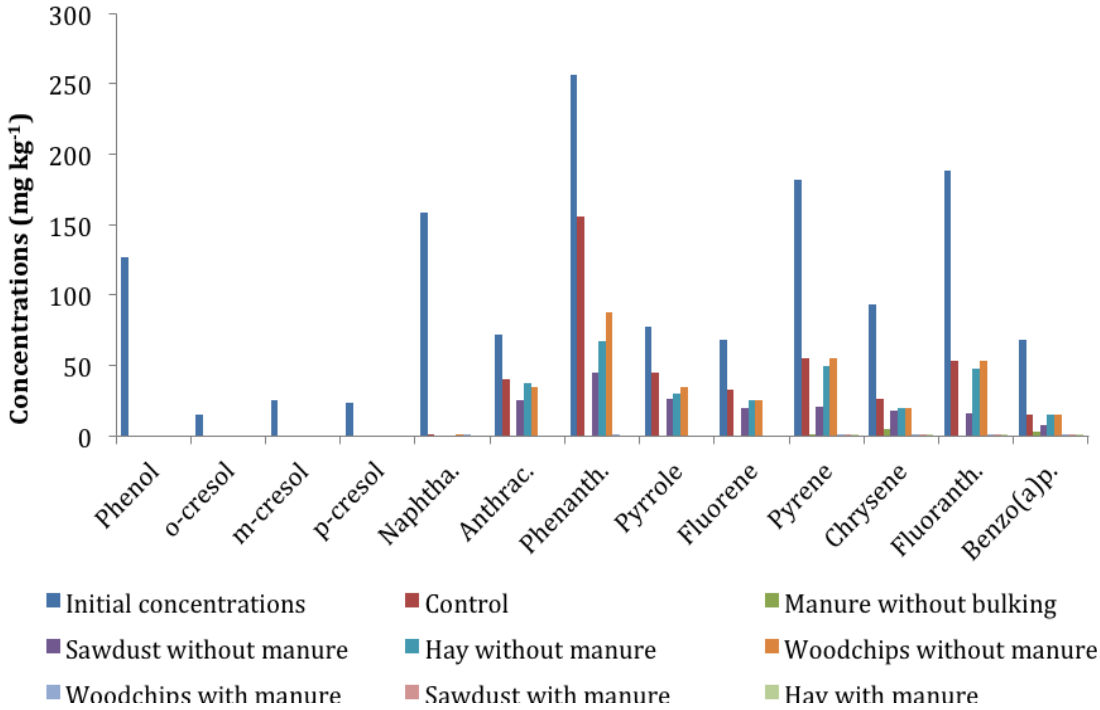

Figure 2. Final concentrations (mg kg^{-1}) of PAHs in compost. Values are means of three ± standard deviation.

poultry manure enhanced the rate of oil sludge degradation in the compost systems. The experiments containing bulking materials but no manure showed a reduction of between 39 and 58%. This is an indication that the bulking materials enhanced aeration, which was vital for the oxidation of the compounds present in the oil sludge. The bulking enhanced aearation, helped to maintain aerobic degradation of the sludge. However, the physical and chemical properties of the bulking agent played a role in enhancing degradation. The small particulate materials of the sawdust and hay, which provided large surface area for microbial attack degraded more rapidly compared to the woodchips. This microbial degradation of the bulking agent offered additional nutrient that allowed the co-metabolisation of the sludge.

Results of analyses of selected PAHs showed that the phenols and the low molecular weight PAHs were removed in all the experiments. However, the high molecular weight PAHs continued to remain till the end of the incubation period in the experiments that did not contain manure and also in the control experiments (Figure 2). This again is a clear indication that the presence of the manure was vital for the degradation of the oil sludge components.

The level of reduction in TPH and PAH concentrations in the experiments correlates with the level of microbial

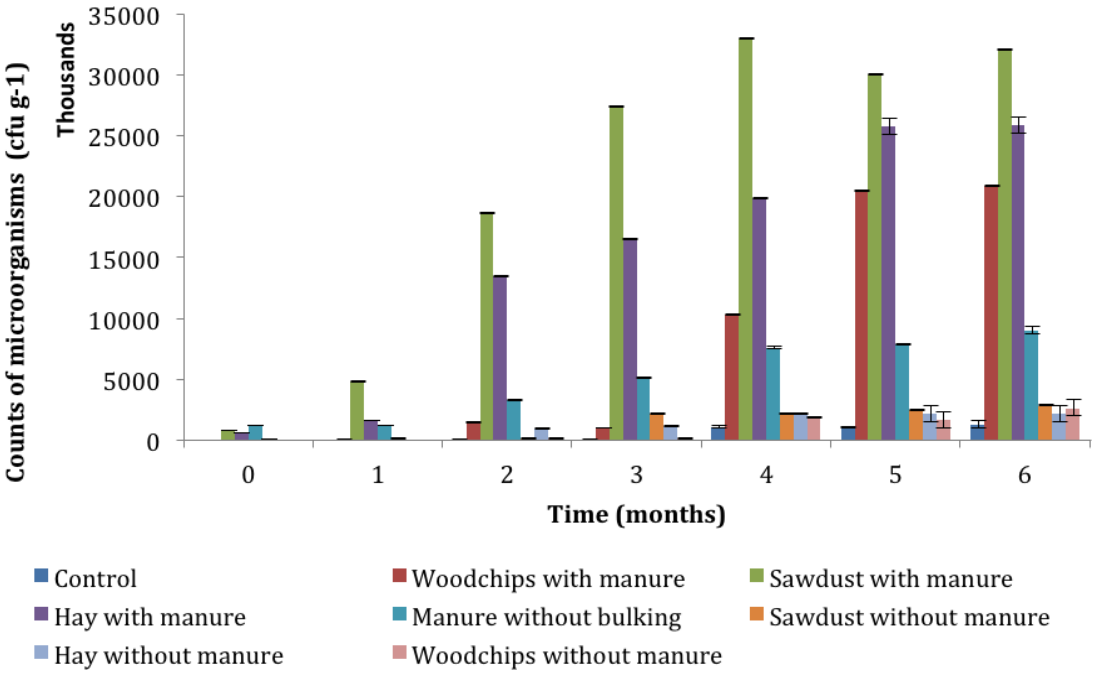

Figure 3. Counts of heterotrophic microorganisms (CFU g^{-1}) in compost. Values are means of three ± standard deviation.

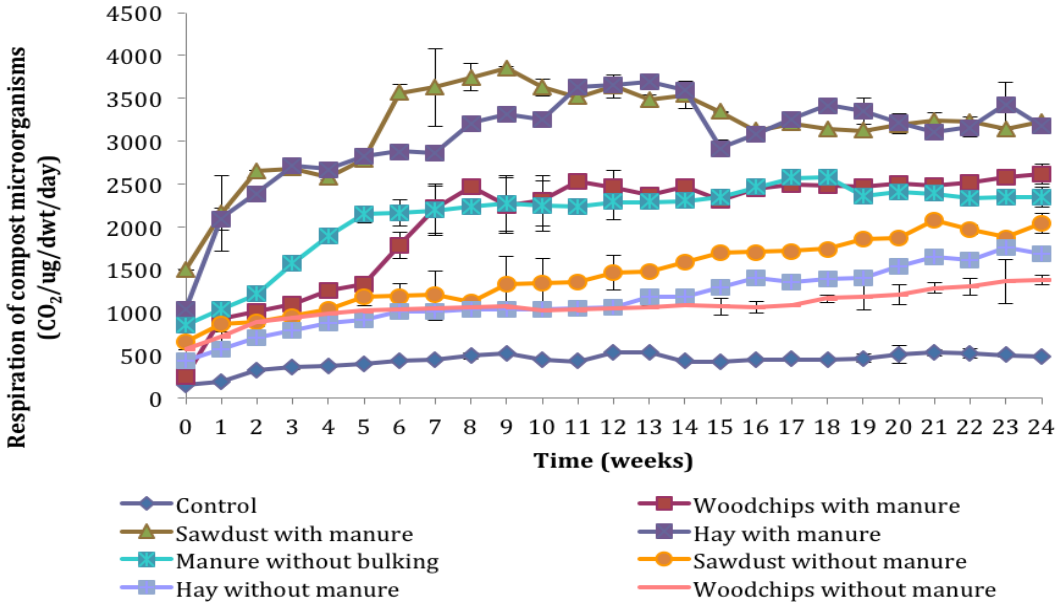

Figure 4. Respiration (CO_2/µg/dwt/day) of compost microorganisms during composting. Values are means of three ± standard deviation.

acrtivities in the compost. The sawdust + manure compost showed a much higher microbial activity, as shown in the counts of heterotrphic organisms (Figure 3) and the respiration of compost organisms (Figure 4). Although, there was no significant difference between the respiration of organisms in the hay and sawdust composts, microbial counts were consistently higher in the sawdust than in the hay. These high levels of microbial activities observed in the composts are attributed to the initial elevated microbial load supplied by the poultry manure and

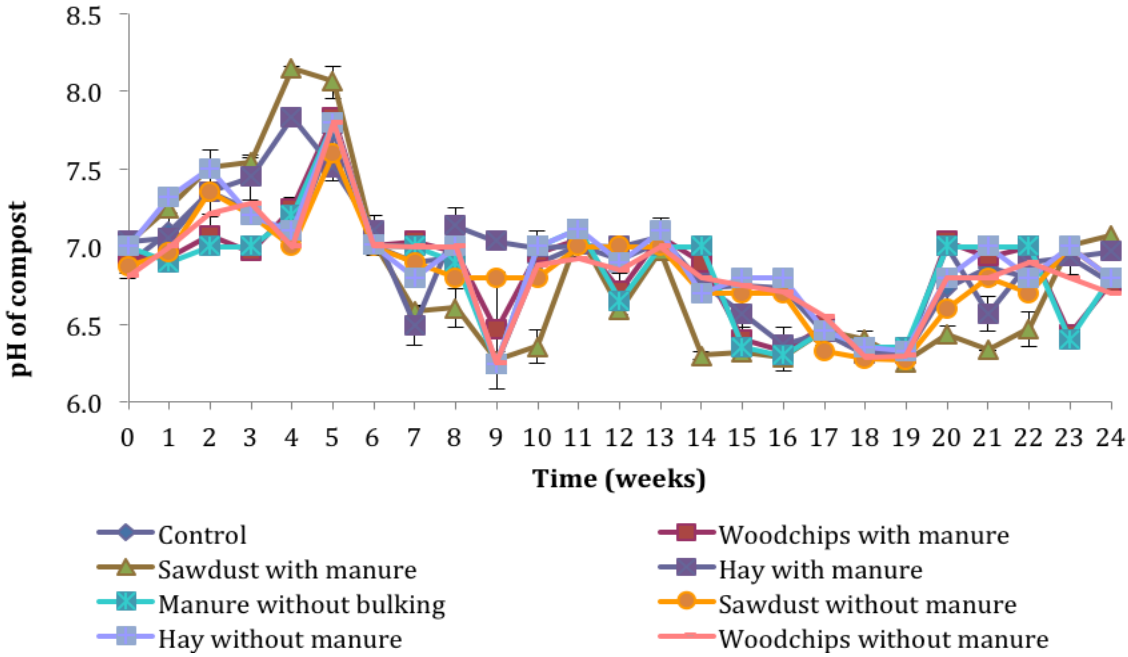

Figure 5. pH of compost during incubation. Values are means of three ± standard deviation.

the aeration, which was supplied by turning the compost heaps weekly. This can be seen from the low levels of activities in the control experiments, which showed low microbial activities throughout the incubation period.

The initial high levels of TPH was expected to impede the activity of compost microorganisms but this did not seem to happen probably due to the diluting effect of the bulking materials and the amount of soil used in the preparation of the compost. This can also be attributed to the available carbon and nutrients provided by the compost system as well as the initial large population of microorganisms. Compost systems have been reported to enhance the degradation of a number xenobiotic compounds including hydrocarbon compounds (Giles et al., 2001; Atagana, 2003). However the reduction of TPH by compost organisms at the levels used in this experiment is remarkable and worth investigating further.

The counts of compost inhabiting microorganisms (Figure 3) and the results of the respiration of microorganisms (Figure 4) supports the large amounts of reduction in TPH. Although relatively lower microbial activities were shown by the woodchip compost when compared with the sawdust and hay experiments, reduction in TPH in the woodchip compost is comparable with the latter two. This may be due to the type of organisms present in the woodchip compost, which may consist of lignin degrading organisms that are known to adapt readily to hydrocarbon substrates (D'Annibale et al., 2006; Marin et al., 2006; Atagana, 2009). The pH of the compost systems rose sharply in the first four weeks of incubation reaching 8.1 in the sawdust compost and 7.8 in the hay and woodchips in the fifth week (Figure 5). The pH lowered to below 7 in most experiments and remained largely below 6.5 for the rest of the experimental period. The drop in pH to slightly acidic range is an indication of the posible production acidic metabolites. However, this did not affect the activities of compost microorganisms. It is assumed that the majority of the microorganisms present are acid tolerant, as some of these are known to be hydrocarbon and and lignin degraders.

Changes in C:N ratio of the compost matrix reflects the reduction in TPH (Table 2). The ratio increased with time as the TPH lowered. This can only be attributed to the lowering of carbon source due to the degradation activities. The changes in electrical conductivity (EC) were not significant (Table 3). This is an indication that the system was stable during the composting period. Stable electrical conditions, when conducive for microbial activities promote microbial growth and subsequently enhance degradation of substrates. This is also associated with the relatively stable pH observed after week five during the incubation period.

The high thermophilic temperatures (63°C) recorded during the incubation period (Figure 6) is an indication of high metabolic activities. The drop in temperature to about 35°C for more than half of the incubation period could be attributed to aeration an activity, which was carried out by turning of the compost heaps and the regular watering. This lowering of temperature was probably instrumental to the high and stable microbial population observed in the compost matrix.

Some of the bacteria identified in the compost include the following: *Arthrobacter* spp., *Bacillus aryabhattai*,

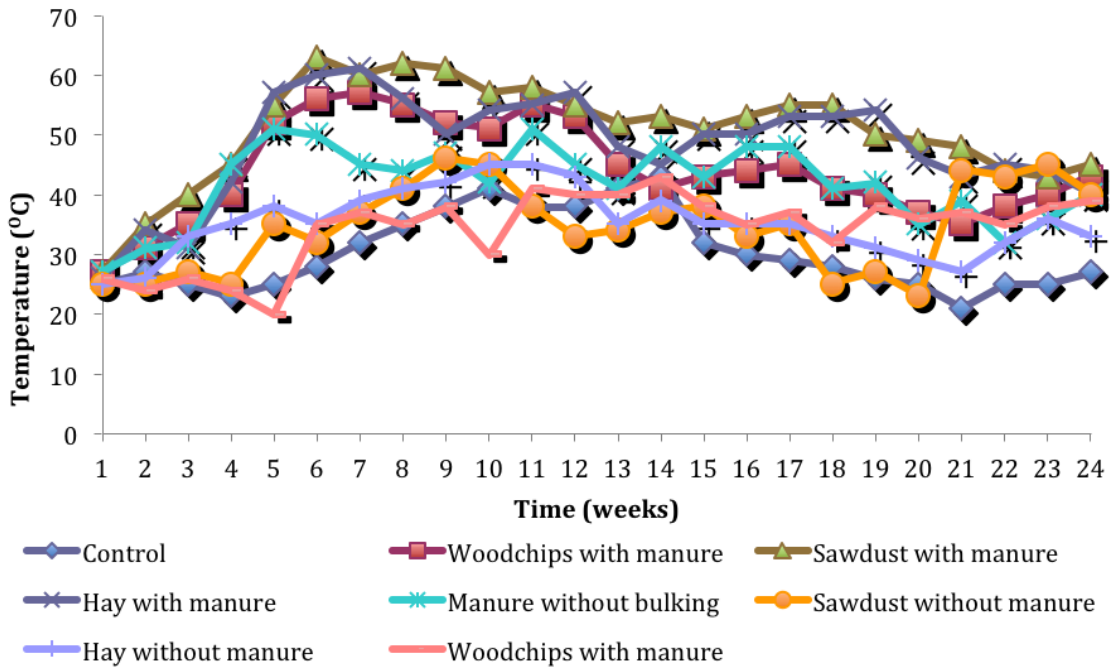

Figure 6. Changes in temperature (°C) of compost. Values are means of three ± standard deviation.

Table 3. Electrical conductivity (dS cm^{-1}) of compost system during incubation. Values are means of three.

Composts treatment	Time (month)						
	0	1	2	3	4	5	6
Control	0.90	0.91	0.95	1.50	1.00	0.92	1.10
Woodchips without manure	0.93	0.93	0.93	0.95	1.50	1.10	1.20
Sawdust without manure	0.95	09.96	1.05	1.80	1.60	1.20	1.40
Hay without manure	0.93	0.95	1.01	1.80	1.50	1.30	1.80
Manure without bulking	1.68	1.90	2.10	2.20	2.10	2.25	2.50
Woodchips with manure	0.95	1.20	1.25	2.50	2.7	2.55	2.75
Sawdust with manure	2.15	2.20	2.35	2.50	2.65	2.80	3.15
Hay with manure	2.40	2.25	2.35	2.75	2.80	2.80	3.20

Bacillus Licheniforms., *Bacillus subtilis, Bacillus* spp., *Brevibacterium frigoritolerans, Corynebacterium* spp., *Geobacillus* spp., *Norcadia* spp., *Paenibacillus* spp. *Pseudomonas spp., Ralstonia* spp., *Rodococcus* spp., *Staphylococcus* spp., *Staphylococcus* spp., *Staphylococcus saprophyticus*, *Streptomuyces* spp., *Thermobifida* spp., *Thermomonospora* spp., *Variovorax* spp.

Although, *Bacillus, Pseudomonas, Arthrobacter* and *Staphylococcus* species were dominat in all the treatments at all temperature regimes during the period of incubation, all other genera were present in all the treatments at all times except for *Thermobifida* spp., *Thermomonospora* spp., *Geobacillus spp*. and *Norcadia* spp., which were not encountered in the two months of incubation. While the absense of *Thermobifidia* and *Thermomonospora* in the first two months could be justified, as they are thermophiles and the temperature of the composts only reached thermophilic stages in the second month of incubation, the absence and appearance of *Geobacillus and Norcadia* at the later stages of the incubation could not be explained. *Thermobifidia* and *Thermomonospora* were not present in the control experiments and this could be attributed to the temperature of the control experiments which did not exceed the mesophilic range at any time during the experiment. The abundance of these organisms in most of the compost piles including the control is an indication of the ecology of the genera, being predominantly soil borne. The organisms identified in this study are known

to inhabit hydrocarbon contaminated soils and utilise oil as soul carbon source (Das and Mukherjee, 2007; Bayoumi, 2009; Koukkou et al., 2009).

Conclusions

The results from these experiments have shown that the different parameters operating in the compost affect the overal functioning of the compost system and consequently the degradation of the hydrocarbon compound present. While the bulking materials, woodchips, sawdust and hay aided initial aeration in the compost, they also provided addition substrate and nutrient for the organisms to grow on, which in turn enhanced the overal degradation of the high concentrations of oil sludge present in the compost matrix within reasonable timeframe. The results have further showed that sawdust can compost faster than hay and woodchips to provide the necessary supplementary carbon substrate that is required by the microbial population to co-metabolise the sludge hydrocarbons. The results suggests that despite the rapid increase in temperature the population of the organisms present in the compost system continued to increase and additional organisms added over time. Aeration through periodic turning of the compost and watering proved to be advantageous in keeping the temperature stable and maintaining a viable population of microorganisms.

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Full Length Research Paper

Optimization of ethanol production from food waste hydrolysate by co-culture of *Zymomonas mobilis* and *Candida shehatae* under non-sterile condition

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A complete conversion of the hexose and pentose sugars in the food wastes hydrolysates (FWH) to ethanol is a prerequisite for maximizing the profitability of an industrial process for bioethanol production. Response surface methodology (RSM) was employed to optimize the effects of nitrogen source [$(NH_4)_2SO_4$], phosphorus source (KH_2PO_4), yeast extract and inoculum size on ethanol production from FWH by co-culture of *Zymomonas mobilis* and *Candida shehatae* under non-sterile condition. The optimal conditions for ethanol production were 1.15 g/L of $(NH_4)_2SO_4$, 0.95 g/L of KH_2PO_4, 1.38 g/L of yeast extract and 14.75%v/v of inoculum. The results indicate that the most significant parameters affecting ethanol production from FWH by co-culture under non-sterile condition was yeast extract. Ethanol production of 77.6 g/L obtained under optimized condition was 56% increased as compared with the use of raw FWH (34 g/L) and was in good agreement with the value predicted by quadratic model (79.98 g/L), thereby confirming its validity. Ethanol yield of FWH in batch fermentation by co-culture was 0.15 g-ethanol/g-food waste (77.6 g/L), which was 94.6% of the theoretical yield while *Z. mobilis* alone yielded 0.11 g-ethanol/g-food waste (54.2 g/L) and *C. shehatae* alone yielded 0.09 g-ethanol/g-food waste (48 g/L). Ethanol production from FWH in 1-L fermentor by co-culture also gave ethanol yield of 0.16 g-ethanol/g-food waste (78.8 g/L) which was 96% of the theoretical yield. Despite of being a waste, an ethanol yield of 0.16 g-ethanol/g-food waste demonstrated the potential of food waste as a promising biomass resource for ethanol production.

Key words: Co-culture, food waste hydrolysates, non-sterile fermentation, response surface methodology, optimization.

INTRODUCTION

Food waste is a kind of organic solid waste with higher percentage of moisture, and it is usually discharged from restaurants, kitchens and cafeterias (Wang et al., 2004). Generally, municipal solid waste includes approximately 35 to 40% organic waste, of which the dominating fraction is kitchen waste (Uncu and Cekmecelioglu, 2011) whereas, the amount of food waste generated in Thailand is approximately 600,000 kg/day, accounting for 80 to 90% of total municipal solid waste (PCD, 1994). The disposal of food waste became a major concern in Thailand when the direct animal feeding of food wastes was banned completely by Thailand government in 2005

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Abbreviations: CCD, Central composite design; **FWH,** food waste hydrolysates; **RSM,** response surface methodology; **SS,** suspended solid; **VSS,** volatile suspended solid; **COD,** chemical oxygen demand; **HPLC,** high performance liquid chromatograph; **ANOVA,** analysis of variance; **TISTR,** Thailand Institute of Scientific and Technological Research.

due to the uncertainty with regard to the safety of its utilization as animal feed (Moon et al., 2009). Currently, these food wastes are disposed of by various methods such as land-filling, incineration, and recovery or recycle. Most of the food wastes are land-filled, causing ground water contamination. In addition, landfill space is limited and uncontrolled fermentation of organic wastes in landfill causes emission of greenhouse gases, such as methane and carbon dioxide (Camobreco et al., 1999), moreover, it is difficult to find new sites and the leachate generated by these materials require secondary wastewater treatments (Masaaki et al., 2008). Hence, food waste management has been an important issue for protecting the environment as well as for conserving natural resources.

Starch and cellulose materials are the major components of the food waste. It also contains some protein materials. The starch and cellulosic components of the food waste can be hydrolyzed to monomeric sugars. The sugars then can be used as substrates in the ethanol fermentative production (McMillan, 1997). But there is little information on the research of the utilization of food wastes for ethanol production. The bioethanol industry has developed rapidly in recent years to cope with the depletion of fossil fuel. Because of its environmental benefits, bioethanol is regarded as a promising biofuel substitute for gasoline in the transportation sector. It can be produced from a variety of raw materials containing fermentable sugars. The utilizations of edible starch material, such as corn and cassava for bioethanol production have caused undue pressure on the global food supply (Kim and Dale, 2004; Katz, 2008). Therefore, it is essential to research alternative and inexpensive substrate for ethanol production at a reduced cost (Hahn-Hägerdal et al., 2006).

Materials unsuitable for human consumption are considered ideal substrates for bioethanol production such as food wastes. For instance, bread residues can be fermented to get the ethanol yield of around 0.35 g/g substrate (Ebrahimi et al., 2007). Wilkins et al. (2007) reported that the citrus peel waste can undergo steam explosion process to remove the D-limonene and subsequently can be consumed by the *Saccharomyces cerevisiae* to get ethanol yield of around 0.33% (v/v). Reports also exist on the production of ethanol by fermentation of fresh kitchen garbage using *S. cerevisiae* as inoculum (Tang et al., 2008; Wang et al., 2008). Open fermentation of ethanol production has various merits compared with conventional sterile and closed-system fermentation. The non-sterile open fermentation of food waste could be carried out on-site at localized storage sites before collection to centralized processing plants. Furthermore, autoclave process could cause bad effect on desired product, such as degradation of substrate sugars and other nutritional elements. Some negative reactions would also take place, such as the Maillard reaction; it could cause decreases in the amounts of functionally useful sugars and amino, and increase the production of unfavorable furfural compounds, which inhibited bacterial growth (Akao et al., 2007; Sakai and Yamanami, 2006).

