African Journal of Biotechnology

Volume 16 Number 31, 2 August, 2017

ISSN 1684-5315

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Vol. 16(31), pp. 1646-1649, 2 August, 2017
DOI: 10.5897/AJB2013.13259
Article Number: 5BE68A765589
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic and phenotypic variations in egg shell colour of two ectotypes of Giant African Land Snail (*Archachatina marginata* var. *saturalis*)

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Received 11 September, 2013; Accepted 18 July, 2016

Data from the snail farm of the University of Calabar, Nigeria were collected and utilized to investigate the distribution and gene frequencies of egg shell colour of two ectotypes of Giant African Land Snails *(Archachatina marginata* var. *saturalis*), black skinned (BS) × black skinned mating group, and white skinned (WS) × white skinned mating group. The eggs collected were scored for the presence of yellow (Y_p), light yellow (L_p) and milky white (M_p) egg shell colour. The egg shell colour distributions between the two ectotypes (BS × BS and WS × WS) were 46.50 vs. 3.77%, 42.68 vs. 48.11% and 10.83 vs. 48.11% for yellow, light yellow and milky white, respectively. In both ectotypes, the dominant gene for yellow, light yellow and milky white egg shell colour segregated at low frequencies (0.26 vs. 0.02, 0.24 vs. 0.31 and 0.06 vs. 0.31). The lowest value being yellow shell of the white skinned × white skinned mating group with frequency of 0.02, followed by milky white shell of the black skinned × black skinned mating group with frequency of 0.06. These values were much lower than the Mendelian value of 0.75. This is an indication that snails have not been purified through artificial breeding. Estimate of genetic distance between the two ectotypes were 0.060, 0.005 and 0.063 at yellow, light yellow and milky white loci, respectively. This shows that the ectotypes are closely related at the egg shell colour loci.

Key words: Genotype, phenotype, snail egg, shell colour, variations.

INTRODUCTION

Snail farming in Nigeria has received numerous attentions in the past decades (Akinnusi, 2002). Snail meat is widely consumed all over the world by both the rich and the poor (Murphy, 2001; Ebenso, 2003). The flesh of the Giant African Land Snail is of remarkable nutritive value, with high iron content (Ogbeide, 1974), and a protein content of 37.00 to 51.30 g/100 g dry matter (Udedibie, 1989). It is possible that snail eggs might also be of high nutritive value, though it is not consumed by humans in Nigeria at the present. Okon et al. (2013) reported that snail eggs are good sources of protein and basic minerals (K^+, Na^+, Ca^{++}, Fe^{++}, Mg^{++} and

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Zn^{++}) that compare favourably with the flesh and chicken eggs. With growing awareness of the role of cholesterol in various heart and arterial diseases, the demand for low cholesterol meat like snails and by extension snail eggs will become more acute. Characterising snail eggs will help consumers' choice and/or preference, just as does chicken eggs colour.

Among the most common land snails in West Africa are *Achatina achatina, Achatina fulica, Archachatina marginata* and *Limicolaria aurora* (Ejidike, 2002; Smith and Fowler, 2003). According to Smith and Fowler (2003), among the most common snails, *A. marginata* and *A. fulica* are truly Giant African Land Snails.

A. marginata is the second largest snail and most popular breed of snail kept and reared in Nigeria (Venette and Larson, 2004; Okon et al., 2012b). *A. marginata* produces a peristome with a reflected lip and reaches maturity 2 to 4 months later than *A. fulica* (Raut and Barker, 2002). The columella and parietal callus of *A. marginata* are either white or red in colour (Venette and Larson, 2004).

The physiological adaptability to the environment and genetic variation among and within breeds has a marked effect on the performance and productivity of snails than other factors (Okon et al., 2012a). The diversity of gene pool, natural selection and free mating among individuals has given rise to different population of snails. In genetic analysis, knowledge of relatedness or variation is used to estimate the genetic parameters such as heritability and genetic correlations (Falconer and Mackay, 1996). In artificial selection, estimation of breeding values relies on the knowledge of relatedness of individuals (Lynch and Walsh, 1998).

Characterisation of breed is the first approach to sustainable use of animal genetic resources. Studies on diversity and variability between and within breeds of Giant African Land Snails (GALS) on the basis of quantitative and qualitative variables have become inevitable (Okon et al., 2011; Ugwu et al., 2011; Okon et al., 2012b; Ibom et al., 2012). Information on the egg shell colour of this animal species is not available in the literature. The present study is aimed at providing information on the variation of egg shell colour among eggs produced from the mating between black skinned × black skinned and white skinned × white skinned ectotypes of *A. marginata var. saturalis* snails.

MATERIALS AND METHODS

The study was carried out at the Botanical Garden, University of Calabar, Calabar, Nigeria. Calabar is located within the geographical area of longitude 8°17' and 10°43' E of the Greenwich meridian, and latitude 4°58' and 15°39' N of the equator. The annual rainfall and temperature ranges between 1260 and 1280 mm and 25 to 30°C, respectively. The botanical garden is described in Okon et al. (2009a,b).

Eighty (80) adult snails comprising of 40 black skinned and 40 white skinned ectotypes of *A. marginata* snails were used for the study. The snails were purchased from a local market in Calabar. They were allowed to acclimatize for 35 days. This was to allow them shed the eggs they came with from the wild. They were allotted into two mating groups (black skinned × black skinned) and (white skinned × white skinned) on the basis of skin (foot) colour. There were two snails per cell and replicated 20 times to ensure that any egg emerging from the cell is a product of mating(s) between the two. The snails were housed in wooden cells measuring 40 × 40 cm by 30 cm within a hutch compartments.

The cells were filled with treated soil up to 15 cm depth from the bottom. Eggs were collected within 24 h of lay as the soil is turned and moistened. Moistening the soil helped maintain the humidity and moisture content.

The snails were fed a combination of concentrate and pawpaw leaves. The diet was formulated to contain 24% crude protein and 2580.36 Kcal/kg.

Eggs collected from the mating groups were classified according to their colours. The white skinned × white skinned mating group had a total of 106 eggs, comprising of 4 yellow shelled eggs, 51 light yellow shelled eggs and 51 milky white shelled eggs. The black skinned × black skinned mating group recorded a total of 157 eggs in the order of 73: 67:17 (yellow shelled eggs: light yellow shelled eggs: milky