Research on ethanol production from starch by open fermentation had been carried out successfully (Tao et al., 2005). If the open fermentation could be done on food waste to produce ethanol, a lot of energy and cost would be saved. The starch and cellulosic components of the food waste can be hydrolyzed to monomeric sugars, which composed mainly of mixture of glucose and xylose. A complete and efficient conversion of these hexose and pentose sugars present in the food wastes hydrolysates to ethanol is a prerequisite for maximizing the profitability of an industrial process for bioethanol production (Vanmaris et al., 2006). Since there is no wild type microorganism that could efficiently accomplish this process, the utilization of two microorganisms and the construction of genetically modified biocatalysts have been two common approaches. The bacterium *Zymomonas mobilis* is known for better ethanol productivity and tolerance compared to *S. cerevisiae* (Davis et al., 2006), it has rarely been employed in such a co-culture process. A sequential culture of *Z. mobilis* and *Pachysolen tannophilus* has been previously reported (Fu and Peiris, 2008). Co-culture of *Z. mobilis* and *Pichia stipitis* for efficient ethanol production on glucose/xylose mixtures are also reported (Fu et al., 2009). Bansal and Singh (2003) reported a comparative study of ethanol production from molasses using *S. cerevisiae* and *Z. mobilis*. However, no study has been reported on the co-culture *Z. mobilis* and *Candida shehatae* on ethanol production from glucose/xylose mixtures substrates.

Recently, many statistical experimental design methods have been employed in bioprocess optimization. Response Surface Methodology (RSM) is one such scientific approach that is useful for developing, improving and optimizing processes and is used to analyze the effects of several independent variables on the system response. This method has been successfully applied to optimize alcoholic fermentation process (Castillo et al., 1982; Ratnam et al., 2003). The present study reports for the first time the new strain combination of *Z. mobilis* and *C. shehatae* for ethanol production from food wastes hydrolysates and to determine the optimum level of fermentation variables, nitrogen source [$(NH_4)_2SO_4$], phosphorus source (KH_2PO_4), yeast extract and inoculum size for ethanol production under non-sterilized condition using RSM.

MATERIALS AND METHODS

Microorganisms

Z. mobilis TISTR0548 and *C. shehatae* TISTR5843 were obtained from the Thailand Institute of Scientific and Technological Research (TISTR) culture collection. Cultures were maintained on agar plates

Table 1. Characteristics of food waste hydrolysate.

Parameter	Concentration (g/L)
Total carbohydrate	232
Total reducing sugar	164
Total nitrogen	6.5
Ammonium–nitrogen	0.45
Total phosphorus	643
Oil	10.6
Total solids	323
Volatile solid	261
Glucose	111
Xylose	23
Sucrose	21
Lactic acid	2.3
Formic acid	1.5
pH	4.2

at 4°C with subculture to fresh media every 2 weeks. Glucose agar for *Z. mobilis* consisted of 20 g/L glucose, 10 g/L yeast extract, 1 g/L KH_2PO_4, 1 g/L $MgCl_2$, 1 g/L $(NH_4)_2SO_4$, 15 g/L agar and pH 6.0. Xylose agar for *C. shehatae* was as previously described (Sreekumar et al., 1999). Inoculum medium consisted of 10 g/L yeast extract, 1 g/L $MgCl_2$, 1 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, with 20 g/L glucose for *Z. mobilis* and 20 g/L xylose for *C. shehatae*. Media were sterilized at 121°C for 15 min and sugars were separately autoclaved from yeast extract and inorganic salts solution. The strains were grown at a temperature of 35°C and cultured for 48 h before being used in the fermentation.

Food waste hydrolysate (FWH)

The food waste was collected from Tong-Song municipal waste management plant, Nakhonsrithammarat Province, Southern Thailand. Food waste was fermented by fungi from Look-Pang for 24 h. It was mixed with water at ratio 1:1 (v/v) and crushed into small particles using liquidizer. It was subsequently incubated at 55°C for 12 h and then hydrolysate was generated richly in sugar content (164 g/L) (Table 1).

Inoculum preparation

Inoculum size was expressed as volume of inoculum medium that the cells were from to volume of fermentation medium that the cells were inoculated into. Cells from the corresponding volume of inoculum medium were firstly centrifuged to exclude the inoculum medium and concentrate the cells, and then the cell pellets were re-suspended with the sterilized yeast extract and inorganic salts solution to be inoculated into each flask. All inocula were incubated in 250 ml conical flasks with 25 ml of inoculum medium at 30°C, with a 24 h stationary incubation for *Z. mobilis* and a 36 to 48 h shaking incubation at 150 rpm for *C. shehatae*. Multiple flasks were simultaneously cultured to get the desirable volume of inoculum medium and subculture up to three times was carried out to ensure that all inoculum flasks contained an identical culture.

Ethanol production

RSM batch fermentation

FWH was neutralized with 1 N NaOH adjusting the pH to 5.0, and then FWH without sterilization was used as a fermentation medium. The nitrogen source $[(NH_4)_2SO_4]$, phosphorus source (KH_2PO_4) and yeast extract were added into FWH according to Table 2 varying between 0.5 to 2.0, 0.5 to 2.0 and 0.3 to 2.0 g/L, respectively. Batch experiments were carried out in a series of 250 ml Erlenmeyer flasks, containing 100 ml of FWH. 5 to 25% inoculum culture was dispensed to each flask. The flasks were shaken at 180 rpm in a thermostat controlled incubating shaker at temperature of 35°C for 72 h. Experiments were carried out in triplicate for all the runs and the average values were subjected to model analysis. Besides, as a statistical measure, six experiments were conducted at the center point to check for any error. For confirmation of optimization conditions, fermentations were done in 1-L fermentor (BIOFLO 3000, New Brunswick Scientific, Edison, NJ, USA) with pH monitoring. Fermentation parameters and FWH components were carried out with the optimized conditions. Fermentation studies were done with *Z. mobilis* alone, *C. shehatae* alone and a mixed culture of both. Time course experiment was also done. Reproducibility of the process was checked in repeat runs with the aforementioned conditions.

Experimental design and data analysis

A central composite experimental design was used to optimize the nitrogen source (X_1), phosphorus source (X_2), yeast extract (X_3) and inoculum size (X_4) on ethanol production from FWH. Ethanol production was used as dependent output variables. 21 experiments were performed in triplicate according to Table 2 to optimize the parameters. A quadratic model (Box et al., 1978) was used to evaluate the optimization of environmental factors as the following equation (equation 1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 + \beta_{11} X^2_1 + \beta_{22} X^2_2 + \beta_{33} X^2_3 + \beta_{44} X^2_4 \quad (1)$$

Where, Y = predicted response; X_1, X_2, X_3 and X_4 = parameters; β_0 = offset term; β_1, β_2, β_3 and β_4 = linear coefficients; β_{11}, $\beta 22$, β_{33} and β_{44} = squared coefficients; and β_{12}, β_{23}, β_{13}, β_{14}, β_{24} and β_{34} = interaction coefficients.

The response variable (Y) was fitted using a predictive polynomial quadratic equation in order to correlate the response variable to the independent variables (Lay, 2000). The Y values were regressed with respect to nitrogen source, phosphorus source, yeast extract and inoculum size. Design expert software version 6.0 (Stat-Ease. Inc., MN, USA) was used for regression and graphical analysis of the experimental data obtained. The optimum levels of the selected variables were obtained by solving the regression equation and by analyzing the response surface contour and surface plots. The quality of the fit of quadratic model was expressed by the coefficient of determination R^2, and its statistically significance was checked by the *t*-test in the same program.

Analytical methods

Ten milliliters of fermentation broth was centrifuged at 5000 rpm for 30 min at 4°C and the supernatant was used to determine the ethanol, reducing sugars concentration and soluble end products. Fermentation end products (volatile fatty acids and ethanol), lactic acid, formic acid, xylose, fructose, sucrose and glucose were analyzed with a high performance liquid chromatograph (HPLC; Agilent 1200 series), equipped with Aminex® HPX-87H ion exclusion column (Hniman et al., 2011). Oil concentration and pH were determined in accordance with the standard methods (Clescerl et al., 1998). Total nitrogen, ammonium-nitrogen, total

Table 2. Experimental design for ethanol production from food waste hydrolysate using RSM.

Run	$X_1(NH_4)_2SO_4$ (g/L)	$X_2KH_2PO_4$(g/L)	X_3yeast extract (g/L)	x_4Inoculum size (%)	Ethanol production (g/L)
1	0.5	2	2	20	31
2	2	0.5	2	20	48
3	1.25	1.25	1.15	12.5	79
4	2	2	2	5	31.78
5	1.25	1.25	1.15	20	69.94
6	2	0.5	0.3	20	56.75
7	0.5	0.5	0.3	5	33.56
8	1.25	1.25	1.15	12.5	79.45
9	0.5	2	0.3	20	38
10	1.25	1.25	1.15	12.5	79.5
11	1.25	1.25	1.15	12.5	79.2
12	1.25	0.5	1.15	12.5	76.02
13	1.25	1.25	1.15	12.5	79.6
14	2	2	0.3	5	22.7
15	2	1.25	1.15	12.5	64.97
16	1.25	1.25	2	12.5	67.67
17	0.5	0.5	2	5	20.43
18	1.25	1.25	1.15	5	60.86
19	1.25	1.25	0.3	12.5	74.48
20	0.5	1.25	1.15	12.5	65.4
21	1.25	2	1.15	12.5	74.48

phosphorus and phosphate concentration were analyzed using commercial test kits from Spectroquant (Merck Ltd., Germany). The total carbohydrate in FWH was analyzed using anthrone method (Morris, 1948). The reducing sugars concentration in FWH and fermentation broth was assayed by the Somogyi-Nelson method (Somogyi, 1952).

RESULTS AND DISCUSSION

Optimization conditions for ethanol production

Food waste is an important municipal waste and mainly composed of carbohydrate. Hydrolysis of food waste by microbial digestion (Look-Pang) generated hydrolysates with high concentration of reducing sugar (164 g/L). Food waste hydrolysate (FWH) characterized abundance in nutrition (Table 1). Ethanol production from food waste was analyzed as low-cost feedstock by co-culture of *Z. mobilis* and *C. shehatae* fermentation. In the present paper, a central composite design (CCD) of response surface methodology (RSM) has been used to optimize conditions for transforming FWH to ethanol by co-culture of *Z. mobilis* and *C. shehatae* under non sterilized condition. Sreekumar et al. (1999) has proved that the most important chemical factors, which affected the ethanol production were the nitrogen source, phosphorus source, yeast extract and inoculums size. To evaluate the effect of each parameter on ethanol production from FWH by co-culture, 21 experiments were conducted according to the CCD method. Table 2 shows the actual parameters and concentration of ethanol. The maximum ethanol concentration was 79.5 g/L, corresponding to ethanol yield of 0.158 g-ethanol/g-food waste revealed 96.8% of the theoretical yield (theoretical yield of ethanol was calculated by the equations used by other researchers (Keating et al., 2004). The results from this study helped to frame a second order polynomial equation (Equation 2) that relates the ethanol concentration (Y) to the concentrations of $(NH_4)_2SO_4$ (X_1), KH_2PO_4 (X_2), yeast extract (X_3) and inoculums size (X_4).

The regression coefficients and significance levels are given in Table 3. This equation was used to predict the ethanol concentration at optimum condition. Although, the model showed a satisfactory explanation (R^2 = 0.99), not all the effects of factors and their interactions on ethanol concentration were significant ($P < 0.01$). Thus, the ethanol concentration was adequately explained by the model equation (Equation 2). Table 3 illustrates the main effect of each variable upon ethanol production (Y). Yeast extract (X_3) and inoculum size (X_4) showed a significant positive effect for ethanol production; whereas, K_2HPO_4 (X_2) and $(NH_4)_2SO_4$ (X_1) had only positive effect on ethanol production. The significant positive variables (X3 and X4) with confidence levels at 99% ($P < 0.001$) were considered as significant variables in model for ethanol prediction. X_1 and X_2 variables should not be used in equation 2 for individual variable prediction but could be used for interaction variables:

Table 3. Summary of model coefficient estimate by multiples linear regression.

Relationship	Factor	Coefficient	F value	Prob >F
Model	-	-	-	<0.0001
	Intercept	-48.2	-	-
Main effects (linear)	X_1	62.5	-0.34	0.7445
	X_2	34.9	-1.22	0.2674
	X_3	18.4	-9.45	<0.0001
	X_4	7.8	7.21	<0.0001
Interactions (pure quadratic)	X_1^2	-27	-27.21	<0.0001
	X_2^2	-9.1	-9.16	<0.0001
	X_3^2	-12.9	-16.65	<0.0001
	X_4^2	-0.3	-26.8	<0.0001
Interactions (cross product)	X_1X_2	-6.4	-5.14	0.0021
	X_1X_3	4	8.12	<0.0001
	X_1X_4	0.7	5.17	0.0021
	X_2X_3	4.7	9.51	<0.0001
	X_2X_4	-0.8	-6.74	0.0005
	X_3X_4	0.2	-4.64	<0.0001
Lack of fit	-	-	1.26	0.4039

*Coefficient of determination (R^2) of this model was 0.97.

$$Y_{ethanol} = -41.2 + 62.5X_1 + 34.9X_2 + 18.4X_3 + 7.8X_4 - 6.4X_1X_2 + 4X_1X_3 + 0.7X_1X_4 + 4.7X_2X_3 - 0.8X_2X_4 - 0.2X_3X_4 - 27X_1^2 - 9.1X_2^2 - 12.9X_3^2 - 0.3X_4^2 \quad (2)$$

Each item in the regression model (Equation 2) has an identified effect on the ethanol concentration. F value can be used to quantify the intensity of parameters on the ethanol concentration, while P values signify the pattern of interaction among the parameters. The larger the value of F value and the smaller the value of P, the more significant is the corresponding coefficient term (Douglas, 2001). The regression coefficients and F value and P values for all the linear, quadratic and interaction effects of the parameters are given in Table 3. A positive sign in the F value indicated a synergistic effect, while a negative sign represented an antagonistic effect of the parameters on the ethanol concentration. The significant of the regression coefficients of the model, indicating that yeast extract (X_3) and inoculum size (X_4) had highly positive effect on ethanol production ($P<0.001$). The effect of the interaction of nitrogen source and yeast extract, interaction of phosphorus source and yeast extract, and interaction of inoculum size and yeast extract were significant ($P<0.001$). Besides, the quadratic terms (X_1X_2, X_2X_2, X_1X_3, X_1X_4, X_2X_3 and X_3X_4) showed the synergistic effects on the ethanol concentration, typically (X_1X_3, X_3X_4 and X_2X_3) ($P < 0.001$). Surface and contour plots demonstrating the effects of different parameters, two parameters varied while keeping the third parameters at middle level, on the ethanol concentration were shown in Figure 1. The stationary points were examined by analyzing these plots. Generally, circular contour plots indicate that the interactions between parameters are negligible. On the contrary, elliptical ones indicate the evidence of the interactions (Muralidhar et al., 2003). From the plots, it was easy and convenient to understand the interactions between two nutrients and also to locate the optimum levels. Figure 1A showed the effect of $(NH_4)_2SO_4$ (X_1) and yeast extract (X_3) on the ethanol concentration. The convex response surface suggested well-defined optimum variables [$(NH_4)_2SO_4$ and yeast extract].

The ethanol concentration increased to the peak with the increasing of yeast extract and $(NH_4)_2SO_4$ to 0.9 and 1.44 g/L, respectively; then declined with the further increase of these two parameters. Figure 1B shows the effect of yeast extract (X_3) and K_2HPO_4 (X_2) on ethanol production. The equation demonstrated that interaction between yeast extract and KH_2PO_4 showed highly significance. At the middle concentration of yeast extract (0.9 g/L) and middle concentration of K_2HPO_4 (0.93 g/L) gave maximum ethanol production (79.5 g/L), a further increase in concentration of yeast extract and K_2HPO_4, the trend was reversed. In a relative low concentration yeast extract and K_2HPO_4, optimum ethanol production could be attained. Figure 1C showed the effect of inoculum size (X_4) and yeast extract (X_3) on the ethanol concentration. The convex response surface suggested

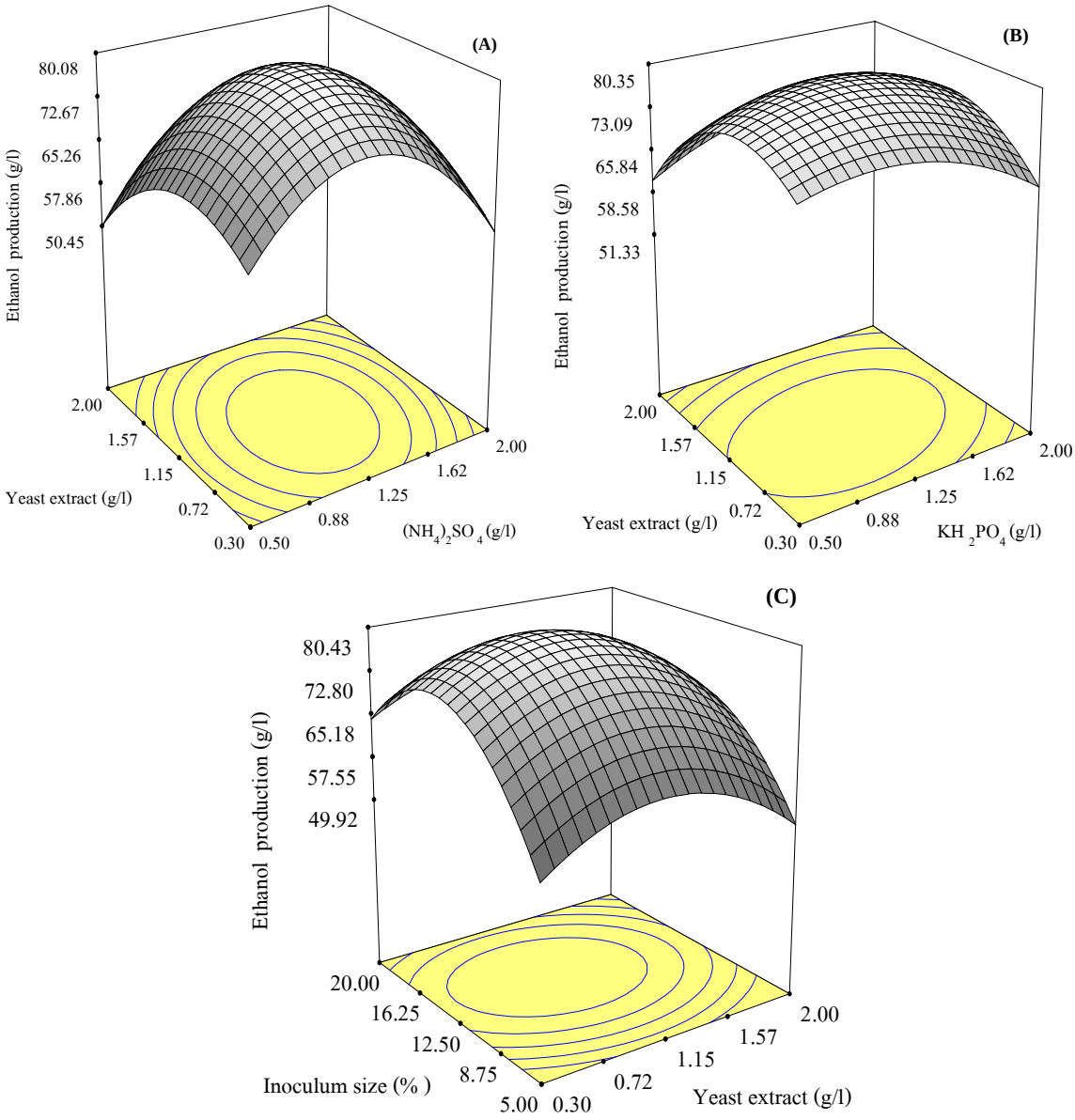

Figure 1. Response surface plot of ethanol production (Y) from FWH under non sterile condition. **A)** The effect of nitrogen source and yeast extract. **B)** The effect of phosphorus source and yeast extract. **C)** The effect of inoculum size and yeast extract on ethanol production.

well-defined optimum variables (inoculum size and yeast extract) and that the ethanol concentration increased to the peak with the increase of inoculum size and yeast extract up to 15% and 0.9 g/L, respectively; then declined with the further increase of these two parameters. This result demonstrated that the response surface had a maximum point for ethanol production. The contour plot described by the model Y is represented in Figure 1, which shows that the maximum concentration of ethanol was 79.5 g/L. The optimal concentration obtained from the maximum point of the model was 1.15 g/L $(NH_4)_2SO_4$, 0.95 g/L KH_2PO_4, 1.38 g/ yeast extract and 14.75%v/v inoculums size.

Ethanol yield in batch fermentation using co-culture was predicted at 0.16 g-ethanol/g-food waste (79.98 g/L), which was 97% of the theoretical yield. The ethanol production increased by 57.2% as compared with the use of raw food waste hydrolysates (34 g/L).

Model validation and confirmation

To confirm the validity of the statistical experimental strategies and gain a better understanding of ethanol production from FWH, a confirmation experiment with triplicate set was performed at the specified optimum

Table 4. Comparison of ethanol fermentation among individual and mixed strains fermentation in 250 flask and 1-L fermentor.

Parameter	250 ml flask fermentation			1-L fermentor
	Z. mobilis	*C. shehatae*	*Z. mobilis + C. shehatae*	*Z. mobilis + C. shehatae*
Ethanol production (g/L)	54.2	48	77.6	78.8
Ethanol yield (g-ethanol/ g-food waste)	0.11	0.09	0.15	0.16
Ethanol yield (g-ethanol/ g-reducing sugar)	0.33	0.29	0.47	0.48
Theoretical ethanol yield (%)	65	58.6	94.6	96

condition representing the maximum point of the concentration of ethanol. Experiments conducted at the optimum condition [1.15 g/L $(NH_4)_2SO_4$, 0.95 g/L KH_2PO_4, 1.38 g/ yeast extract and 14.75%v/v inoculums size] demonstrated that the ethanol concentration (77.6 g/L) was closer to the predicted value (79.98 g/L). This is improvement of concentration by 57.2% relative to that obtained from raw food waste hydrolysate. The good correlation between predicted and experimental values after optimization justified the validity of the response model and the existence of an optimum point. Corresponding to the ethanol concentration of 77.6 g/L, the ethanol yield was calculated as 0.15 g-ethanol/g-food waste. This showed that the model was useful to predict the ethanol concentration as well as optimize the experimental conditions. *Z. mobilis* alone yielded 0.11 g-ethanol/g-food waste (54.2 g/L), which is 65% of the theoretical yield and *C. shehatae* alone yielded 0.09 g-ethanol/g-food waste (48 g/L), reaching more than the published value for *C. tropicalis* with starch (Nellaiah et al., 1988).

Scale up experiment

The process scaled up with FWH had shown higher ethanol yield than batch fermentation (Table 4). Ethanol yield in batch fermentation using mixed culture was 0.15 g-ethanol/g-food waste in 72 h, which was 94.6% of the theoretical yield. Ethanol yield by *Z. mobilis* alone was 0.11 g-ethanol/g-food waste (54.2 g/L), which is 65% of the theoretical yield and *C. shehatae* alone yielded 0.09 g-ethanol/g-food waste (48 g/L) which was 58.6% of theoretical yield in 72 h (Table 4). However, in the 1 L fermentor using mixed culture, the ethanol yield was 0.16 g-ethanol/g-food waste (78.8 g/L) which was 96% of the theoretical yield. Reproducibility of the process was checked in repeat runs with the aforementioned conditions.

Conclusions

A significant improvement in ethanol yield (0.48 g/g-reducing sugar) was demonstrated, resulting in very low sugar and fewer by-products. CCD design have shown that yeast extract and inoculum size are the key parameters that influence ethanol production from FWH, while $(NH_4)_2SO_4$ and KH_2PO_4 showing a little effect. Maximum ethanol concentration of 79.98 g/L was obtained at the optimum condition of 1.15 g/L $(NH_4)_2SO_4$, 0.95 g/L KH_2PO_4, 1.38 g/L yeast extract and 14.75 %v/v inoculums size. The ethanol concentration at the optimum experimental condition (77.6 g/L) agreed well with the predicted one (79.5 g/L). This indicated the suitability of the model employed and the success of RSM to optimize the conditions of ethanol production from FWH. The ethanol yield was reproduced in 1 L fermentor with 0.16 g-ethanol/g-food waste (78.8 g/L) which was 96% of the theoretical yield. The results from the investigation showed that FWH can be used as an alternative substrate for ethanol production, in comparison to virgin biomass resources such as energy-rich crops, if sterilized suitably prior to fermentation by some low cost energy sources such as excess heat or waste heat from some industrial processes adjacent to ethanol production facility.

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Full Length Research Paper

Optimization of aqueous extraction process to enhance the production of phytase by *Rhizopus oryzae* using response surface methodology coupled with artificial neural network

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Aqueous extraction process was optimized to reduce endotoxins from mixed substrate (1:1) for further phytase production by *Rhizopus oryzae* in solid state fermentation. 2^3 factorial design of experiment was combined with either a back-propagation artificial neural network (ANN) or the response surface methodology (RSM) for optimizing the process variables (length of extraction time, substrate loading and different pH of extraction solvent) to predict and simulate phytase production and phosphorus release. ANN was found to be a more powerful tool than RSM, for modeling and optimizing variables for the aqueous extraction process and can be used for predictive simulations of a process. A 2.37-fold increase in phytase production (37.65 U/gds) was achieved at the model predicted optimum concentration of extraction time of 29.78 min, substrate loading at 11.04 g and pH of extraction solvent at 7.1 as compared to the phytase yield in untreated substrate (15.91 U/gds). Moreover, the reduction in phytic acid after aqueous extraction of substrates was validated after high performance liquid chromatography (HPLC) characterization study. The results suggest that aqueous extraction process can be used efficiently for reducing the endogenous anti-nutritional factors from substrates eventually leading to enhanced phytase yield.

Key words: *Rhizopus oryzae*, high performance liquid chromatography (HPLC), phytic acid, solid state fermentation, optimization.

INTRODUCTION

Oil cakes/meals and agricultural by-products (various cereal brans and husks) have long been considered as the most inexpensive and high energy rich substrates for fermentation industry. Effective utilization of these residues not only helps to curb looming environmental pollution due to its disposal but also paves the way for solid waste management and minimizes the initial capital costs for the production processes. Linseed meal is a byproduct of linseed cold-pressing. The solid residue is used as a protein supplement and contains more Total Digestible Nutrients (TDN, 72%) than wheat bran (64%). Combination of these two substrates, with final moisture content of about 40%, was suggested to be an economic alternative substrate for phytase production by solid-state fermentation (Rani and Ghosh, 2011). Linseed and its byproducts have also been practiced in the animal feed industry and as the nutraceutical food for human due to its unlimited potential in reducing the risk of several diseases (Omah and Mazza, 1998; Ridges et al., 2001). Despite being a rich source of dietary proteins and fibres,

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presence of various endogenous antinutrients such as phytic acid, linamarin (a cyanogen) and linatine (an antipyridoxine factor) in linseed meal is the important factor limiting its use as value added substrate for economical production of industrial enzymes and as feed supplement at higher levels (>3%). Phytic acid (D-myo-inositol 1, 2, 3, 4, 5, 6 hexakisphosphate) is the principle source of phosphorus present in plant feedstuffs (Lott et al., 2000). Apart from containing a major portion of plant phosphorus, it also acts as a strong chelator, having the ability to form protein and mineral-phytic acid complexes and resulting into reduced protein and mineral bioavailability (Hossain and Jauncey, 1993; Erdman, 1979; Ketola, 1985; NRC, 1993; Spinelli et al., 1983). Phytase (myo-inositol hexakisphosphate phosphohydrolases), which hydrolyzes the phytate, helps in preserving the non-renewable phosphate source by replacing the lavish supplementation of additional phosphates into animal diets to meet their nutritional requirements. The negative effects of various anti-nutritional factors can be reduced by addition of exogenous phytase or by the removal of phytic acid from the oilseed meals and wheat bran by the use of appropriate feed processing techniques (Boutwell, 1917; Beleia et al., 1993; Han, 1988; Prendergast et al., 1994). For instance, Klosterman et al. (1967) showed that the linatine is a polar compound and can be extracted with water.

Similar results have also been found where the nutritive value of linseed meal was markedly improved by soaking the meal for 18 h prior to drying to remove HCN and phytic acid (Hossain and Jauncey, 1990). However, with the exception of this study, no comparative studies have been conducted to date, so as to optimize the process conditions for the reduction of phytic acid in linseed meal and its subsequent utilization for phytase production to ascertain the applicability of the aqueous extraction as well as the phytase production processes. Response surface methodology (RSM) has been thoroughly used as an optimization technique in a wide range of biotechnology applications including optimization of bioprocesses and enzyme production from microorganisms. Nowadays, many researchers have shifted towards artificial neural networks (ANN). The architecture of a multi-layered ANN, consisting of highly interconnected neurons, weights and biases, is normally composed of an input layer, a hidden layer and an output layer (Rumelhart et al., 1986). It offers an alternative to the RSM and can replace the quadratic polynomial models for solving regression problems in process modeling. In the present study, RSM and ANN were used to investigate the interdependence of the process parameters and models for the reduction of phytic acid in terms of phosphate release from mixed substrate (linseed meal + wheat bran, 1:1) for phytase production by *R. oryzae* in solid state fermentation.

To the best of our knowledge, there are no studies dealing with comparative analysis of RSM and ANN modeling techniques for the optimization of aqueous extraction process in substrate treatment and its affect on phytase production by *R. oryzae*. Furthermore, the reduction in phytic acid in the substrate was demonstrated by using an efficient and reproducible high performance liquid chromatography (HPLC) method.

MATERIALS AND METHODS

Microorganism and chemicals

Strain MTCC 1987 of *R. oryzae* was procured from microbial type culture collection (MTCC), Chandigarh, India. All chemicals were of analytical grade and at the highest purity, procured from Hi-Media laboratories P. Ltd., Mumbai, India. Agro-industrial by-products such as wheat bran (WB) and linseed oil cake (LOC) were purchased from local retail feedstuff outlets in Roorkee.

Inoculum preparation

The fungal strain was routinely grown on potato dextrose agar (PDA, Himedia, India) slants for 4 days at 30°C. Viable spores from slants were harvested by washing with 0.1% (v/v) Tween 80 (Himedia, India) and the spore suspension adjusted to ~1 × 10^6 cfu/ml (colony forming unit per milliliter) was used as inoculum for subsequent fermentations.

Pretreatment of mixed substrate and phytase production

The aqueous extraction of substrate was carried out to reduce endogenous anti-nutritional factors and to evaluate its effect on phytase production. The experiments were conducted in 250 ml Erlenmeyer flasks containing linseed meal and wheat bran (1:1) as the mixed solid substrate in SSF. The substrate was firstly subjected to a sieving procedure employing mesh-size sieves of 4, 8, 12, 16 and 20 prior to extraction with solvent of different pH. The smallest particles were of ~1.0 mm size, collected from fractions between meshes 16 and 20 (-16, +20), intermediate particles (~1.5 mm) were collected from fractions (-8, +12) and finally, heterogeneous oilcake (0.5 to 5.0 mm) was also used as substrate. Aliquots of mixed substrate (~1.0 mm) were soaked in five times their weight of extraction solvent of different pH (adjusted with HCl/NaOH) and were kept at room temperature for different length of time. The substrates were then filtered and dried at 37°C using an electric oven. The substrate free supernatants obtained were used for estimation of released inorganic phosphate and HCN using standard phytase assay and AOAC analysis method, respectively.

After pre-treatment, the dried substrate was supplemented with 20% (v/w) of mineral solution [(w/w), 0.3% NaCl, 0.3% $MgSO_4.7H_2O$, pH 5.6] with the moisture level adjusted to 40%. Medium sterilized at 121°C for 20 min was inoculated with 20% (v/w) inoculum and fermentation was carried out at 30°C for 96 h. The fermented medium was extracted with Tween 80 [0.1% (v/v)] at 30°C on an orbital shaker at 200 rpm for 1 h. Cell free extract was used for phytase activity assay. All experiments were performed in triplicate.

Phytase assay

Phytase activity was determined by estimating the inorganic phosphate released from sodium phytate (Bae et al., 1999). One unit of phytase is defined as the amount of enzyme required to release 1 nmol of inorganic phosphate (Pi) per second under the standard assay conditions. The phytase yield was expressed as a function of dry substrate weight (U/gds).

Table 1. Design matrix for PBD with coded levels of independent factors.

Run	A	B	C	D	E	F	G	Phytase activity[a] (U/gds)
1	1	1	1	-1	1	-1	-1	14.96
2	-1	1	1	1	-1	1	-1	21.78
3	-1	-1	1	1	1	-1	1	16.08
4	1	-1	-1	1	1	1	-1	12.36
5	-1	1	-1	-1	1	1	1	20.91
6	1	-1	1	-1	-1	1	1	13.54
7	1	1	-1	1	-1	-1	1	15.74
8	-1	-1	-1	-1	-1	-1	-1	16.99

[a]Results represent the mean of three experiments.

Inorganic phosphate and hydrocyanic acid determination

Spectrophotometric quantification of inorganic phosphate was performed according to Bae et al. (1999) in triplicate. HCN in the substrate was determined according to the AOAC method of analysis (1980).

HPLC method

Reduction in phytic acid content was validated by reversed-phase high performance liquid chromatography (RP-HPLC) using Agilent 1200 series (Hewlett Packard, Palo Alto, CA, USA) liquid chromatograph equipped with a variable wavelength detector (VWD 1200) and Agilent XDB eclipse C18 (250 × 4.6 mm) column (Rani and Ghosh, 2011). Phytate (IP6) dissolved in the 100 mM sodium acetate buffer (pH 5.1) was used to calibrate the standard curve. A 100 mM sodium acetate solution (pH 5.1) was used as mobile phase with a flow rate of 1.0 ml/min.

Identification of significant process variables using PBD

The prerequisite for optimization of a process involving multiple inputs is to screen out the most influential inputs to determine the model output. PBD was employed for screening the most significant variables in extraction process influencing the phytase production mostly. Based on single-factor experiment, suitable conditions and their ranges were preliminarily determined. In the present study, seven assigned factors were screened in a total of 8 runs. Dummy variables were introduced into the experiment to estimate the experimental error of an effect. The variables, whose effects were negligible under high and low concentrations, were considered as dummy variables. The detail of the design with the response (phytase activity) is given in Table 1.

Central composite designs (CCD)

To determine the mutual interactions among the selected variables (length of extraction time, substrate loading and different pH of extraction solvent) and their corresponding optimum levels, central-composite design (CCD) of response surface methodology (RSM) was used. A 2^3 factorial design having eight factorial points, six axial points and six replicates at the centre point with a total number of 20 runs was formulated. The details of experimental design with coded and actual levels of each factor are summarized in Table 2. A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response to the independent factors. The complete second-order polynomial model (Equation 1) to be fitted to the yield values was:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i=1, i\neq j}^{n-1} \sum_{j=2}^{n} \beta_{ij} x_i x_j \quad (1)$$

Where Y is the observed value of the response (phytase production), x_i (i = 1, 2 and 3) is the controlling factors, b_0 is the offset term, and b_i (i = 1, 2 and 3), b_{ii} and b_{ij} (i = 1, 2 and 3, j = 2 and 3) are the model linear, quadratic and interaction coefficient parameters, respectively.

Artificial neural network (ANN)

The most commonly used network architecture of ANN that is, multilayer feed-forward neural network was used to build predictive models consisting of three inputs (extraction time substrate loading and extraction solvent pH) and two outputs (predicted phytase yield and released phosphate). The method used for the training phase was the back-propagation (BP) based on Levenberg-Marquardt algorithm (LMA) with the aim to fit the outputs of the network to be closer to the desired target and to minimize the performance function in terms of mean squared error (MSE). A backpropagation neural network typically uses sigmoid transfer function and a linear output layer. Therefore, in the present study, the tan sigmoid transfer function, 'tansig' and the 'purelin' transfer function were used for hidden layer and the output layer, respectively. Both input variables and targets were normalized to a range of (-1, 1) before being implemented in the ANN model to avoid any overflows due to very large or very small weights, by using Equation 2:

$$Y = \frac{(Y_{max} - Y_{min})(X - X_{min})}{(X_{max} - X_{min})} + Y_{min} \quad (2)$$

Where Y, Y_{max}, Y_{min}, X, X_{min}, and X_{max} denote the normalized value, maximum value of normalized values (+1), minimum value of normalized values (-1), value of variable, minimum value of variable, and maximum value of the variable, respectively.

The developed topology of ANN was trained several times until the network error (MSE) becomes sufficiently small or equal to the set error goal ($E_0 = 10^{-2}$). After successful termination of training phase of ANN, the predicted model was tested for statistical significance by using analysis of variance (ANOVA).

Table 2. Experimental design used in CCD and ANN with observed and predicted responses.

Run	Time	Substrate loading	pH	Phytase activity (U/gds)			Phosphate released (µmol/ml)		
				Observed	RSM predicted	ANN predicted	Observed	RSM predicted	ANN predicted
1	20(-1)	15(1)	5(-1)	28.34	28.66	29.21	0.184	0.190	0.187
2	20(-1)	15(1)	9(1)	26.57	27.37	26.64	0.106	0.120	0.107
3	30(0)	10(0)	10.36(2)	25.87	25.98	25.86	0.036	0.042	0.035
4	30(0)	18.41(2)	7(0)	31.78	31.39	31.70	0.208	0.207	0.209
5	30(0)	10(0)	7(0)	36.32	36.63	36.37	0.224	0.230	0.229
6	40(1)	5(-1)	5(-1)	25.09	24.25	25.14	0.218	0.230	0.217
7	40(1)	15(1)	5(-1)	25.98	26.04	26.08	0.212	0.220	0.205
8	30(0)	10(0)	7(0)	37.04	36.63	36.37	0.239	0.230	0.229
9	20(-1)	5(-1)	9(1)	25.19	25.09	25.35	0.101	0.096	0.097
10	46.82(2)	10(0)	7(0)	25.67	26.65	25.67	0.166	0.168	0.166
11	30(0)	10(0)	3.64(-2)	24.84	24.78	25.18	0.240	0.220	0.218
12	20(-1)	5(-1)	5(-1)	26.21	26.73	26.18	0.186	0.190	0.187
13	30(0)	10(0)	7(0)	36.79	36.63	36.37	0.207	0.230	0.229
14	30(0)	1.59(-2)	7(0)	27.53	27.97	27.52	0.197	0.192	0.208
15	40(1)	15(1)	9(1)	29.66	29.10	29.58	0.103	0.100	0.102
16	30(0)	10(0)	7(0)	35.98	36.63	36.37	0.226	0.230	0.229
17	40(1)	5(-1)	9(1)	27.32	26.97	27.24	0.095	0.095	0.098
18	30(0)	10(0)	7(0)	37.31	36.63	36.37	0.232	0.230	0.229
19	13.18(-2)	10(0)	7(0)	28.22	27.28	27.89	0.132	0.130	0.136
20	30(0)	10(0)	7(0)	36.34	36.63	36.37	0.230	0.230	0.229

Values in brackets show the corresponding coded values of each factors.

Table 3. Results of PBD analysis.

Factors (code, unit)	Low level (-1)	High level (+1)	SS[a]	Effect	Cont.[b] (%)	t-value
Extraction time (A, min)	20	60	45.89	-4.79	59.92	55.65**
Substrate loading (B, g)	5	10	25.99	3.61	33.94	41.74**
Solvent volume (C, ml)	30	50	0.016	0.09	0.021	1.98
Substrate particle size (D, mm)	1.0	>2.0	0.024	-0.11	0.032	2.23
Rotation of flask (E, rpm)	0	100	1.75	-0.94	2.28	10.69
Solvent pH (F)	3	7	2.90	1.21	3.79	13.92**
Extraction temperature (G,°C)	25	37	0.004	0.05	0.005	0.89

R^2 = 99.94%, R^2 (adj) = 99.86%, R^2 (pred) = 99.59%, coefficient of variation (CV) = 0.74%. [a]Sum of squares;[b]Contribution.

Statistical analyses

The statistical software package 'Design-Expert_8.0.5, Stat-Ease Inc., Minneapolis, MN, USA was used for experimental design and subsequent regression analysis of the experimental data. All experiments were done in triplicate, and the average phytase yield and released inorganic phosphate were taken as the responses. The Neural Network Toolbox V7.13 for MATLAB mathematical software was used for construction of the ANN model.

RESULTS

Selection of significant process variables using PBD

PBD was used for investigating the relative importance of seven independent factors for phytic acid reduction from substrate to be utilized for phytase production. The corresponding effects of these factors on the response (phytase activity) are given in Table 3. From the regression analysis, it was evident that A (extraction time), D (substrate particle size) and E (rotation of flask) enhanced the phytase production at their low level whereas, high level of B (substrate loading), C (solvent volume), F (solvent pH) and G (extraction temperature) supported high phytase yield. Based on analysis of total sum of squares and percent contribution, the most significant factors influencing phytase production were found to be A (extraction time), B (substrate loading) and F (solvent pH), respectively (Table 3). The regression

Table 4. ANOVA analysis for RSM model.

Source of variation	Phytase activity (U/gds)		Phosphate released (µmol/ml)	
	F– value	p–value Prob > F	F– value	p–value Prob > F
Intercept	85.52	< 0.0001	57.55	< 0.0001
A	0.84	0.3798	6.71	0.0269
B	25.07	0.0005	0.39	0.5460
C	3.06	0.1108	307.43	< 0.0001
AB	0.017	0.8977	0.000	1.0000
AC	16.79	0.0022	4.57	0.0582
BC	0.11	0.7484	0.47	0.5108
A^2	298.38	< 0.0001	94.25	< 0.0001
B^2	154.45	< 0.0001	11.45	0.0070
C^2	404.68	< 0.0001	121.06	< 0.0001

model gave a model F-value of 1291.33 with a corresponding model p-value (>F) of 0.0001, which shows the model to be highly significant. Also, the coefficient of determination (R^2) indicates that the model could explain 99.94% of the total variations in the response. A very low value of coefficient of variance (CV, 0.74%) further confirms the reliability of the model.

Predictive modeling using RSM

The experimental design output (Table 2) was analyzed using analysis of variance (ANOVA) which shows that the regression was statistically significant ($p<0.0001$) at 95% of confidence level. The results for ANOVA analysis for RSM for both responses have been summarized in Table 4. Application of multiple regression analysis on the RSM experimental data resulted in the following quadratic model (Equations 3 and 4) explicitly explaining the phytase production and phosphate released as a function of initial values of selected process parameters:

$$\text{Phytase activity } Y_1 = 36.63 - 0.19A + 1.02B + 0.36C - 0.035AB + 1.09AC + 0.088BC - 3.42A^2 - 2.46B^2 - 3.98C^2 \quad (3)$$

$$\text{Phosphate released } Y_2 = 0.23 + 0.008A + 0.002B - 0.054C + 0.000AB - 0.008AC + 0.00275BC - 0.029A^2 - 0.010B^2 - 0.033C^2 \quad (4)$$

Where Y_1 and Y_2 represents phytase activity (U/gds) and phosphate released (µmol/ml), respectively, and A, B and C are the coded factors of extraction time (min), substrate loading (g) and extraction solvent pH, respectively.

In this case, linear term (B), interaction terms (AC) and all the quadratic terms (A^2, B^2 and C^2) were found to be the most significant for phytase production. The statistical significance of the model equation was supported by the model high F-value of 85.52. Correspondingly, ANOVA for phosphate release indicated the F-value to be 57.55, which implied that the model is significant. Again, the quality of fit of the regression model was justified by high value of coefficient of determination (R^2) of 0.9872 and 0.9811, respectively, for responses Y_1 and Y_2, which indicates an excellent correlation between the independent factors. At the same time, the predicted R^2 (correlation coefficient) value of 0.9180 and 0.9073 were found in concordance with the adjusted R^2 value of 0.9756 and 0.9640, respectively, suggesting a strong agreement between the experimental and predicted values of phytase production and phosphate released. The coefficient of variation (CV) indicates the degree of precision with which the treatments are evaluated and a lower value of CV namely, 2.51 and 6.44%, respectively for Y_1 and Y_2, demonstrates that the performed experiments were highly reliable. Furthermore, high values of adequate precision (23.314 and 22.878, respectively, for Y_1 and Y_2) that represents signal (response) to noise (deviation) ratio, indicates an adequate signal and suggested that the model can be used to navigate the design space.

Predictive modeling using ANN

The feed-forward back propagation network used for fitting the same experimental data (Table 2) resulted in an optimum topology of ANN model with 3 inputs, one hidden layer with 4 neurons and 1 output layer involving single neuron in case of both responses (Figure 1). The results of the design of experiments and ANN are given in Table 2. The training phase was carried out for different set of conditions, for instance, number of neurons in the hidden layer, learning rate, random initialization etc. In the present study, the training was stopped after 9 epochs. At the final point of training, the performance function was observed to be 1.3×10^{-2}.

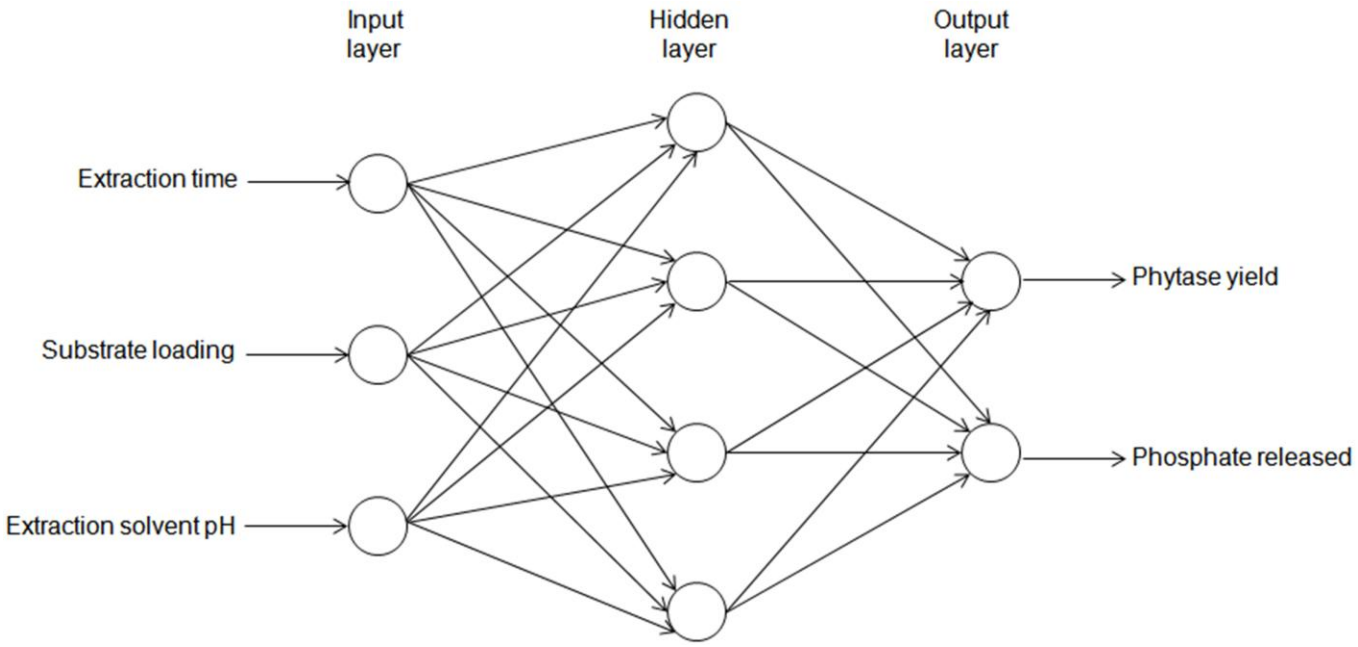

Figure 1. Schematic illustration of the ANN architecture.

Table 5. ANOVA analysis for ANN model.

Source	SS[a]	MS[b]	CV[c] (%)	F-value	p-value	R^2	R^2_{adj}	R^2_{pred}
For phytase yield								
Model	407.16	45.24	1.97	130.93	< 0.0001	0.992	0.984	0.931
Residual	3.46	0.69						
Total	410.62							
For released phosphate								
Model	0.067	7.49e-03	3.17	236.86	< 0.0001	0.995	0.991	0.965
Residual	3.17e-04	3.17e-05						
Total	0.068							

[a]Sum of squares. [b]Mean square; [c]Coefficient of variation.

The performance function (MSE) for training, validation and test data was found to have approached the set goal. The regression R values between the model predicted and experimental phytase yield related to the training, validation, test, and all datasets are illustrated in Figure 2A. The comparable values of MSE and R for each set outputs reveal that the feed-forward-based model possesses good approximation characteristics. Statistical results for the developed ANN model, calculated in a similar way as for the RSM model, have been summarized in Table 5, depicting a significant ANN model that can be used for predictive simulations of aqueous extraction process. The results also showed that the ANN based prediction were found to be more accurate as compared to the RSM model (Figure 2B).

In order to gain the better understanding of the effects of the significant factors on phosphate release and phytase production, the RSM predicted model and trained ANN model were represented as 3D response surface curves shown in Figure 3. The elliptical response surfaces implied that there were perfect interaction between the independent variables, however, the circular surfaces suggested that the optimized values may not vary widely from the single variable conditions. Significant interaction effect between extraction time and pH of extraction solvent for phytase production was predicted by both modeling techniques, however, neural network predicts a sharper ridge surface than RSM (Figure 3A). Figure 3B shows the interaction between extraction time and pH of extraction solvent on release of phosphate.

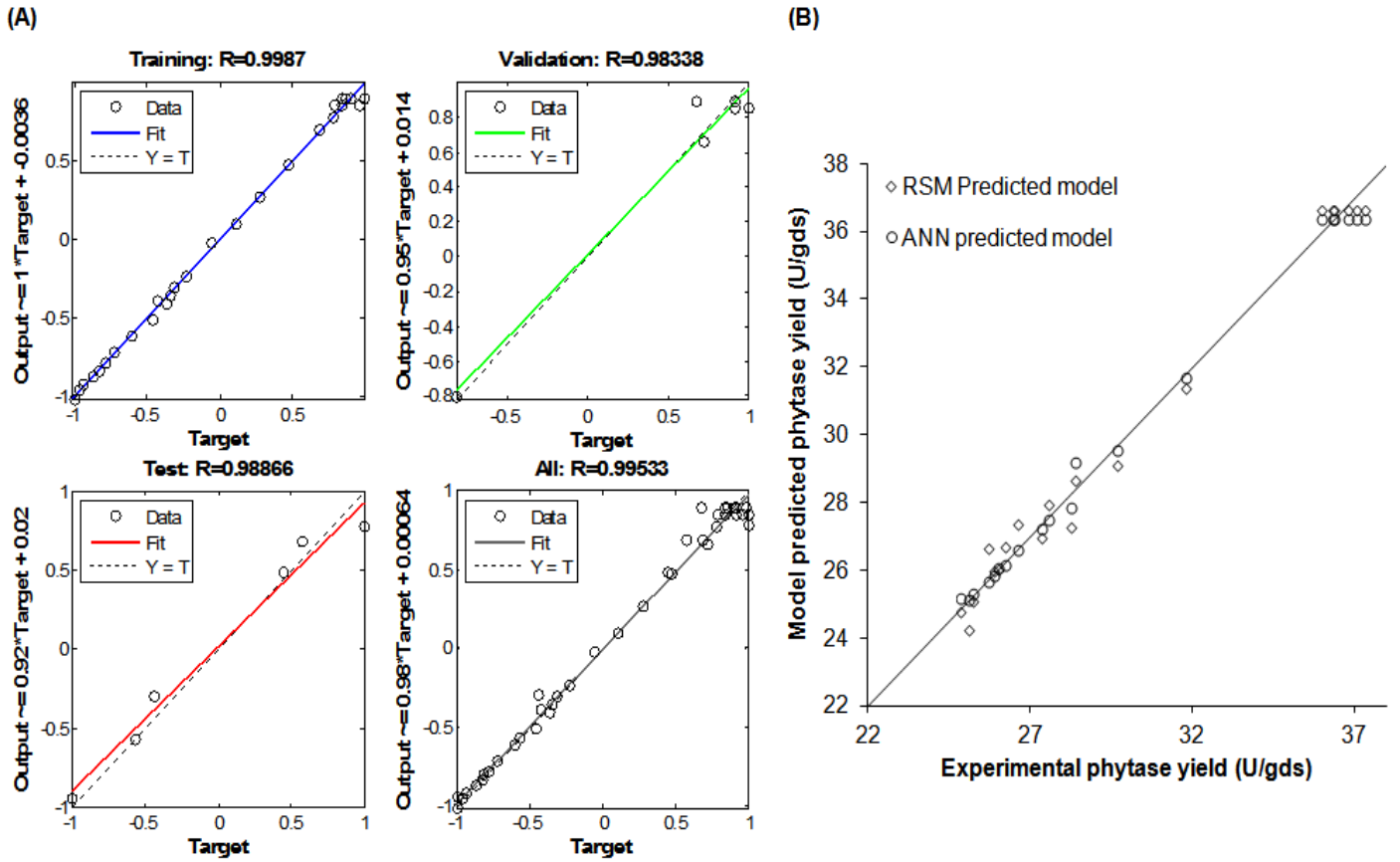

Figure 2. Regression analysis for training, validation, test, and all datasets for phytase production and phosphate released (A) and comparative analysis of RSM and ANN predicted models for phytase yield (B).

Increase in extraction time from 20 to 32 min with solvent of lower pH enhanced the release of phosphate from the substrate. It could be explained that, decreasing solvent pH may enhance the release of phosphate in aqueous extraction process.

As shown in Figure 3C, the interaction of substrate loading and pH of extraction solvent had a much weaker effect on the yield of phytase. The effect of combination of extraction time and substrate loading on the phosphorus release is shown in Figure 3D. It may be observed that increase of extraction time from 20 to 32 min and substrate loading from 5 to 11 g, the release of phosphorus was increasing gradually. How-ever, this interactive effect of extraction time and sub-strate loading on the phosphorus release was not very significant ($p = 0.465$). The maximum phytase production was observed after treating substrate (11.04 g) with extraction solvent adjusted at pH 7.1 for 29.78 min.

Validation of experimental model

The results from validation experiments showed a strong agreement between the maximum predicted response and the experimental response of 36.59 and 37.65 U/gds, respectively, thus supporting the high adequacy of the model. Moreover, the statistical optimization of aqueous extraction process for phytase production resulted in an overall 2.37-fold increase in phytase yield. Reduction in phytic acid content from mixed substrate was validated by the HPLC characterization study. The chromatogram of standard sodium phytate was found to be linearly proportional to the concentrations throughout, with R^2 value and retention time (R_t) of 0.991 and 1.23 ± 0.02 min, respectively, and was found to be in complete agreement with our previous report. Chromatogram profile for untreated and aqueous extracted substrate for phytic acid reduction is illustrated in Figures 4A and 4B, respectively.

DISCUSSION

The ANOVA analysis showed the effect of process variables on each response for both statistical models. Interestingly, the efficiency of each model for both responses was found to be different. The F-value of the RSM predicted model were 85.52 and 57.55 for the phytase

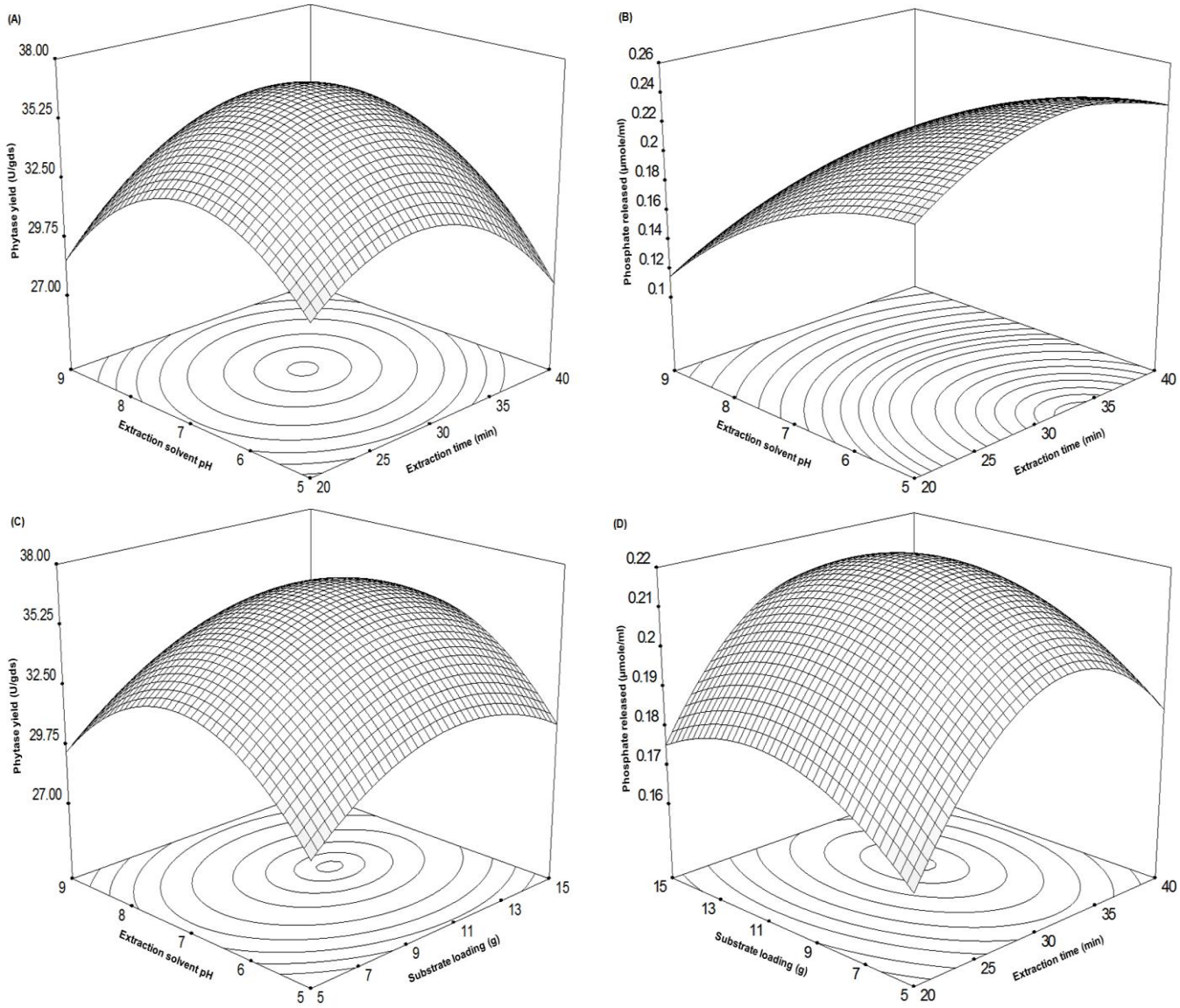

Figure 3. 3D response surface plots showing effect of interactions of, extraction time and extraction solvent pH on phytase production (A), extraction time and extraction solvent pH on phosphate released (B), substrate loading and extraction solvent pH on phytase production (C) and extraction time and substrate loading on phosphate released (D).

yield and phosphate released, respectively (Table 4), whereas, for ANN predicted model, the corresponding values were found to be 130.93 and 236.86, respectively. The comparative study between RSM and ANN, clearly revealed that the ANN based model for the extraction process was superior than the RSM model. Additionally, the experimental values were found to be very close to the ANN predicted theoretical values, which further showed that the ANN model could be used for the process optimization of the detoxification process in water. We conclude that aqueous treatment resulted in the reduction of HCN (data not shown) and inorganic phosphorus concentration and hence, reduction in phytic acid content from the substrate. The results were in full agreement with the findings by Hossain and Jauncey (1990), where a significant reduction in phytic acid along with HCN content was observed, with higher reduction efficiency in water extracted linseed meal as compared to the heat treated. Furthermore, an increasing trend in release of inorganic phosphate from the substrate treated with the extraction solvent at lower pH and for longer time, with no further increase in phytase yield suggested that initial inorganic phosphate concentration in the substrate is well correlated with the phytase production in SSF.

This result is in good conformity with the previously reported findings (Vats and Banerjee, 2002; Vohra and Satyanarayana, 2003) where, higher concentration of

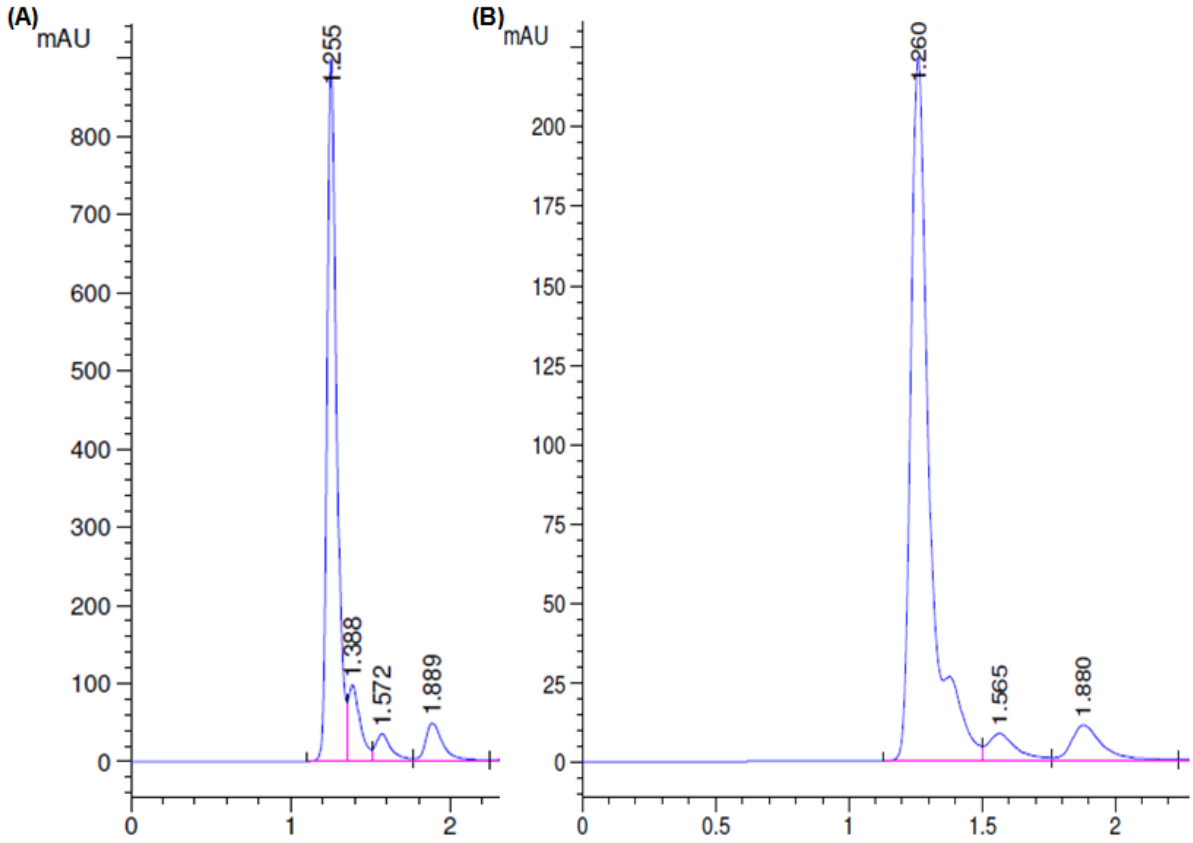

Figure 4. HPLC characterization studies showing phytic acid content in, untreated substrate (A) and aqueous extracted substrate (B).

inorganic phosphates resulted in a repression of phytase synthesis.

Longer extraction time, higher substrate loading and very high/low pH resulted in a significant lower phytase production during SSF. The results clearly suggested that the low inorganic phosphorus substrate stimulates phytase synthesis and excess of inorganic phosphorus causes repression of phytase synthesis, although the presence of traces of inorganic phosphorus is an essential ingredient of phytase production medium and induces its production (Soni and Khire, 2007).

Conclusion

Artificial neural network (ANN) and response surface methodology (RSM) methods were compared for their optimization efficiency in an aqueous extraction process. Both models preformed well and suggested stable responses in envisaging the interactions of the independent process variables and their optimal concentrations with respect to the responses, however, the ANN based approach was found to be more robust and accurate in fitting the computed responses when compared to the RSM based model. Treatment of the substrate prior to fermentation was shown to affect phytase production during solid-state fermentation with an overall increase in phytase yield.

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Toxicity study of diethyl phthalate on *Clarias gariepinus* fingerlings

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Diethyl Phthalate (DEP) is used as a plasticizer, a detergent base, in aerosol sprays, as a perfume binder and after shave lotion. It is known to be a contaminant of fresh water and marine ecosystem. Therefore, a study was designed to determine the acute toxicity effects of DEP on a fresh water fish, *Clarias gariepinus* fingerlings. The fish was treated with 50, 75, 100 and 150 µg/l. DEP was dissolved in distilled water to determine the LC_{50}. There was 100% mortality observed in 150 µg/l. The LC_{50} of DEP was estimated at log toxicant concentration as 2.217, 2.734, 3.435 and 3.931 µg/l at 24, 48, 72, 96 h and 1.871µg/l for the total death. This shows that the impacts are dose and time dependent with respect to marked reduction in mortality rate. At sub-lethal concentrations of the test substance at 30, 40, 60 and 80 µg/l in a renewal bioassay system, the water and the test compound were changed intermittently. One group was maintained as a control in dechlorinated water. There was significant difference ($P < 0.05$) in brain and muscle AchE activity compared to the control. The liver ACP activity was statistically significant ($P < 0.05$) at day 15 while the muscle ACP in other treatment groups showed no significant difference ($P > 0.05$). Liver AST showed no significance in all treated groups ($P > 0.05$) and liver ALT activity was statistically significant ($P < 0.05$) at day 30 only. The haematological parameters (HB, PCV, RBC and WBC) carried out showed that haemoglobin and erythrocyte levels estimated in all treatment groups to the duration of exposure showed no significant difference ($P > 0.05$) compared to the control. The park cell volume showed a significant difference ($P < 0.05$) at day 30 only. The leucocyte count throughout the exposure period showed that the mean values are statistically significant ($P < 0.05$) at day 15 only compared to the control. The mean cell volume (MCV) showed a significant difference at day 15 ($P < 0.05$) whereas mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) showed no significant difference ($P > 0.05$) throughout the exposure period. No significant difference was seen between the lymphocytes and the neutrophils. In day 0 and 15 only, the monocytes and the lymphocytes showed a significant difference ($P < 0.05$). The gill damages indicated toxicity of DEP with raised lamella, oedema of the lamella epithelia, loss of lamellar epithelium, mild oedema and raising of the filament. The liver damage showed focal necrosis and vacuolization, hepatocyte degeneration in the liver. These alterations may have long term effects on that that are continuously exposed to DEP in the aquatic environment.

Key words: Diethyl phthalate, *Clarias gariepinus*, acute toxicity, sublethal toxicity, heamatology, biochemical, histopathology, environment.

INTRODUCTION

Diethyl Phthalate (DEP) is an industrial chemical that originated from a variety of compounds of anthropogenic origin such as pesticides, detergents and plasticizers (Nivedita et al., 2002) used in products such insecticides,

mosquito repellants, camphor substitute, plasticizer for cellulose, bathing soaps, cosmetics, pharmaceutical coatings, after shave-lotion, detergent, esterplastic film and sheets, etc. (Kamrin and Mayor, 1991; Huang et al., 2008). Diethyl phthalate in aquatic environment originates from a variety of compounds of anthropogenic origin such as pesticides, detergents and plasticizers (Fatoki et al., 2010). Many reports have discussed the impact of man-made xenoestrogenic compounds on man and wildlife (Fatoki et al., 2010; Sekizawa et al., 2003).

A study carried out on Puerto Rican girls, for example, revealed that seven samples collected from the girls contained high levels of phthalate esters, which were suspected to be responsible for premature breast development (Colon et al., 2000). It has also been estimated that approximately 1% the phthalate ester content of plastic materials in direct contact with water or other liquids are released into the aquatic environment (Peakall, 1975). Besides, DEP may be discharged from industries as effluent.

The empty DEP containers are washed in freshwater bodies such as rivers and lakes and the container are used for domestic water storage. There is a growing awareness of the critical role, which changes in the blood parameters, biochemicals and tissue damage of fish could play in the assessment of the pollution status in aquatic environment. This is predicted on the fact that blood parameters respond rapidly to changes in water quality (Oluah and Njoku, 2001; Nussey et al., 1995; Van Vuren, 1986).

Vosylienė (1999) noted that changes in the hematological parameters, biochemicals are useful tools in assessment of the physiological status of fish. The use of biochemical and physiological responses in the biological assessment of the environmental impact of chemical substances has increased in the past few years. The present study was conducted to determine the toxic effects of DEP contamination of fresh water through an experiment on 150 fish *Clarias gariepinus*.

MATERIALS AND METHODS

Collection of test organism

One hundred and fifty fingerlings of mean wet weight, 13.13 ± 2.27 g were obtained from Aquafish Limited, Awka, Anambra State, Nigeria, identified using taxanoic key of Reed et al. (1967) and were treated with 0.05% $KMnO_4$ solution for 2 min to avoid any dermal infections. Fish was acclimated for 21 days and fingerlings were randomly distributed into fifteen 25 L glass containers (75 × 45 × 45 cm) filled to 20 L mark with unchlorinated water each containing ten fingerlings. Each container was covered on top with nylon mesh tied firmly around the top of the container with rubber band to prevent the fish from jumping out. Every effort was made to provide optimal conditions for fish; no mortality occurred during this period. Feeding was discontinued 48 h before the commencement of the experiment to minimize the production of water in the test container (Ezekiel and Benedict, 2008). The handling of the test organism conforms to the policy on Animal use by the American College of Toxicology.

Test chemicals

Analytical grade diethyl phthalate (99.97% purity) obtained from Sigma Chemical, Ohio, USA was used in this study. The chemical is usually used in manufacture of insecticides etc.

Range finding test

The exploratory range of concentration of test chemicals was determined with a series of range finding experiment (American Public Health Association, 1998). This was initially conducted using the geometric series of concentration values to identify the highest concentrations that will kill 100% of the test organisms and the least concentration that will have no effect on them. Thereafter, definitive acute toxicity bioassays (continous flow-through system) were conducted by exposing fish to different concentrations of DEP.

Test solutions

Stock solution was prepared by dissolving 1 g of DEP in 1000 ml of distilled deionized water because DEP is soluble in water and serial dilutions made from which different concentrations (0, 50, 75, 100 and 150 µg/L) corresponding to the treatments were made. Water quality of the experimental tank was determined according to standard procedures (APHA, 1998).

Acute toxicity tests

Acute toxicity test were conducted according to standard proce-0dures (American Society for Testing of Materials, 19990). The fish were monitored for 96 h and any mortality, morphological, behavioral changes and mortality were recorded. The definitive test was conducted using concentrations (0, 50, 75, 100 and 150 µg/L) of DEP earlier determined for the range finding test. Mean mortality from a particular dose and its replicate was calculated. A fish was considered dead when it did not respond after prodding with a glass rod; dead fish were removed from the tank immediately. Fish behavior was also observed during exposures. Fish were not fed during the experimentation period as recommended by Ward and Parrish (1982) and Reish and Oshida (1987). To ensure proper oxygenation of water throughout the experimentation period, during experimentation, it was observed that an enhanced concentration of particular test chemicals was required as a lethal dose in flow-through systems as compared with static tanks. As such flow-through system posseses many advantages over static tanks, the toxicity in flow-through system, however, provides chemical parameters consistency, which is not achievable in static. A continous-flow system not subject to build up of metabolites and depletion of toxicants clearly represents a more appropriate model of an open system in nature than does a static culture (Porcella, 1969; Davis, 1978). Flow-through toxicity tests are especially important for fish (Solbe, 1974; Spraque, 1964).

Mortality

Mortality was recorded at an interval of 24 h over a period of 4 days (96 h). Fingerlings were taken dead when they turned upside down

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Table 1. Water quality parameters of exposure aquaria (30 days).

Dissolved oxygen (mg/L)	Concentration of DEP (µg/L)		
	Day 1	Day 15	Day 30
Control	4.60±0.10	4.30±0.20	4.20±0.10
30	1.40±0.10	1.80±0.10	2.00±0.10
40	1.70±0.10	2.20±0.00	1.90±0.00
60	1.90±0.10	2.20±0.00	2.50±0.10
80	2.50±0.10	2.30±0.10	2.40±0.10
pH			
Control	6.90±0.00	6.70±0.10	6.70±0.10
30	6.20±0.00	6.10±0.00	6.10±0.00
40	6.10±0.00	6.10±0.00	6.10±0.00
60	6.20±0.10	6.10±0.00	6.10±0.00
80	6.40±0.10	6.10±0.00	6.00±0.00
Temperature(°C)			
Control	28.0±3.01	28.40±3.03	28.50±3.03
30	28.0±3.03	28.00±3.03	28.30±3.03
40	28.0±3.03	28.2±2.03	28.20±2.03
60	27.0±2.00	28.2±3.03	28.20±2.03
80	27.0±2.71	28.5±3.03	28.00±3.03

*Each value is mean±SD of 3 observations.

and sank to the bottom of the tank or when their tail showed no form of movement even prodded with a glass rod (Mgbaeruhu, 2002).

Chronic toxicity tests

One hundred and eighty fingerlings (13.13 ± 2.27 g) were used and the fish were randomly divided into five treatments groups (A to E) of thirty six fish. Each group was further randomized into three replicate experiments containing twelve fish each. The fish in group A and B were exposed to 30 and 40 µg/L of DEP, respectively. Similarly, the fish in groups C and D were treated with 60 and 80 µg/L, respectively. The fifth group (control) was exposed to tap water as the control. The experiment lasted for 30 days, and at the beginning of every week fingerlings were collected to assess their heamatological, biochemical and histopathological changes. Water temperature, pH and dissolved oxygen, DO of the exposure aquaria were monitored using meecury in-glass thermometer, dissolved oxygen meter (Table 1) (Jenway, 9071) and digital pH meter (mettle Toledo 320).

Heamatological evaluation

Heamatological examination of fish followed the method described by Svobodova et al. (1991). 11/2 ml of blood were collected at the beginning (initial) of the experiment through weekly to the end of the experiment (week 4) from the caudal peduncle of both the test and the control fish as described by Stoskopf (1993) and Joshi et al. (2000). The blood samples were dispensed into tubes containing lithium heparin anticoagulant. Heamoglobin was estimated by cyanomethemoglobin method. Red blood cells (RBC) and white blood cell (WBC) were counted by Neubauer's improved heamocytometer using Hyems (Shah and Alltinday, 2005) and Turks solution (Mgbenka et al., 2003) as a diluting fluid respectively. Packed cell volume (PCV), mean corpuscular heamoglobin concentration (MCHC), mean corpuscular heamoglobin (MCH) and mean cell volume (MCV) were calculated respectively using standard formula described by Dacie and Lewis (1991) and Joshi et al. (2002).

Biochemical profile

At the end of each week, the fish were stunned by being placed on a block of ice and then dissected to obtain the brain, muscle and liver of the test animal (*Clarias gariepinus*); 10% homogenate in ice cold saline were prepared. The homogenates were used for estimating enzyme activity. The liver and muscle homogenates were used for estimating the activity of acid and alkaline phosphatase (ACP and ALP) (Bassey et al., 1946), aspartate and alanine aminotransferase (AST and ALT) (Reitman and Frankel, 1957). The muscle and brain homogenates were used for estimation of acetylcholinesterase (AchE) activity (Bergmeyer, 1974).

Histopathology

The tissue samples (liver and gill) were quickly excised from the fingerlings and fixed at 10% formal-saline. Slices of the organs were quickly prepared for histological examination to show if there were morphological change in the organs during the treatment (intoxication). Processing started with parking of the tissues in the tissue capsule. The tissues were dehydrated in graded levels of ethanol (70 to 100%) in ascending order. The alcohol was changed after soaking the tissues in them for 1 to 2 h. The tissues were cleared in chloroform and impregnated with paraffin wax and sectioned at 4 to 5 µ thickness using rotary microtone. The sections were floated on a water bath maintained at 2 to 3°C below melting point of paraffin wax. They were on a hot plate thermostatically maintained at a temperature of 2 to 3°C above the midpoint of paraffin wax. When properly dried (15 to 30 min), they were stained with haematoxylin and eosin (H and E), dehydrated, cleared and

Table 2. Cumulative percentage mortality of *Clarias gariepinus* exposed to graded concentrations of diethyl phthalate (DEP) for a maximum of 96 h.

DEP (µg/L)	% mortality (24 h)	% mortality (48 h)	% mortality (72 h)	% 96 h mortality	Total % mortality
Control	0	0	0	0	0
50	3.3	13.3	10	3.3	29.9
75	6.7	16.7	13.3	10	46.7
100	23.3	23.3	6.7	3.3	56.6
150	43.3	26.7	20	10	100

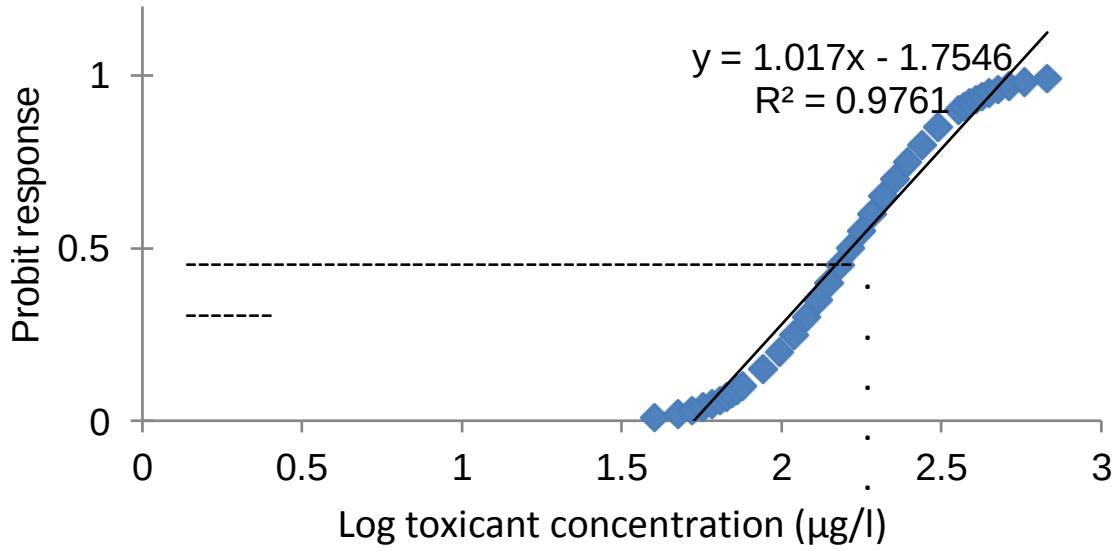

Figure 1. Probit transformed responses for 24 h exposure of *Clarias gariepinus* fingerlings to graded concentrations of diethyl phthalate (DEP).

mounted (D.C.M.) in a mountant, avoiding air bubbles. E staining was used for the demonstration of general tissue structures in various colours. The nuclei as well as some calcium salts and ureates were to take blue colour.

Other tissue structures were to appear red, pink or orange in color (eosinophilic). The permanent slides prepared were mounted one after the other and were viewed at different magnifications of the microscope. Photographs of each slides was taken and the results are shown.

Statistical analysis

Mean values were analyzed for significant differences ($P \leq 0.05$) using the ANOVA. Differences between means were partitioned using the Duncan New Multiple Range test. The Statistical Package for Social Sciences (SPSS) version 16 was used. The probit value was determined from the probit model developed by Finney (1971).

RESULTS

Acute test result

The mortality rate of *C. gariepinus* fingerlings exposed to diethyl phthalate for the period of 96 h is shown in Table 2. Mortality of fish treated with DEP concentrations (50, 75, 100 and 150 µg/L) was dose-dependent. The LD_{50} of DEP for the fish at 24, 48, 72 and 96 h are shown in Figures 1 to 5. The LD_{50} values for DEP were 2.22, 2.73, 3.44 and 3.93 µg/L after 24, 48, 72 and 96 h, respectively. The mean LD_{50} was 1.87 µg/L after 96 h. A chi-square goodness-of-fit did not indicate significant difference ($P>0.05$) between the observed and expected responses. The observed percentage mortality which was dose dependent increased from 29.9 to 46.7% for the fingerlings exposed to 50 and 75 µg/L DEP while the mortalities were 56.6 and 100% for the fish exposed to concentrations of 100 and 150 µg/L, respectively. No mortality was recorded in the control group. The r^2 value was 97.6% indicating the adequacy of the model. Haemorrhaging of the gill of some fish was observed in the fish exposed to 100 and 150 µg/L of the compound. Rapid opercula movement, restlessness, erratic swimming and loss of balance were observed in the fish exposed to DEP. The results of the study are shown in Tables 3 and 4. The haematological parameters in the control fish did not alter significantly throughout the study period. The haemoglobin concentration in the treatment groups were significantly lower than the control ($P < 0.05$) and also differed with between the treatment groups ($P < 0.05$). There was inverse relationship between the haemoglobin concentration and DEP concentration at day 15 (Y = -0.025x

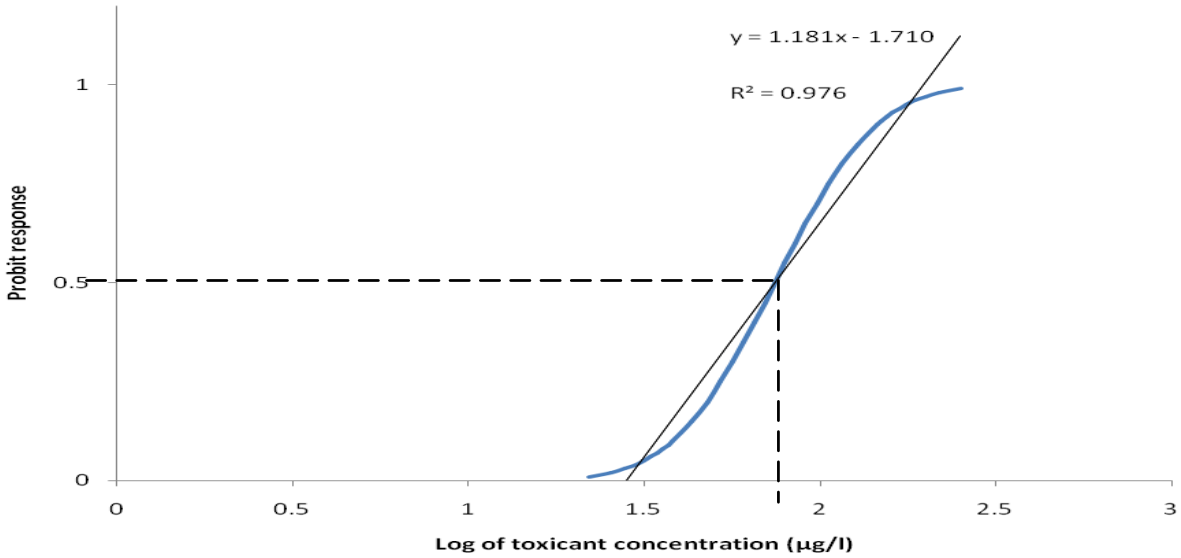

Figure 2. Probit transformed responses for total death of *Clarias gariepinus* fingerlings exposed to graded concentrations of diethyl phthalate (DEP).

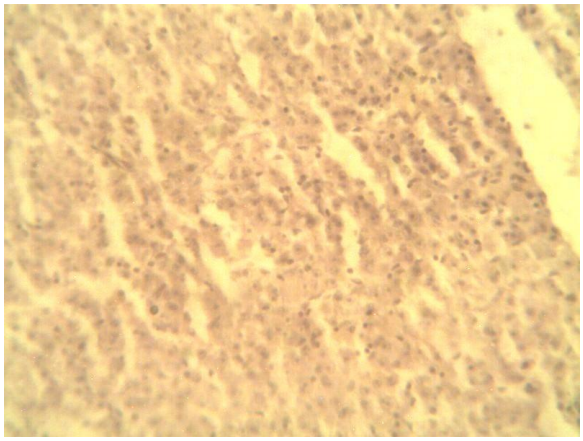

Figure 3. Photomicrograph of liver section from control juvenile *C. gariepinus* 80 µg/L diethyl phthalate at day 30 showing the central vein (V) and normal place of hepatocytes (arrows). H&E (mag ×100).

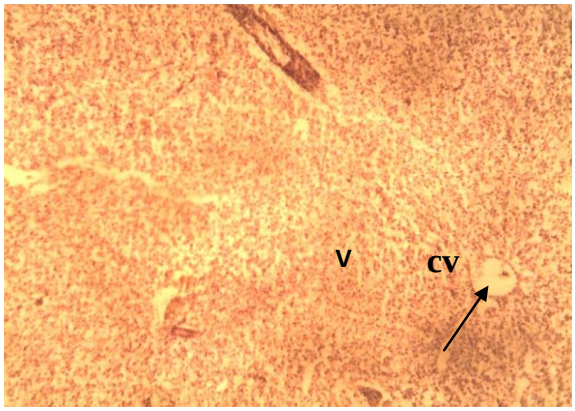

Figure 4. Photomicrograph of histologic section of liver of *Clarias gariepinus* treated with 30 µg/L of diethyl phthalate day 7 showing mild vacuolation of hepatocytes. Note the central vein (V). H&E (mag ×100).

+ 4.92; R = 0.051) and day 30 (Y = -0.0214x + 4.29; R = 0.937). This was an indicative that haemoglobin concentration decreased with increasing DEP concentration and with duration. There was a non-significant increase in Hb concentration at day 15 in fish exposed to 60 µg/L DEP when compared to the group treated with 30 µg/L DEP. The packed cell volume (PCV) of the DEP exposed fish was significantly lower ($P < 0.05$) than the control. The decrease in PCV was concentration dependent and with increasing duration of expo-sure. The erythrocyte count decreased significantly in the treatment group when compared with the control ($P < 0.05$). Also, the erythrocyte count differed in the treatment groups ($P< 0.05$). Similarly, the rate of decrease was DEP concentration dependent and with exposure time.

The morphological indices of the fish exposed to DEP were affected adversely. Both the MCV and MCH of the DEP exposed fish were lower than the control ($P <0.05$). The decrease in the morphological indices of the fish was neither DEP concentration dependent nor influenced by the duration of exposure. The leucocyte count in the fish exposed to DEP was significantly higher than the control ($P < 0.05$) although the rate of increase does not seem to be concentration dependent. Results of the biochemical changes of *C. gariepinus* exposed to the four doses (30, 40, 60 and 80 µg/L) of DEP were as follows. There was a significant increase in liver and muscle ACP levels compared to the controls (Tables 5 and 6) interestingly, liver ACP levels were significantly increased in treated groups (30 to 80 µg/L) compared to the control; whereas, muscle ACP levels also increased significantly in the experimental

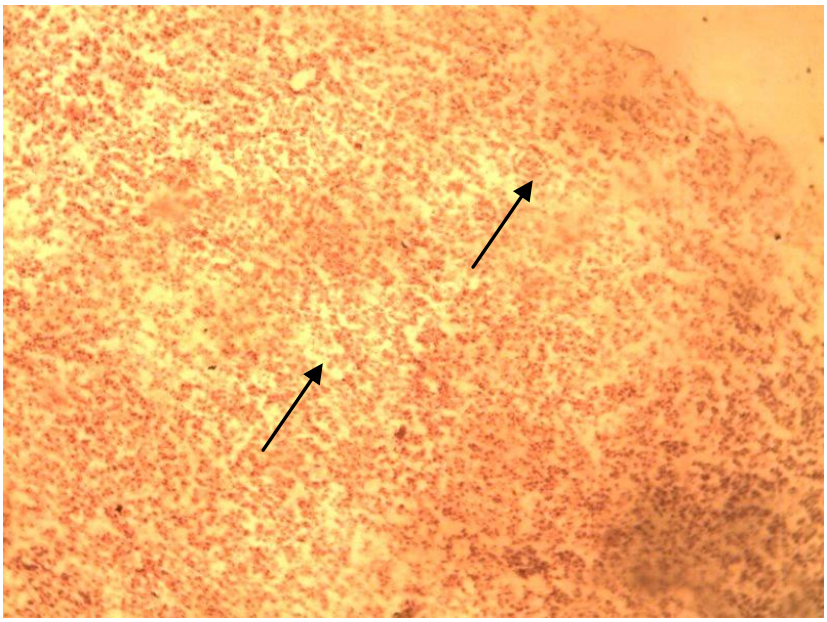

Figure 5. Histologic section of liver of *Clarias gariepinus* treated with 30 µg/L diethyl phthalate at day 15 showing multifocal areas of hepatocyte degeneration. (H&E) mag ×100.

Table 3. Changes in the haematological parameters of *Clarias gariepinus* fingerlings exposed to diethyl phthalate (DEP) for 30 days[1, 2, 3].

DEP (µg/L)	Time (day)	HB (g/dl)	PCV (%)	RBC (× 0^6/mm^3)	WBC (×10^4/mm^3)	MCV (fl)	MCH (pg)	MCHC (g/dl)
Control	1	4.92±1.47^a	18.2±1.30^a	2.714±0.59^b	1.3±0.10^a	9.70±4.25^a	18.56±5.10^a	0.24±0.07^a
	15	4.92±1.61^c	18.4±4.15^c	2.694±0.45^c	2.12±0.35^{ab}	9.6±.64^a	18.32±4.90^a	0.27±0.07^a
	30	4.42±.45	18.2±1.64	2.27±0.33	2.73±0.19	8.12±1.04	19.56±1.45	0.24±
30	1	4.64±.98^a	19.6±2.40^a	2.20±0.58^{ab}	2.04±0.24^{ab}	9.20±1.7^a	21.6±4.92^a	0.24±0.08^a
	15	3.64±1.25^{ab}	17.2±2.38^{ab}	2.34±0.70^{ab}	2.26±0.50^b	7.70±1.85^{ab}	14.31±5.40^a	2.02±0.96^a
40	1	4.76±1.40^a	16.4±3.91^a	2.12±0.37^a	1.83±0.26^a	8.56±2.12^a	22.42±4.93^a	0.296±0.08^a
	15	2.76±0.95^a	15.2±4.4^{ab}	1.612±.47^a	2.05±0.19^{ab}	6.79±2.60^b	17.46±4.60^a	0.192±0.07^a
60	1	4.70±0.62^a	17.8±3.11^a	2.36±0.21^{ab}	1.92±0.18^{ab}	7.68±4.14^a	24.20±2.32^a	0.274±0.05^a
	15	3.96±1.33^{ab}	14.8±3.70^{ab}	2.15±0.61^{ab}	2.35±0.13^b	6.30±0.16^a	16.51±1.79^a	0.27±0.03^a
80	1	4.96±1.24^a	19.6±1.94^a	2.54±0.70^{ab}	1.92±0.98^{ab}	8.09±1.90^a	20.28±9.50^a	0.241±0.06^a
	15	2.86±1.98^{ab}	12.4±4.39^a	2.014±0.67^{ab}	2.38±0.40^b	6.50±2.02^a	14.40±8.20^a	0.26±0.22^a

[1]Means within the same column followed by different letters are significantly different (P ≤ 0.05). [2]DEP = concentration of diethyl phthalate, HB = haemoglobin, PCV = packed cell volume, RBC = red blood cells, WBC = white blood cells, MCV = mean cell volume, MCH = mean cell haemoglobin, MCHC = mean cell haemoglobin concentration. Each value is mean±SD of 3 observations.

groups compared to the control. The liver ACP activity was statistically significant (P < 0.05) at day 15 respectively compared to the control. The muscle ACP in other treatment groups showed no significant difference (P > 0.05). Liver AST levels were significantly increased in DEP treated fish compared to the control. The liver AST levels were dose-dependent (Tables 5 and 6). On the other hand, liver ALT only increased significantly on day 15 and significantly decreased in day 30. Liver AST showed no significance in all treated groups (P > 0.05) and liver ALT activity was statistically significant (P < 0.05) at day 30 only. There was significant increase in brain AchE in day 15 among the treated groups and decreased at day 30. It can be seen that the initial values of AchE was maintained at the end of the day 30 which shows that DEP inhibits AchE activity for some period or remains in a constant level throughout the exposure. There was significant difference (P < 0.05) in brain and muscle

Table 4. Changes in the haematological parameters of *Clarias gariepinus* fingerlings exposed to diethyl phthalate (DEP) for 30 days[1, 2].

DEP (µg/l)	Duration (day)	Hb (g/dl)	PCV (%)	RBC (10^6/mm^3)	WBC (10^4/mm^3)	MCV (µm^3)	MCH (pg)	MCHC (g/dl)
Control	1	4.92±1.47^a	18.2±1.30^a	2.714±0.59^b	1.3±0.10^a	9.7±4.25^a	18.56±5.1^a	0.2442±.07^a
	30	4.42±.449^b	18.2±1.64^d	2.27±0.327^c	2.73±0.19^a	8.12±1.04^a	19.56±1.45a	0.24±.03^a
30	1	4.64±.978^a	19.6±2.4^a	2.20±.578^{ab}	2.26±0.5^b	9.2±1.7^a	21.6±4.92^a	0.24±.08^a
	30	3.36±1.2^{ab}	13.4±2.79^c	1.93±.477^a	2.58±0.30^a	7.1±1.2^a	17.32±2.4^a	0.25±.07^a
40	1	4.76±1.4^a	16.4±3.91^a	2.12±.374^{ab}	2.05±0.19^{ab}	8.56±2.12^a	22.42±4.93^a	0.30±.08^a
	30	3.5±1.17^{ab}	13.8±3.34^c	1.84±.450^a	2.64±0.36^a	7.8±2.95^a	18.76±1.67^a	0.26±.083^a
60	1	4.7±.621^a	17.8±3.11^a	2.36±0.21^a	1.92±0.18^{ab}	7.68±4.14^a	24.2±2.32^a	0.27±.054^a
	30	2.96±1.10^{ab}	11.6±2.96^{ab}	1.94±.501^a	2.45±0.48^a	5.42±1.02^a	14.76±7.8^a	0.29±.081^a
80	1	4.96±1.24^a	19.6±1.94^a	2.54±.695^{ab}	1.92±0.98^a	8.09±1.9^a	20.28±9.5^a	0.24±.062^a
	30	2.66±1.7^a	8.8±3.44^a	1.62±.644^a	2.87±0.32^a	6.24±2.7^a	17.8±9.4^a	0.3±.11^a

[1]Means within the same column followed by different letters are significantly different (P ≤ 0.05). [2]DEP = concentration of diethyl phthalate, HB = haemoglobin, PCV = packed cell volume, RBC = red blood cells, WBC = white blood cells, MCV = mean cell volume, MCH = mean cell haemoglobin, MCHC = mean cell haemoglobin concentration. Each value is mean±SD of 3 observations.

Table 5. Effect of diethylphthalate on the biochemical parameters of *Clarias gariepinus* fingerlings [1, 15].

Tissue	parameter	Duration	Control	30 µg/L	40 µg/L	60 µg/L	80 µg/L
Liver	AST	1	15.30±2.89^a	13.09±3.82^a	16.20±3.64^a	19.05±5.06^a	15.80±1.69^a
		15	35.80±2.83^{ab}	32.40±3.82^{ab}	27.0±.01^c	39.15±9.55^c	47.30±1.25^a
	ALT	1	11.70±3.67^a	18.15±3.71^a	18.15±3.71^a	22.10±2.58^a	21.80±2.54^a
		15	28.15±3.98^a	14.55±5.16^a	14.55±5.16^a	17.30±1.27^a	14.55±2.62^a
	ACP	1	1.11±.14^a	1.21±.14^a	1.62±.28^a	1.16±.21^a	1.67±.35^a
		15	1.54±.34^{ab}	2.22±.94^b	3.11±.097^c	1.09±.097^a	1.84±.36^{ab}
Muscle	ACP	1	1.06±.07^a	3.59±.78^a	3.84±.43^a	2.58±.78^a	2.53±.14^a
		15	2.39±.62^a	2.12±.27^a	2.55±.78^a	3.93±.21^a	2.02±.14^a
	AChE	1	1110.4±55.7^a	3322.10±2.54^a	1697.2±6.81^{ab}	2527.70±5.01^{cd}	2879.8±1.47^{cd}
		15	1184.4±8.9^{bc}	2446.4±8.63^a	1263.80±3.53^c	9505.90±20.78^b	5777.6±4.61^a
Brain	AChE	1	5615.10±96.35^b	2049.20±25.7^a	6048.5±11.96^{bc}	9226.10±14.53^c	8327±16.12^{cd}
		15	2903±6.30^d	5777.70±4.43^a	13433±19.71^b	6086.53±10.21^a	22014±8.53^c

[1]Means within the same column followed by different letters are significantly different (P ≤ 0.05). [2]ALT, AST, ACP, ALP and AchE are indicated as IU/l. Abbreviations: ALT, Alanine transaminace; AST, aspartate aminotransfarase; ALP, alkaline phosphotase; ACP, acid phosphatase. Each value is mean±SD.

AchE activity compared to the control.

Histopathological studies

Liver

The histology of control fish liver revealed normal (Figure 3) typical paranchymatous appearance. The liver was made up of hepatocytes, which were polygonal cells with a central spherical nucleus. There were degeneration of hepatocytes (Figures 4 and 5) in 30 µg/L treated fish at day 30 and mild vacuolation of hepatocytes and multi-focal areas of hepatocytes degeneration at day 15 and 30, respectively. Fish exposed to 40 µg/L DEP (Figures 6 and 7) showed a centrilobular degeneration of hepato-cytes for day 15 and 30, respectively. At 60 and 80 µg/L concentration, mild vacuolation of hepatocytes and severe necrosis were observed accordingly as shown in Figures 8 and 9, respectively. The histopathological changes were more pronounced after 30 days exposure period. The liver cells were degenerated with necrosis which appeared as focal areas with lymphocytic infiltration (Figure 10).

Table 6. Effect of diethylphthalate on the biochemical parameters of *Clarias gariepinus* fingerlings[1, 30].

Tissue	Parameter	Duration	Control	30 µg/L	40 µg/L	60 µg/L	80 µg/L
Liver	AST	1	15.30±2.89^{a}	13.08±3.82^{a}	16.20±3.64^{a}	19.05±5.06^{a}	15.30±1.69^{a}
		30	37.75±9.55^{a}	27.80±9.42^{a}	64.85±3.64^{a}	62.15±2.98^{a}	81.50±1.9^{a}
	ALT	1	11.70±3.67^{a}	18.15±3.76^{a}	18.15±3.71^{a}	22.10±2.55^{a}	21.85±2.54^{a}
		30	11.80±1.27^{a}	2.70±1.27^{c}	6.00±.56^{a}	7.30±2.55^{ac}	7.30±2.55^{c}
	ACP	1	1.11±.14^{a}	1.21±.14^{a}	1.62±.28^{a}	1.16±.21^{a}	2.53±.14^{a}
		30	1.53±0.31^{c}	143.70±5.27^{a}	127.7±5.27^{a}	136.63±17.7^{ab}	153.7±20.37^{ab}
Muscle	ACP	1	1.06±.0.7^{a}	3.59±.78^{a}	3.84±.43^{a}	2.58±.78^{a}	2.53±.14^{a}
		30	121.23±.051^{b}	175.30±7.29^{b}	142.2±9.87^{a}	140.65±13.64^{a}	153.70±20.37^{ab}
	AchE	1	1110.40±55.74^{a}	3322.10±2.54^{a}	1697.20±6.81^{ab}	2527.70±5.01^{cd}	2879±1.47^{cd}
		30	1110.40±26.74^{a}	3322.1±22.54^{d}	1697.2±26.80^{ab}	2527.7±15.01^{cd}	2879.8±11.47^{cd}
Brain	AchE	1	5615.10±96.35^{b}	2049±25.7^{a}	6048.50±11.96^{bc}	9226.10±14.53^{c}	8327±16.12^{cd}
		30	5615.10±96.35^{b}	2049±25.72^{a}	6048.50±11.96^{bcd}	9226.10±4.53^{b}	8327±16.12^{bdc}

[1]Means within the same column followed by different letters are significantly different (P ≤ 0.05). [2]ALT, AST, ACP, ALP and AchE are indicated as IU/l. Abbreviations: ALT, Alanine transaminace; AST, aspartate aminotransfarase; ALP, alkaline phosphotase; ACP, acid phosphatase. Each value is mean±SD.

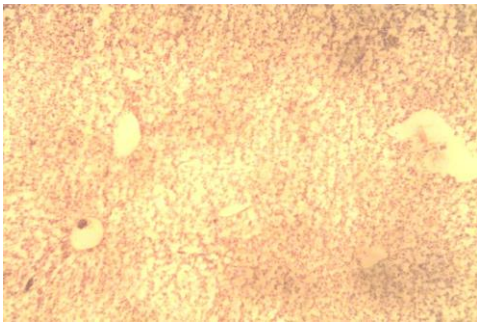

Figure 6. Photomicrograph of juvenile *Clarias gariepinus* treated with 30 µg/L diethyl phthalate at day 30 showing degeneration of hepatocytes. (H&E) mag ×100.

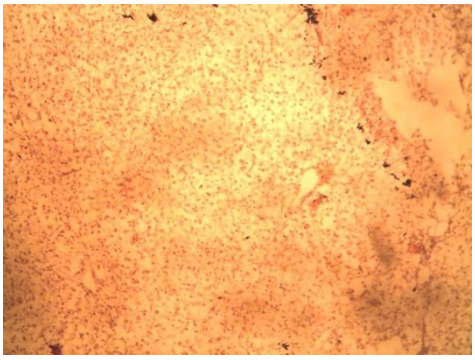

Figure 8. Photomicrograph of liver of *C. gariepinus* treated with 40 µg/ diethyl phthalate at day 30 showing centrilobular vacuolation of hepatocytes. H&E (mag ×100).

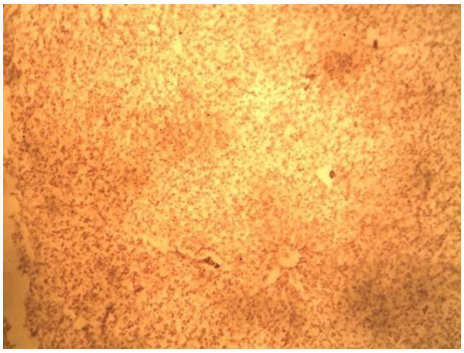

Figure 7. Liver section of juvenile fish treated with 40 µg/L diethylphthalate at day 15 showing centrilobular degeneration of hepatocytes. H&E 9mag ×100).

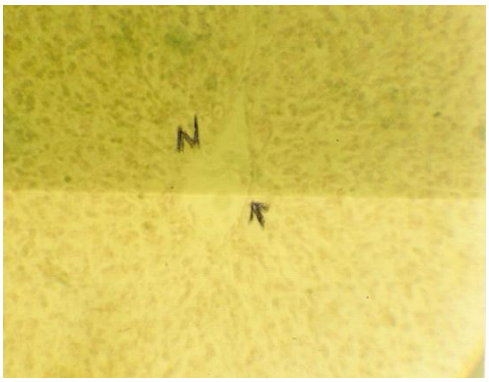

Figure 10. Liver section exposed to 80 µg/l diethyl phthalate for 30 days mag ×40 (severe necrosis).

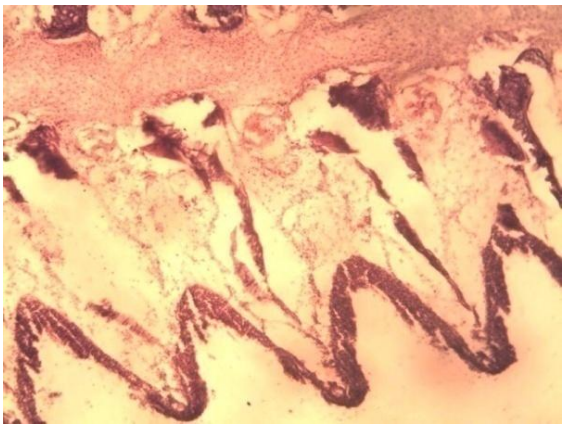

Figure 12. Photomicrograph of gills of adult *Clarias gariepinus* treated with 30 µg/ diethyl phthalate at 7 day showing raised lamellar. H&E (mag ×100).

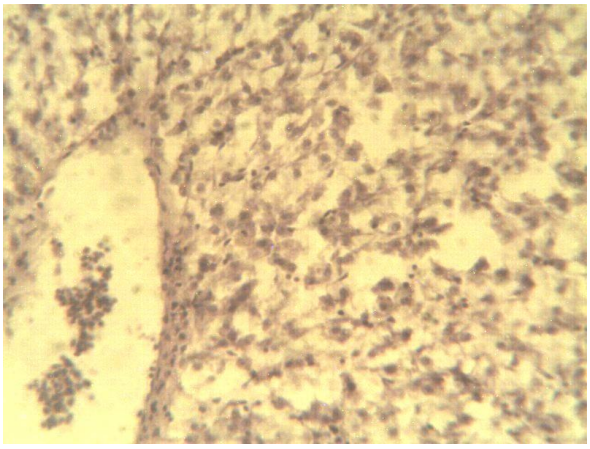

Figure 9. Photomicrograph of liver section of *C. gariepinus* treated with 60 µg/L for day 30 diethylphthalate showing mild vacuolation of hepatocytes.H&E (mag × 400).

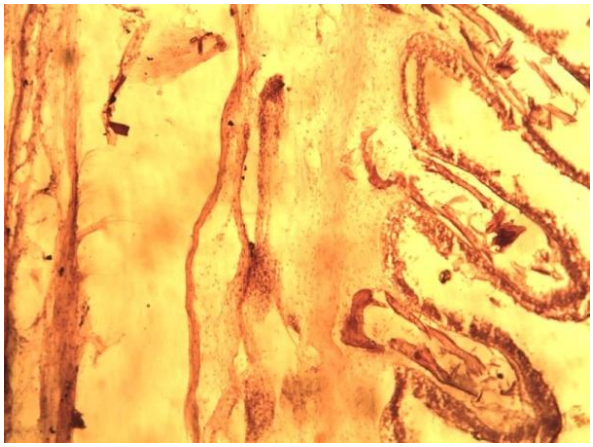

Figure 11. Histologic section of gills of control *C. gariepinus* showing normal filaments and well separated lamellar. H&E (mag ×100).

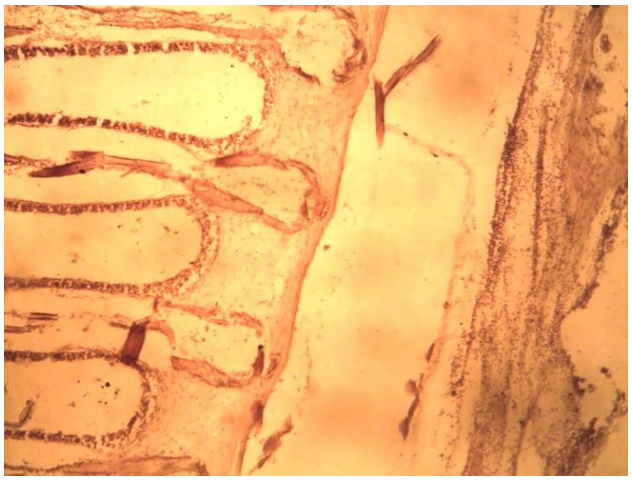

Figure 13. A section of gills of juvenile *Clarias gariepinus* treated with 30 µg/L diethyl phthalate at day 14 showing oedema (D) of the lamellar epithelia. H&E (mag ×100).

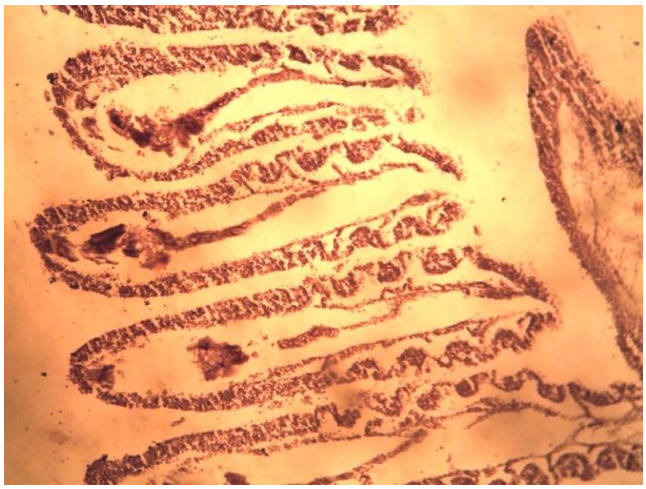

Figure 15. Photomicrograph of transverse section of gills of *C. gariepinus* treated with 60 µg/L diethyl phthalate day 21 showing oedema (E) and raising of the filaments. H&E (mag ×100).

Gill

No recognisable changes were observed in the gills of the control fish. Each gill consisted of a primary filament and secondary lamella (Figure 11). At different concentration of DEP, there were raised lamellar (Figure 12). Most of the primary and secondary lamellae were distended with oedema in the lamellar epithelia (Figure 13). However, mild oedema and raising of the filament (Figures 14 and 15) and loss of lamella epithelim (Figure 16). These changes depicts possible gill damage that have affected the respiratory system of the fish. The de-grees of the pathological changes in the gills were directly concentration-dependent.

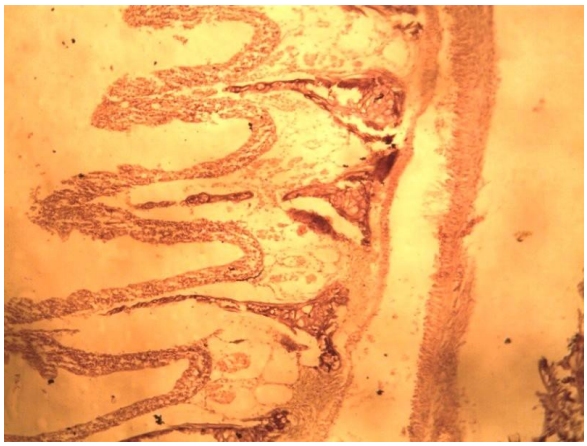

Figure 14. Photomicrograph of gills of juvenile *Clarias gariepinus* treated with 40 µg/L diethyl phthalate showing mild oedema and raising of the filaments. H&E (mag ×100).

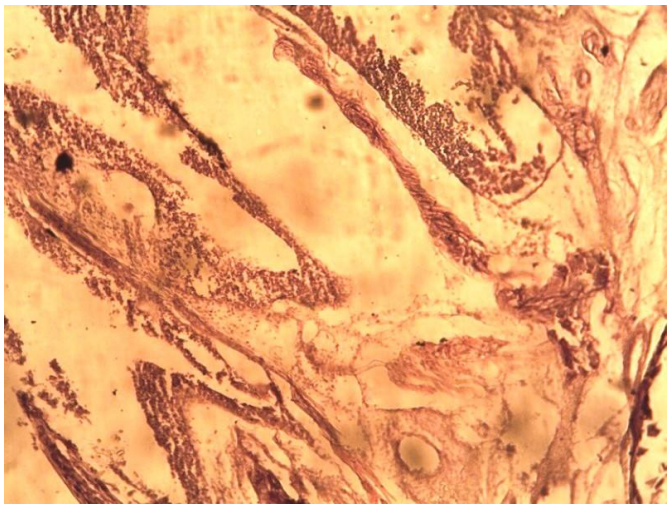

Figure 16. Histologic section of gills of *C. gariepinus* treated with 80 µg/L diethyl phthalate at day 21 showing loss of lamellar epithelium. H&E (mag ×100).

DISCUSSION

Results obtained from this study show that percentage mortality of *C. gariepinus* fingerlings increased with increase in concentration of diethyl phthalate and was dose dependent. This is in consonance with a similar study by Nivedita et al. (2002) on toxic effect of DEP on a freshwater fish, *Cirrhina mrigala*. The LD_{50} reported in this study is less than the observed field concentration in the water column (0.16 to 3.53 mg/L) and sediment (0.16 to 0.32 mg/L) of DEP in the Venda region of South African waters (Fatoki et al., 2010) where similar indiscriminate discharge of DEP-laden effluents and wastes take place as in Nigeria. In Nigeria, there is dearth of information about the field levels of DEP but it is expected to be higher than the LD_{50} reported in the present study. The LD_{50} of the carbofuran to juvenile fathead chubs was 1.96 mg/L (Fisher et al., 1999), a data that is comparable to the result in this study. Since DEP binds to the sediment and remains in the water column, it is possible that it could pose serious threat to fish and other aquatic life. The rapid opercula movement, erratic swimming and loss of balance observed in this study suggested possible nervous disorder. Haemorrhaging of the gill when the test fish were exposed to 100 and 150 µg/L of the compound is indicative of toxicity of the chemical. This is probably due to rupture of blood vessels of the gills and possible reduction in the haemotological parameters of erythrocyte count, haematocrit and mean corpuscular volume of the fish. Mgbenka et al. (2005) reported similar toxic effects with clogging of the gills with mucus of *C. gariepinus* due to lindane exposure. These observations are also in agreement with earlier reports by Okoli-Anunobi et al. (2002) on the lethal effect of the detergent (Elephant blue) on the Nile tilapia.

Haemorrhaging has also been reported in fathead minnows exposed to organic-based insecticide (Buckler et al., 1981). At higher concentrations of 0.16 to 4.04 mg/L DEP in the water of some rivers in the Venda region of South Africa, Fatoki et al. (2010) have suggested that DEP is toxic and could be carcinogenic to aquatic organisms and man though it is less harmful than di-(2-ethylhexyl) phthalate (DEHP). The observed decrease in the haemoglobin concentration is in agreement with some earlier studies. Van Vureen (1986) reported that metasystox caused decreased haemoglobin concentration in *Labeo umbratus* while Omoregie et al. (1990) observed decrease in haemoglobin in *O. niloticus* exposed to gammalin 20 and actellic 25EC. Other studies (Santhakumar et al., 1999; Mgbenka et al., 2003) reported that monocrotophos and acetellic exposures decreased the haemoglobin concentration in *Anabas sp.* and *C. albopunctatus*, respectively. The decreased haemoglobin concentration observed in this study is an indication of impaired oxygen delivery to the tissues. The reduction in erythrocytes count in *C. gariepinus* due to DEP exposure is in consonance with the report of Santhakumar et al. (1999) on *Anabas sp.* exposed to monocrotophos and in *C. albopunctatus* treated with actellic 25 EC (Mgbenka et al., 2003). Similar results were reported for *Oreochromis mossambicus* exposed to copper (Nussey et al., 1995). The general reduction in haemoglobin concentration, erythrocyte count and PCV in the fish treated with DEP was an indication of anaemia.

The observed decrease in the PCV, Hb and erythrocyte count of *C. gariepinus* juvenile after 30 day exposure to DEP could be indicative of heamodilution due to erythrocyte sequesteration. Some workers have attributed changes in such blood parameters to erythrocyte swelling (Annune and Ahuma, 1998) or haemolysis (El-Domiaty, 1987; Annune et al., 1994). The fact that MCV similarly

decreased throughout the study suggested that the observed decrease in there parameters may not be due to erythrocyte swelling. Earler studies (B. C. Ikele, University of Nigeria, Nsukka, Msc thesis) showed that the kidney architecture in *C. gariepinus* juvenile was adversely affected by DEP exposure. Erythropoeitin that is produced in the kidney of vertebrates (Gordon et al., 1967) control erythropoiesis and according to Reddy et al. (1992) activates pyridoxal phosphate in immature erythrocyte to facilitate haemoglobin synthesis; thus, the reported ammonia in this study could plausibly be due to impaired erythropoietin production following damage to the kidney tissue in the fish. Thakur and Bais (2000) also reported that erythrocyte count, MCV, MCH and MCHC decreased in *Heteropneutes* sp. treated with insecticides with concomitant decline in oxygen transport. Leucocytosis is a usual response of the vertebrates to conditions or substances that attempt to change their normal physiology; thus, the leucocytosis recorded in this study shows that DEP elicited the stimulation of the immune system of the fish to protect it against possible infection or secondary effect of DEP to predispose it to disease. Similar leukocytosis was reported in *C. albopunctatus* exposed to gammalin 20 (Mgbenka et al., 2003) and in Indian catfish (*Heteropneustes fossilis*) treated with sewage, fertilizer and insecticides (Srivastava and Narain, 1982).

Leucocytosis was also reported in *Anabas testudinus* exposed to monocrotophos (Santhakumar et al., 1999). Also, Trivedi et al. (1990) reported a similar trend in *C. batrachus* exposed to fertilizers. The reduction in the erythrocyte counts and the haemoglobin concentrations in *C. gariepinus* have demonstrated that DEP may have affected the erythropoietic centres in the kidney thereby limiting erythropoiesis in the fish. It was evident that liver and muscle acid phosphatase level were significantly increased in DEP treated fish at various concentrations and also decreased significantly at other groups. This increase is probably due to increased lysosomal activity in the liver and muscle tissues. This goes in consonance of Nivedita et al. (2002) that ACP is an inducible enzyme because its activity goes up when there is a toxic impact and the enzyme begins to counteract the toxic effect. Subsequently, the enzyme may begin to drop either as a result of having partly or fully encountered the toxin or as a result of cell damage. In a study of male Sprague-dawley rats treated with 50 ppm (w/v) DEP in drinking water for four months, there was significant increase in liver ACP (Sonde et al., 2000). It is apparent that DEP causes increased ACP activity in the liver and muscle by interacting with lysosome. Lowe et al. (1992) reported that alteration in the membrane permeability can have severe consequences such as leakage of hydrolytic enzyme including ACP, which could have detrimental effect on the cell.

Liver AST levels were significantly increased in DEP treated fish though not statistically significant compared with the control. This indicates that DEP stimulates glutamate transaminase activity in the liver which could be due to toxic injury caused by DEP, which may stimulate tissue repair through protein turn over and increased respiretion. AST levels were comparatively lower in DEP treated group indicating that DEP does affect mitochondrial function. This agrees with Nivedita et al. (2002). This correlates well with increased AST activity in the liver of DEP treated fish. In this regard, it can be said that DEP toxicity leads to enhanced AST activity, which is indicative of high protein turnover and amino acid metabolism. This is in consonance with Nivedita et al. (2002) and Muthuviveganandavel et al. (2007). Acetylcholinesterase is of interest because it is the target site for organophosphate and carbamate pesti-cides in the central nervous system and its role in choli-nergic synapses is essential for life. It is an enzyme that degrades the neurotransmitter acetylcholine, producing choline and acetate group. It is mainly found in neuro-muscular junction and cholinergic synapses in CNS, where it terminates the synaptic transmission. It is also found in red blood cell membranes, where it constitutes the Yt blood group antigen. Some studies reported evidence that AchE activity may be inhibited by environmental contaminants other than organophosphate and carbamate compounds, including some metals, surface-tants agents and combustion hydro carbons (Guilhermino et al., 1994; Herbert et al., 1995; Payne et al., 1996; Labrot et al., 1996).

It was evident in this study that the AchE activity in the brain and muscle of diethyl phthalate treated fish was found to be significantly increased and also decreased based on the duration, indicating that DEP inhibit AchE activity. This could be due to the lipophilic nature of DEP, which may be taken up faster by the brain tissues. This correlates with the sluggish, non-motile behavior of the DEP treated fish. In previous studies, the sensitivity of AchE to endosulfan was similar to the activity of non-exposed animals. The higher the AchE level in the tissue, the more susceptible it is to inhibition and low concentration of toxicants can inhibits AchE, which leads to an accumulation of acetylcholine at the central cholinergic synapses and neuromuscular junction which was evident in group B. Cholinesterase inhibition in brain and muscle produces effect in the movement of fish because acetylcholinesterase participates in the neuronal and neuromuscular transmission (Fernandev-Vega et al., 1999). The unexposed fishes (control) showed inhibition in the control group in day 15 and higher specific activity in the brain compared to the muscle, this inhibition can be species dependent according to Sancho et al. (1998). Thus, this enzyme seems not to be sensitive to this chemical, results agree with those obtained by Inbaraj and Hainder (1988) in *Channa punctatus*.

The literature on histopathology effects of DEP on fish is still rare. Neskovic et al. (1996) conducted sub lethal toxicity test (14 days) of sub lethal glyphosate concentration on histopathological changes of carp organ such as gill, liver and kidneys. In the present study, damages

of the gills indicated that the sublethal concentration of DEP caused impairment in gaseous exchange efficiency of the gills. The major changes were raised lamellar, oedema of the lamellar epithelia, mild oedema and raising of the filament, and loss of lamellar epithelium. Histopathological changes of gill such as hyperplasia and hypertrophy, epithelial lifting, aneurysm and increase in mucus secretion have been reported after the exposure of fish to a variety of noxious agents in the water such as herbicides, phenols and heavy metal (Nowak, 1992). Liver is especially a useful organ in assessing the possible impact of pollutant in fish. This is because che-mical tends to concentrate there. This is also a major site for biotransformation of toxic chemicals which usually makes them less toxic and more easily excreted. In the study of Risbourg and Bastide (1995), the exposure of fish to atrazine herbicide increased in the size of lipid droplets, vacuolisation in the liver. The most frequent encountered types of degenerative changes are those of hydropic degeneration, cloudy swelling, vacuolization and focal necrosis. This also agrees with Babu et al. (2007) in the exposure of fish to fenevalerate on the liver tissues of *C. mrigala*, when necrosis of tubular epithelium and pycnotic nuclei in the hematopoietic tissue occurred.

Necrosis of the liver tissues in the study was observed, probably resulted from the excessive work required by the fish to get rid of the toxicant from its body during the process of detoxification by the liver. The inability of fish to regenerate new liver cells may also have led to necrosis.

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Biodiesel production from butter factory effluent

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The increase in energy demand coupled with the depletion of fossil fuels has increased the need for renewable and sustainable energy sources. Butter waste effluent was identified as a possible feedstock for biodiesel. The effects of the temperature, alcohol to oil molar ratio, catalyst concentration and the reaction time were investigated to determine the optimal reaction conditions of the transesterification reaction. The optimal reaction conditions according to the results were 50°C, 6:1 alcohol to oil molar ratio, 1.0 to 1.2 wt% catalyst loads and a reaction time of 60 to 90 min. Different methods of purification were investigated in an attempt to decrease waste of a biodiesel plant, including the dry washing agents, Magnesol® D-SOL™ and Purolite® PD-206. The Magnesol® D-SOL™ was found to be the optimum method for lowering the water content and the acid value of the fuel. The biodiesel was tested according to the SANS 1935:2011 standard and did not meet the requirements of the standard with regard to flash point, sulphur content, carbon residue, oxidation stability, free glycerol, total glycerol and cold filter plugging point. In order for the biodiesel to be suitable for commercial use, it should be blended with mineral diesel.

Key words: Transesterification, butter waste, biodiesel purification, reaction kinetics.

INTRODUCTION

With the ever-increasing world population, there is an increasing demand for energy (BP, 2012; United Nations, 2011). The increasing energy demand is putting a strain on the fossil fuel reserves and therefore increasing fuel prices. The need for sustainable, renewable fuel sources cannot be emphasised enough. Biofuels have been investigated as an alternative to fossil fuels. The feedstock for biofuels has changed during the last years from edible to non-edible, and finally to waste sources. The change was driven by the food versus fuel debate, that is, the unethical use of food for fuel (Ewing and Msangi, 2009). The use of waste fats and oils for biodiesel has been the focus of recent research. The waste from a butter factory in Frankfort (Free State Province, South Africa) was identified as a possible feedstock for biodiesel production. The waste is produced during cleaning processes. The effluent (a mixture of oil and water) from the factory is collected in a settling pit. The water is separated from the oil and discarded into the municipal sewerage. The oil is removed and stored. Fines are issued by the municipality for dumping fat in the sewerage system in an effort to reduce the environmental impact of the industries in the area. Pereira et al. (2003) observed toxic conditions in sediments which were polluted with linseed and sunflower oil. The waste should thus be disposed of in an environmentally friendly manner and this increases the production cost.

The fatty waste can be used to produce biodiesel, which can in turn be used to fuel machinery at the factory. This will decrease the overall production cost of the factory by decreasing disposal costs and operating costs. The aim of the study was to develop a complete biodiesel production process from a butter waste that will produce a biodiesel that conforms to the SANS1935:2011

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Table 1. Characteristics of butter waste.

Property	Value
Water content (wt%)	0.17
FFA content (wt%)	0.2
Density (kg/m^3)	914
Viscosity at 40°C (mm^2/s)	46.9

standards.

MATERIALS AND METHODS

The waste oil was dried to reduce the water content to below 0.1 wt%. The dried oil was stored in 25-L containers for ease of transport and handling. There are a few processes for biodiesel production. The most commonly used process is the alkali-catalysed transesterification reaction, requiring a feedstock with a water content of less than 0.1 wt% in order to avoid undesirable side reactions (Cvengros and Cvengrosova, 2004). A feedstock with a free fatty acid (FFA) content of less than 2 wt% (Hayyan et al., 2011), 3 wt% (Meher et al., 2006) and 5 wt% (Van Gerpen, 2005) has been reported to give the best yields. Thus, a feedstock with less than 2 wt% FFA will be acceptable for biodiesel production. The characteristics of the oil are shown in Table 1.

Biodiesel production

The heated and well mixed oil (300 g) was transferred to a 2 L three-neck round-bottom reaction flask. The flask was fitted with a condenser, thermometer and a sampling tube. The flask was heated by means of a heating mantle and the agitation was done with a magnetic stirrer. The stirring speed was kept constant for all reactions at 375 rpm. The samples were extracted into a centrifuge tube by the sampling tube. A hydrochloric acid solution was added to the sample to deactivate the catalyst and stop the reaction. The sample was then centrifuged at 1,200 rpm for 5 min and the bottom water rich layer was removed. The process was repeated four more times with water at 60°C. The sample was then dried in an oven at 105°C for 2 h before analysis. The effect of the alcohol to oil molar ratio was calculated for ratios 3:1, 4:1, 5:1, 6:1, 7:1 and 8:1. The temperature was varied from 45 to 65°C with 5°C increments and the potassium hydroxide loads used in this experiment were 0.8, 1.0 and 1.2 wt%.

Purification of biodiesel

The purification step was done with water, in order to remove the unreacted methanol and remaining catalyst from the biodiesel. This method increases the effluent of a biodiesel plant and increases the production costs since the effluent should be treated before discarding it. The water washing step is also followed by a drying step which can affect the quality of the biodiesel. The use of resins was investigated to eliminate the water washing purification step. The two resins used in this study were Magnesol® D-SOL™ and Purolite® PD-206. These resins have been proven to reduce the water content, acid number, soap content, methanol content and potassium content of biodiesel (Manique et al., 2012; Faccini et al., 2011). The untreated and unwashed biodiesel was heated to 65°C before the resins were added. This is to remove the excess of unreacted methanol. The resins were then added and stirred for 30 min at 65°C. Samples were taken every 10 min and analysed. The experiments were carried out with resin loads of 0.5, 1.0, 1.5 and 2.0 wt%. The resins have also been shown to remove impurities like mono-, di- and triglycerides and free glycerol (Berrios and Skelton, 2008).

Analytical methods

The ester content of the biodiesel was determined with gas chromatography (GC). The filter samples were injected into an Agilent 7890 A. The Agilent HP-88, 100 m (0.25 mm, internal diameter and 0.2 µm film thickness) column was used to separate the esters. The GC was set up according to the following conditions: helium was used as carrier gas with a linear velocity of 35 cm/s; 1 µL samples were injected into the column. The temperature profile was as follows: 250°C inlet temperature; 100°C for 5 min; ramp at 10°C/min up to 120°C and hold for 1 min; ramp at 10°C/min up to 175°C and hold for 10 min; ramp at 5°C/min up to 210°C and hold for 5 min; ramp at 5°C/min up to 230°C and hold for 5 min. A flame ionisation detector (FID) was used at 350°C.

RESULTS AND DISCUSSION

The purified product consists of 96.16 ± 4 wt% esters as analysed by GC and a reaction yield of 87 wt% was achieved. The effects of temperature, alcohol to oil molar ratio, catalyst load and reaction time were determined.

Temperature

The effect of temperature on the ester content can be seen in Figure 1. The reaction temperatures used were 45, 50, 55, 60 and 65°C. There is an increase in ester content with an increase in temperature up to 50°C. A further increase in temperature decreases the ester content. This is due to the boiling point of methanol. An increase in temperature above the boiling point will result in more methanol evaporating. This reduces the alcohol to oil molar ratio in the reaction mixture and thus favours the reverse reaction.

Alcohol to oil molar ratio

The effect of alcohol to oil molar ratio on the reaction yield is shown in Figure 2. There is an increase in ester content with an increase in alcohol to oil molar ratio. This increase is clear up to a ratio of 6:1. Further increase in methanol has no significant change in ester content. This is seen for varying temperatures and catalyst loads. A ratio of 6:1 is double the stoichiometric ratio. An alcohol to oil molar ratio of 6:1 is needed to shift the reaction towards the right for the duration of the reaction and to achieve the highest conversion.

Catalyst load

The effect of catalyst load on the ester content can be

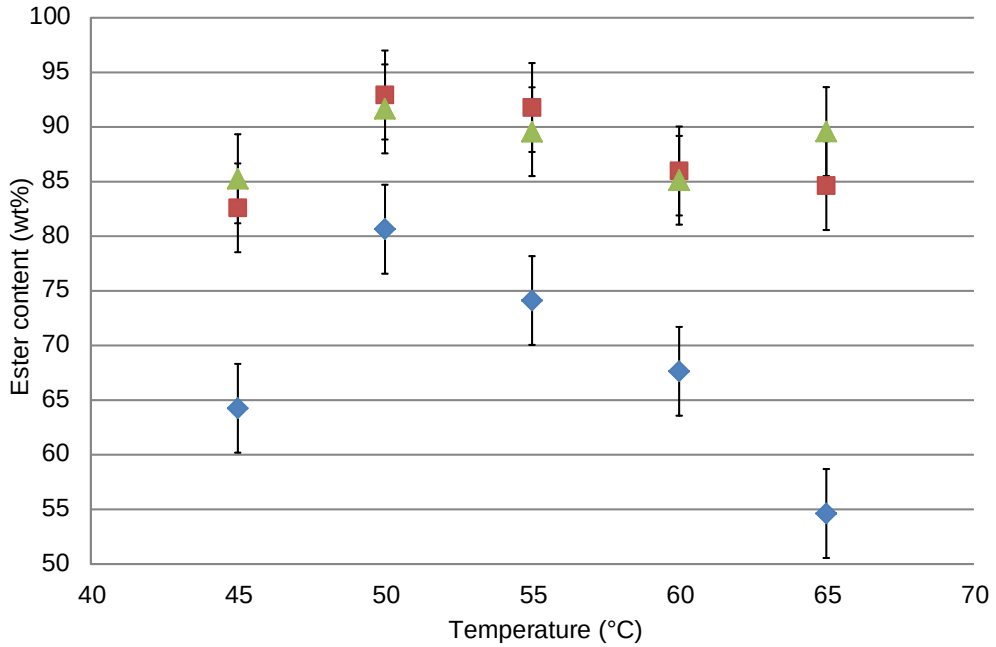

Figure 1. Chemical analyses of extracted water; influence of reaction temperature on ester content with a catalyst load of 1.2 wt%, a reaction time of 120 min, and alcohol to oil molar ratios of 3:1 (◆), 6:1 (■) and 8:1 (▲).

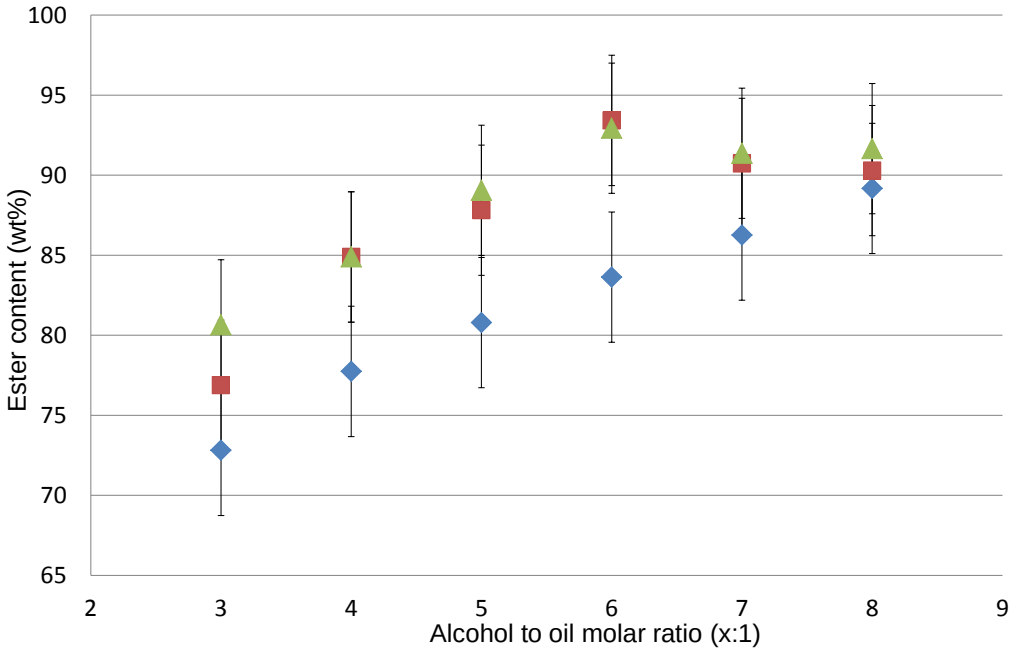

Figure 2. Influence of alcohol to oil molar ratio on ester content with a reaction time of 120 min, a reaction temperature of 50°C and catalyst loads of 0.8 wt% (◆), 1 wt% (■) and 1.2 wt% (▲).

seen in Figure 3. There is an increase in ester content with an increase in catalyst concentration at low temperatures (45°C). The effect of increasing catalyst load is, however, very limited at higher temperatures (>50°C). The catalyst facilitates the forward reaction to reach equilibrium in a shorter amount of time. The equili-

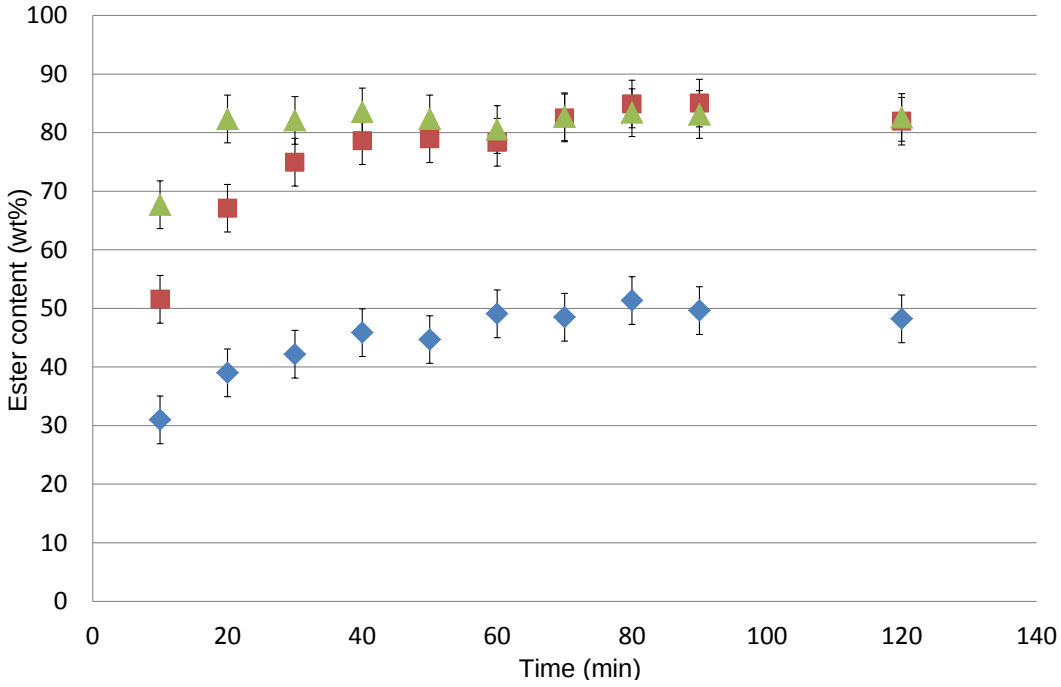

Figure 3. Influence of catalyst load on ester content with a reaction temperature of 45°C, an alcohol to oil molar ratio of 6:1 and catalyst loads of 0.8 wt% (◆), 1 wt% (■) and 1.2 wt% (▲).

brium of the 0.8 and 1.0 wt% reactions is reached after 40 min, but this is lowered to 20 min with a catalyst load of 1.2 wt%. An increase in temperature increases the reactivity of the molecules. The catalyst is used to lower the activation energy. An increase in catalyst load will not affect the ester content at higher temperatures since the activation energy needed is already supplied by the heat source. The optimal reaction conditions for biodiesel production with the alkali-catalysed transesterification reaction are a reaction temperature of 50°C, an alcohol to oil molar ratio of 6:1 and a catalyst load of 1.0 to 1.2 wt%. A reaction time of 60 to 90 min is needed to reach equilibrium and maximum conversion.

Purification

The effect of Magnesol® D-SOL™ can be seen in Figure 4. The initial water content was 1,700 ppm. The water content of the biodiesel is lowered with an increase of time. The efficiency of the Magnesol® D-SOL™ lowers as more is added to the system. This is consistent with literature (Faccini et al., 2011). The acid value of the biodiesel is also lowered by the Magnesol® D-SOL™. The acid value is lowered from 2.78 to 0.13 mg KOH/g with a 0.5 wt% load of the resin, at a reaction time of 30 min. The ester content of the biodiesel was increased from 86.74 to 96.98 wt% with a load of 0.5 wt% and reaction time of 20 min. The optimum load and reaction time for biodiesel purification with Magnesol® D-SOL™ is 0.5 wt% with a reaction time of 20 min. The effect of Purolite® PD-206 as purification agent is seen in Figure 5. A decrease in water content is observed with an increase in Purolite® PD-206 for 1.0, 1.5 and 2.0 wt%. There is no significant change in water content for a load of 0.5 wt%. The water content increases with an increase in load. This is not consistent with literature (Faccini et al., 2011). The acid value of the biodiesel decreases with an increase in load. However, a load of 2.0 wt% is not enough to lower the acid value to the desired value of the SANS 1935:2011 standard. The ester content of the biodiesel increases with an increase in Purolite® PD-206. The ester content is increased from 86.74 to 98.8 wt% with a load of 0.5 wt% and a reaction time of 20 min.

Magnesol® D-SOL™ is the better washing agent since it brings all of the evaluated parameters within the specified range of SANS 1935:2011. The SANS 1935:2011 results of the biodiesel are displayed in Table 2. The parameters that do not meet the requirements (indicated in bold) will be discussed as follows:

Flashpoint

The flash point can be described as the lowest temperature where enough combustible vapour is emitted to create a flame (Carrero and Perez, 2012). The average flash point of biodiesel is above 150°C (Boog et al., 2011; Kumar et al., 2013). The flash point is usually lowered by unreacted methanol and can lower the flash

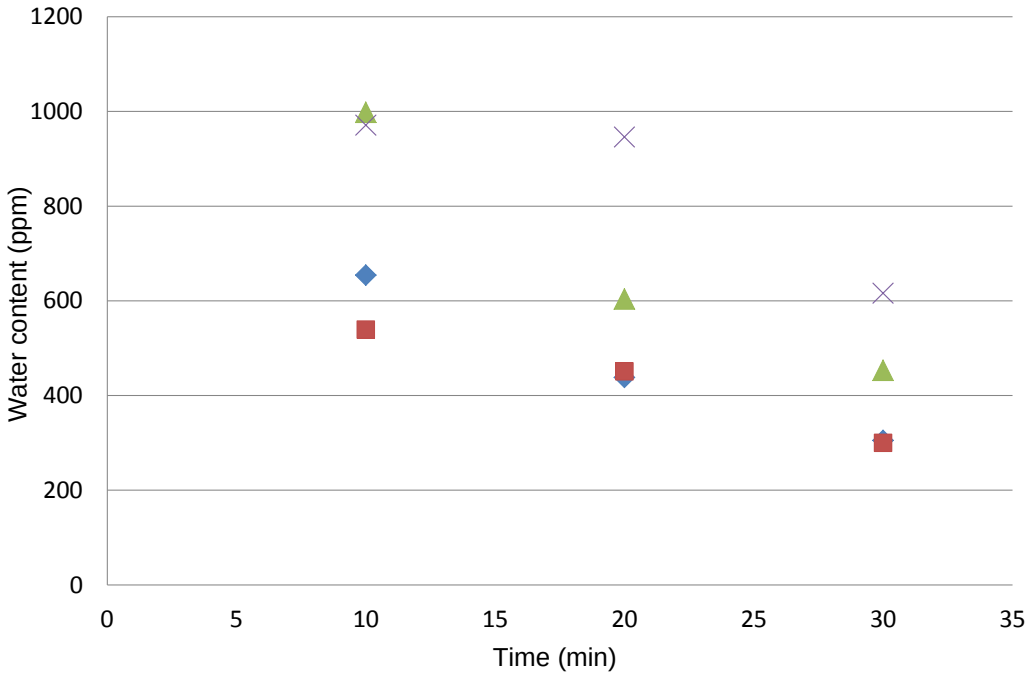

Figure 4. The effect of Magnesol® D-SOL™ on the water content of biodiesel (◆ 0.5 wt%, ■ 1.0 wt%, ▲ 1.5 wt%, ✕2.0 wt%).

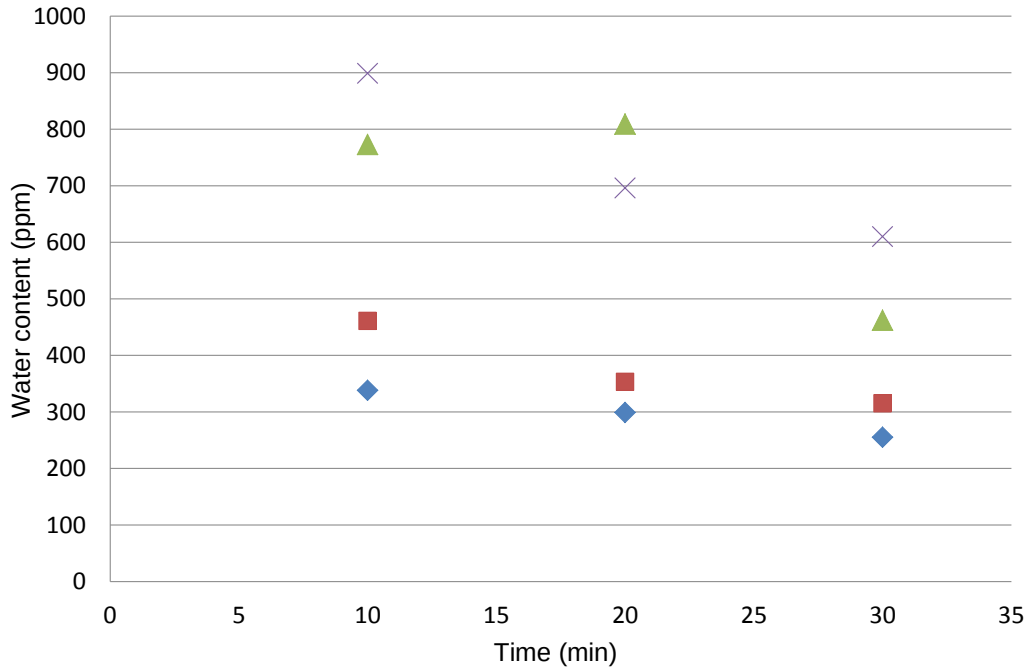

Figure 5. The effect of Purolite® PD-206 on the water content of biodiesel (◆ 0.5 wt%, ■ 1.0 wt%, ▲ 1.5 wt%, 2.0 wt%, SANS 1935:2011 maximum limit).

point from 178 to 105°C with the addition of 0.123 wt% methanol (Boog et al., 2011). No methanol could be detected by means of GC analysis in the biodiesel. The biodiesel produced from the butter waste has a broad range of esters. The biodiesel contains small amounts of short chain esters. The flash points of these esters are

Table 2. The SANS 1935:2011 parameters of biodiesel produced from butter waste.

Property	SANS 1935:2011	Result
Ester content (wt%, min)	96.5	96.16
Density at 15 °C (kg/m$^{3)}$	860 - 900	877.4
Kinematic viscosity at 40°C (mm^2/s)	3.5 - 5.0	4.2
Flash point (°C, min)	101	68
Sulphur content (mg/kg, max)	10	50
Carbon residue (on 10% distillation residue) (wt%, max)	0.3	1.3
Cetane number (min)	51.0	68.8
Sulphated ash content (wt%, max)	0.02	0.001
Water content (mg/kg, max)	500	315
Total contamination (mg/kg, max)	24	5
Copper strip corrosion (3 h at 50°C) (rating, min)	Class 1	Class 1
Oxidation stability, at 110°C (h, min)	6	0.8
Acid value (mg KOH/g, max)	0.5	0.078
Iodine value, g of iodine/100 g of FAME (max)	140	45
Linolenic acid methyl ester (wt%, max)	12	1.328
Polyunsaturated (> = 4 double bonds) methyl ester (wt%, max)	1	N/D
Methanol content (wt%, max)	0.2	N/D
Monoglyceride content (wt%, max)	0.8	N/A
Diglyceride content (wt%, max)	0.2	N/A
Triglyceride content (wt%, max)	0.2	N/A
Free glycerol (wt%, max)	0.02	0.04
Total glycerol (wt%, max)	0.25	0.27
Group 1 metals (total of Na and K) (mg/kg, max)	5	0
Group 2 metals (total of Ca and Mg) (mg/kg, max)	5	2
Phosphorous content (mg/kg, max)	4	1
Cold filter plugging point		
Winter (°C, min)	-4	+8
Summer (°C, min)	+3	

well below the standard and are therefore responsible for the undesired result. A solution to increase the low flash point is to remove the short chain esters by distillation or to blend with mineral diesel. A B10 and B20 blend of biodiesel in diesel gave a flash point of 52 and 55°C, respectively. This is lower than specified by SANS 1935:2011 (SABS, 2011) but the flash point of the B20 blend is above the minimum specified by the SANS 342:2006 standard for diesel (SABS, 2006).

Sulphur content

The sulphur content of the biodiesel is above the maximum level, but is within the range of SANS 342:2006. Thus, a blend of the biodiesel with a low sulphur content (50 ppm) diesel will be within specification.

This might be a problem for future specification for biodiesel since the standard for sulphur content will decrease to 10 ppm by 2017 in South Africa.

Carbon residue

The carbon residue is an indication of the amount of impurities in the biodiesel that will be left behind after combustion of the fuel. The impurities may cause engine failures due to blockage of the fuel injectors (Carrero and Perez, 2012). The biodiesel can be filtered or blended with mineral diesel to lower the carbon residue.

Oxidation stability

The oxidation stability is an indication of the storage conditions needed for the biodiesel. The oxidation stability is measured by heating the biodiesel to 110°C and aerating the sample. The air is then dissolved in water and the change in conductivity is measured. The oxidation stability induction period is determined when a sudden increase in conductivity is measured. Low oxidation stability increases the acid value (Carrero and Perez, 2012). The oxidation stability can be improved by

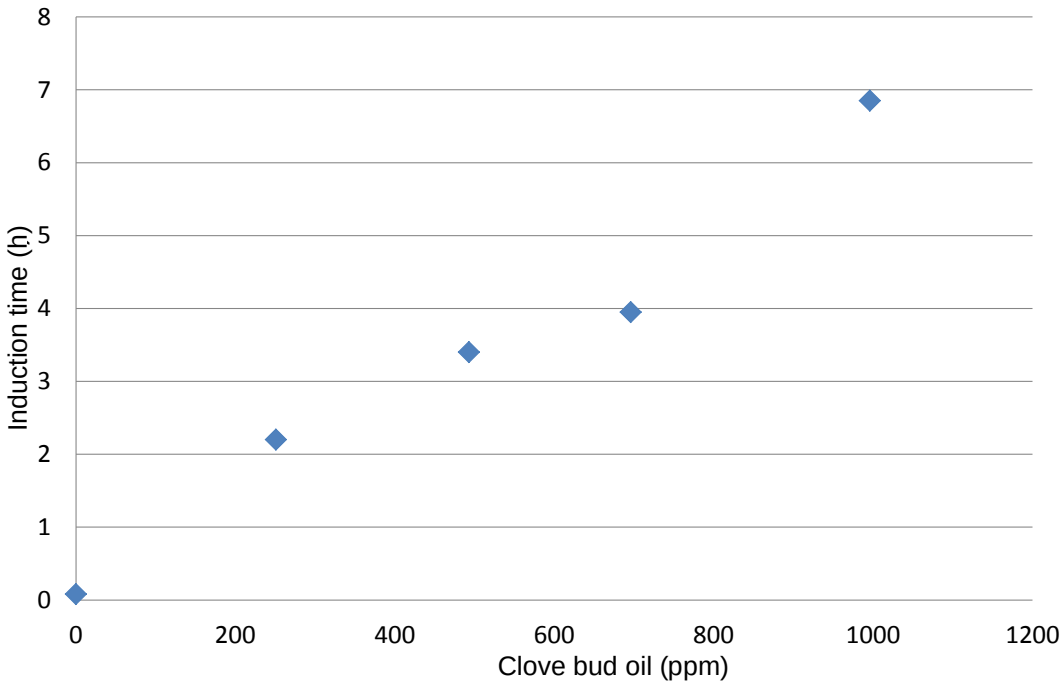

Figure 6. The effect of clove bud oil concentration on the oxidation stability.

the addition of antioxidants or by blending with mineral diesel, which contains antioxidants. A biodiesel/diesel blend of up to 40 vol% can be used to improve the oxidation stability. The use of commercial antioxidants increases the production cost of biodiesel. The essential oil with the highest anti-oxidative properties is clove bud oil (Teixeira et al., 2013). The effect of different clove bud oil concentrations on the oxidative stability is presented in Figure 6. The addition of a 1,000 ppm of clove bud oil improved the oxidative stability to meet the SANS 1935:2011 requirements. A very inexpensive essential oil can be used as an antioxidant.

Free and total glycerol

The use of dry washing resins like Magnesol® D-SOL™ and Purolite® PD-206 can decrease the glycerol content of the biodiesel (Berrios and Skelton, 2008). This is due to the ability of the resins to remove free glycerol and glycerides. The free glycerol content of the biodiesel was reduced from 0.145 to 0.04 wt% with 1.0 wt% Magnesol® D-SOL™ in 30 min and the total glycerol content was reduced from 0.87 to 0.27 wt%. This is still not within the specifications of SANS 1935:2011, but can be reduced with further purification.

Cold filter plugging point

The cold filter plugging point (CFPP) is a temperature similar to the melting point of the biodiesel. The CFPP is determined by cooling the biodiesel until it is unable to flow through a filter within a specified time. This parameter determines the environmental conditions that the biodiesel can be used in. A biodiesel that does not meet the CFPP requirements will block fuel filters and pipelines (Carrero and Perez, 2012). The specific ester concentration is responsible for this parameter. An increase in unsaturated esters decreases the CFPP, while an increase in chain length of esters increases the CFPP. The ester composition and degree of unsaturation is a major factor in the CFPP. The biodiesel can be blended with mineral diesel in order to reduce the CFPP. The CFPP was reduced from 8 to -5 and -9°C with a biodiesel/diesel blend of B20 and B10, respectively, which is well within the range of SANS 1935:2011 and 342:2006.

Conclusions

The butter waste was subjected to various methods in order to determine its use as a feedstock for biodiesel production. The transesterification reaction was used to produce biodiesel with 96 wt% ester content. The use of dry washing agents has the ability to reduce the amount of waste produced at a biodiesel plant. Small amounts of the resins or adsorbents are needed to remove impurities from the biodiesel and can be regenerated. The pure biodiesel does not meet the full range of specifications (flashpoint, sulphur content, carbon residue, oxidation

stability, free and total glycerol, cold filter plugging point), but blending with mineral diesel (B10 or B20) results in an on-specification final product.

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Full Length Research Paper

Effects of some anti-diabetic plants on the hepatic marker enzymes of diabetic rats

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This study was embarked upon in order to evaluate the effects of the chloroform extracts of the leaves of *Psidium guajava*, *Anacardium occidentale* and *Eucalyptus globulus* and fruits of *Xylopia aethiopica* on hepatic marker enzymes of diabetic rats. The degree of hepatic damage caused by diabetes mellitus and the effects of the extracts were assessed using standard methods for assaying the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). All the extracts significantly ($p<0.05$) decreased the activities of ALT, AST and ALP with that of the *P. guajava* being the most protective. In addition, the *P. guajava* extract exerted more hepatoprotection than glibenclamide in terms of the AST and ALP activities. In conclusion, the chloroform extracts of the leaves of *A. occidentale*, *E. globulus* and *P. guajava* as well as the fruits of *X. aethiopica* exhibited remarkable protective effects on alloxan-induced acute liver damage and thus, may be used for treatment of some liver-associated disorders.

Key word: Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), *Psidium guajava*, *Anacardium occidentale*, *Eucalyptus globulus*, *Xylopia aethiopica*, chloroform extracts, diabetes mellitus.

INTRODUCTION

The liver is a vital organ of the body which plays a pivotal role in regulating various physiological processes. It is also involved in several vital functions such as metabolism, secretion and storage. It has a paramount importance in the maintenance and regulation of the homeostasis of the body. It can also detoxify xenobiotics and antibiotics (Ahsan et al., 2009). The liver is capable of utilizing glucose as fuel, storing it in the form of animal starch (glycogen) and synthesizing glucose from non-carbohydrate precursors.

By so doing, it plays a significant role in the regulation of carbohydrate meta-bolism and hence, assisting in the maintenance of normal blood glucose concentrations in both fasting and post-prandial states. Any hepatic damage by way of diabetes mellitus or any other form of metabolic disorder, leads to the distortion of these metabolic functions. Unfortunately, the conventional or synthetic drugs used in the treatment of liver diseases are inadequate and sometimes have serious side effects on the other organs. Herbal drugs or their extracts are prescribed for treatment of liver diseases widely, though their biological active compounds are unknown (Latha and Reddy, 2012).

Insulin dysfunction or lack of it makes the liver undergo glycogenolysis thereby increasing hepatic glucose production. During diabetes mellitus, the unstored fatty acids, abnormal storage of glycerides, together with increased lipolysis in insulin sensitive tissues, such as the liver, results in excess free fatty acids. This has a direct toxic effect on the hepatocytes and in high concentration, culminates in disruption of cell membrane of hepatocytes, leading to the leakage of the hepatic enzymes (Neuschwander-Tetri and Caldweel, 2003). Therefore, some herbal alternatives have proven to be effective in

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providing symptomatic relief and help prevent secondary complications associated with the diseases.

Some plants have also been shown to assist in regenerating beta cells and overcoming insulin resistance (Yasir et al., 2012). This study is directed at investigating the effects of the chloroform extracts of the leaves of *Psidium guajava* (Myrtaceae), *Anacardium occidentale* (Anacardiaceae) and *Eucalyptus globulus* (Myrtaceae) and fruits of *Xylopia aethiopica* (Annonaceae) on hepatic marker enzymes of diabetic rats.

MATERIALS AND METHODS

The plant samples

The leaves of *A. occidentale*, *E. globulus* and *P. guajava* were collected from the premises of University of Nigeria, Nsukka while the fruits of *X. aethiopica* were purchased from a local market in Delta State. The plant samples were identified by Prof. (Mrs.) May Nwosu of the Department of Botany, University of Nigeria, Nsukka where the voucher specimens were deposited in the herbarium.

Preparation of crude extract

The leaves of *A. occidentale*, *E. globulus*, *P. guajava* and fruits of *X. aethiopica* were air dried to constant weight at room temperature and then reduced to powder. Six hundred grams of each plant material was macerated in 2.7 L of analytical grade chloroform. After 48 h, the resulting extracts were filtered and concentrated with rotary evaporator at reduced pressure and the yield of extracts calculated.

A standard weight, 8 g of each extracts was dissolved in 16 ml of 10% dimethyl sulphuroxide (DMSO). The doses of each extracts administered was estimated by the methods of Tedong et al. (2007), where volumes given were calculated as follows:

$$V\ (ml) = \frac{D \times P}{C}$$

Where D = Dose used (g/kg body weight of test animals), P = body weight (kg), C = concentration (g/ml), and V = volume (ml).

Animals

Fifty (50) male Wistar albino rats of weight (180 to 230 g) and 128 male mice of weight (30 to 40 g) were used for this study. The University Animal Research Ethical Committee approved the experimental protocol. The animals were housed and maintained at a 12 h light and dark cycle and fed with rat diet *ad libitum*. The mice were used for the acute oral toxicity study while the rats were made diabetic by a single dose of 180 mg/kg body weight of alloxan monohydrate intraperitonially and 44 rats (grouped into 11) selected for the study, 72 h after diabetes has been established. Treatments were for 40 h and administrations of the extracts were twice daily. After 40 h, rats were sacrificed and their blood and livers collected for further biochemical analyses.

Acute oral toxicity test (LD_{50})

Acute oral toxicity study of each plant extract was carried out by the method described by Lorke (1983).

Experimental procedures

Alanine and aspartate aminotransferase (ALT and AST) activities were assayed using Randox commercial enzyme kit as described by Reitman and Frankel (1957) and Schmidt and Schmidt (1963). Alkaline phosphatase (ALP) activity was estimated using Randox commercial enzyme kit, based on the methods of Rec (1972) and Englehardt (1970).

Statistical analysis

Data generated from this study were represented as mean ± SEM. Variables were analyzed by one-way analysis of variance (ANOVA) and comparison done by multiple comparisons using Duncan test.

RESULTS

Effects of varying doses of the different plant extracts on ALT activity

Figure 1 shows that upon the administration of varying doses of the different plant extracts to diabetic rats, there was significant ($p<0.05$) reductions in ALT activity. *X. aethiopica* (100 and 250 mg/kg body weight) had the greatest reduction in ALT activity in a dose dependent manner when compared in a descending order to those of *P. guajava* (100 and 250 mg/kg body weight), *A. occidentale* (100 and 250 mg/kg body weight) and *E. globulus* (100 and 250 mg/kg body weight). These reductions in ALT activity caused by the extracts were significantly ($p<0.05$) different from that of the glibenclamide (5 mg/kg body weight), except those of the *P. guajava* (250 mg/kg body weight) and *X. aethiopica* (100 mg/kg body weight).

Effects of varying doses of the different plant extracts on aspartate aminotransferase (AST) activity

Figure 2 shows that *P. guajava* (100 and 250 mg/kg body weight) significantly ($p<0.05$) decreased AST activity more than as recorded for the groups administered *E. globulus* (100 and 250 mg/kg body weight), *A. occidentale* (100 and 250 mg/kg body weight) and *X. aethiopica* (100 mg/kg body weight). These decreases were in dose dependent manners with the 250 mg/kg body weight of *P. guajava* extract having the greatest reduction ability. The reduction in AST activity caused by the glibenclamide (5 mg/kg body weight) was not significantly ($p>0.05$) different from those of *A. occidentale* (250 mg/kg body weight), *E. globulus* (100 mg/kg body weight) and *X. aethiopica* (100 and 250 mg/kg body weight).

Effects of varying doses of the different plant extracts on alkaline phosphatase (ALP) activity

As observed in Figure 3, *E. globulus* extract (100 and 250

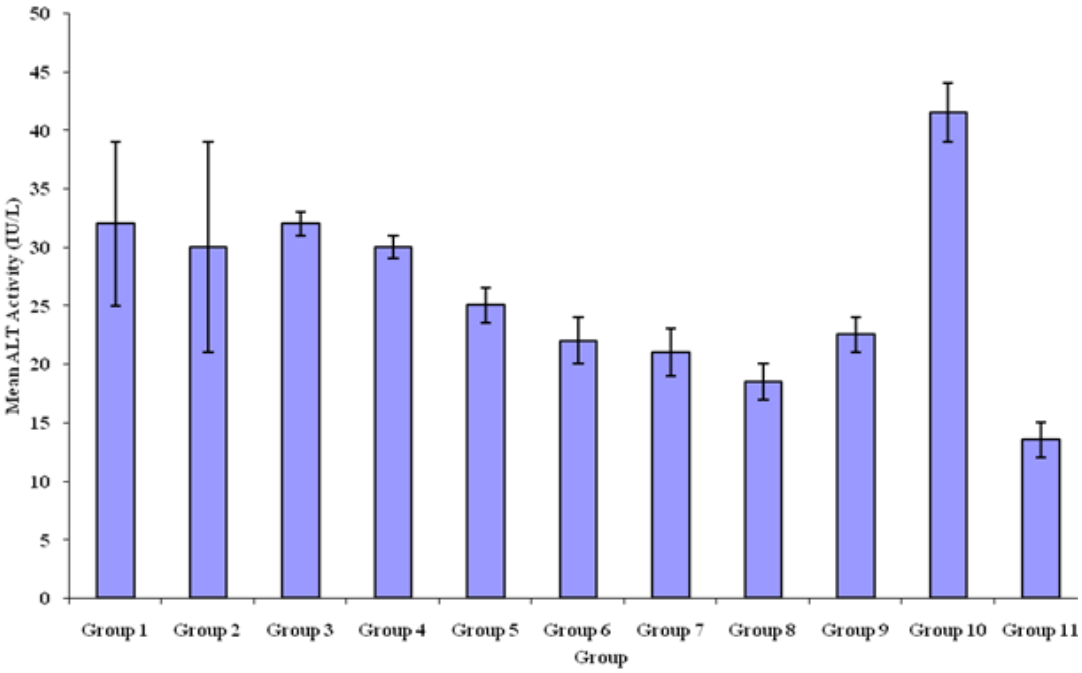

Figure 1. Effect of varying doses of different plant extracts on alanine aminotransferase (ALT) activity in rats. Group 1 = *Anacardium occidentale* (100 mg/kg); Group 2 = *Anacardium occidentale* (250 mg/kg); Group 3 = *Eucalyptus globulus* (100 mg/kg); Group 4 = *Eucalyptus globulus* (250 mg/kg); Group 5 = *Psidium guajava* (100 mg/kg); Group 6 = *Psidium guajava* (250 mg/kg); Group 7 = *Xylopia aethiopica* (100 mg/kg); Group 8 = *Xylopia aethiopica* (250 mg/kg); Group 9 = Glibenclamide (5 mg/kg); Group 10 = Diabetic untreated; Group 11 = DMSO control.

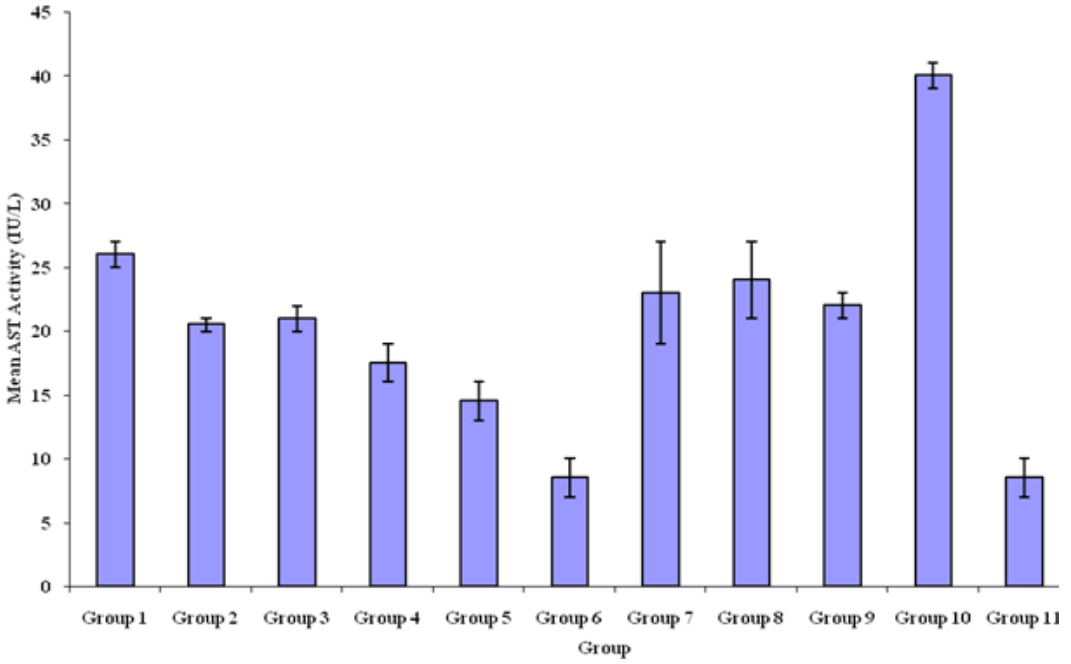

Figure 2. Effect of varying doses of different plant extracts on aspartate aminotransferase (ASP) activity in rats. Group 1 = *Anacardium occidentale* (100 mg/kg); Group 2 = *Anacardium occidentale* (250 mg/kg); Group 3 = *Eucalyptus globulus* (100 mg/kg); Group 4 = *Eucalyptus globulus* (250 mg/kg); Group 5 = *Psidium guajava* (100 mg/kg); Group 6 = *Psidium guajava* (250 mg/kg); Group 7 = *Xylopia aethiopica* (100 mg/kg); Group 8 = *Xylopia aethiopica* (250 mg/kg); Group 9 = Glibenclamide (5 mg/kg); Group 10 = Diabetic untreated; Group 11 = DMSO control.

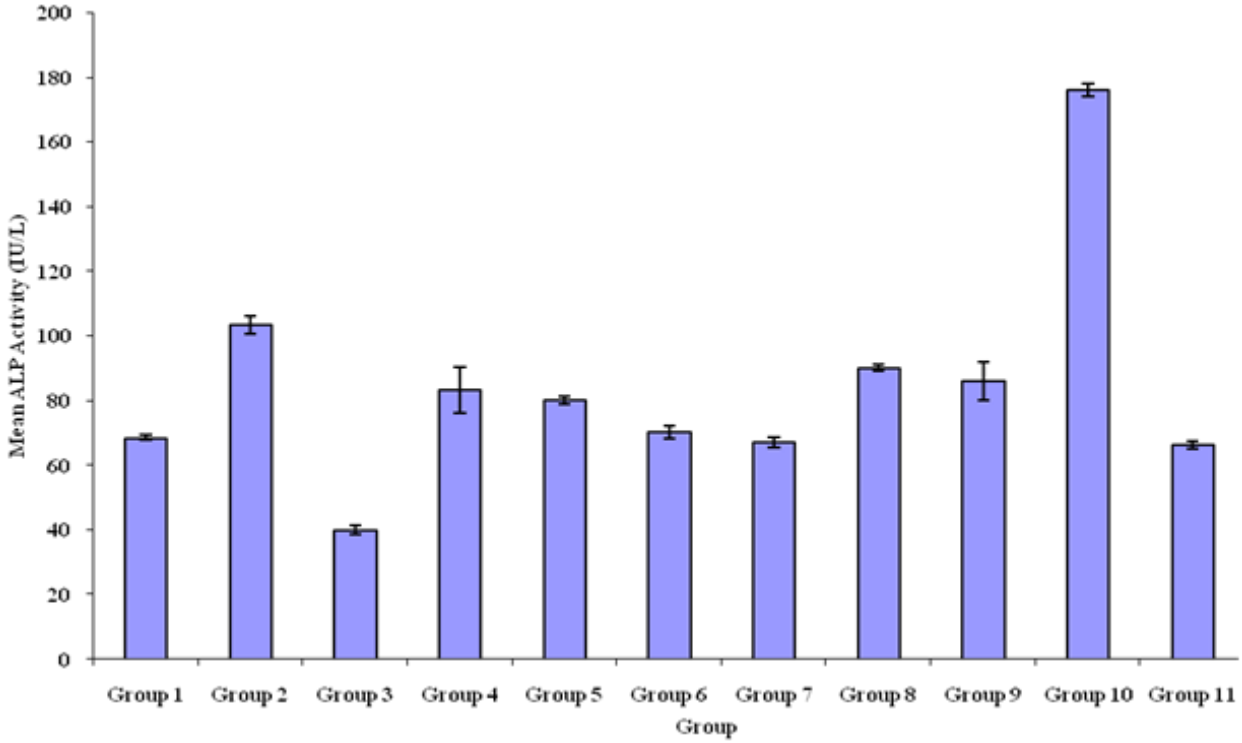

Figure 3. Effect of varying doses of different plant extracts on alkaline phosphate (ALP) activity in rats. Group 1 = *Anacardium occidentale* (100 mg/kg); Group 2 = *Anacardium occidentale* (250 mg/kg); Group 3 = *Eucalyptus globulus* (100 mg/kg); Group 4 = *Eucalyptus globulus* (250 mg/kg); Group 5 = *Psidium guajava* (100 mg/kg); Group 6 = *Psidium guajava* (250 mg/kg); Group 7 = *Xylopia aethiopica* (100 mg/kg); Group 8 = *Xylopia aethiopica* (250 mg/kg); Group 9 = Glibenclamide (5 mg/kg); Group 10 = Diabetic untreated; Group 11 = DMSO control.

mg/kg body weight) significantly ($p<0.05$) reduced ALP activity when compared to the reductions caused by *P. guajava* (100 and 250 mg/kg body weight). However, the decrease in ALP activity caused by glibenclamide (5 mg/kg body weight) was significantly ($p<0.05$) different from those of *A. occidentale* (100 mg/kg body weight), *E. globulus* (100 mg/kg body weight), *P. guajava* (250 mg/kg body weight) and *X. aethiopica* (100 mg/kg body weight). All the results show that the diabetic untreated groups had significant increases in the activities of all the enzymes assayed when compared to every other test group.

DISCUSSION

The effects of the chloroform extracts of the leaves of *A. occidentale*, *E. globulus* and *P. guajava* and fruits of *X. aethiopica* on ALT, AST and ALP in diabetic rats were investigated in the present study. Hepatic and cardiac tissues release aspartate and alanine aminotransferases and therefore, the elevation of plasma concentrations of these enzymes are indicators of hepatic and cardiac damage as in the case of complications in diabetes mellitus (Crook, 2006).

The reductions observed in ALT and AST activities could be said to have been caused by the hepatocellular and cardiac protection offered by these extracts. This is further confirmed by Ogbonnia et al. (2010) who studied the effect of a poly-herbal formulation on liver function enzymes in diabetic rats. It was noted that the administration of the poly-herbal formulation (which has *X. aethiopica* as one of its component) to rats led to a pronounced decrease in ALT and AST activities in the treated rats. The implication of this, is that the extract did not produce harmful effects on the hepatic tissues of the treated rats while in the diabetic untreated group, there were notable elevations in the activities of these two enzymes, an indication of hepatic and cardiac tissue damage.

The reduction in serum ALP activity recorded is suggestive of cellular membrane/hepatocellular membrane protective effects of the plant extracts. ALP functions as a biochemical marker enzyme for maintaining membrane integrity. Increase in its plasma activity indicates peroxidation of cell membrane which occurs during diabetes mellitus (Akanji et al., 1993). Uboh et al. (2010) showed that the aqueous extract of *P. guajava* confers hepatocellular protection in rats. The hepatocellular protection evidenced in the present study

might be due to the presence of flavonoids in the plant extracts. Flavonoids have been reported to possess antioxidant activity (Middleton, 1996) and thus, are capable of protecting cell membranes from peroxidative actions of free radicals.

In conclusion, the present study reveals that the chloroform extracts of the leaves of *A. occidentale*, *E. globulus* and *P. guajava* as well as the fruits of *X. aethiopica* showed potent protection on alloxan-induced acute liver toxicity in diabetic rats.

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UPCOMING CONFERENCES

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7th Annual World Congress of Industrial Biotechnology (IBIO-2014), Dalian, China, 25 Apr 2014

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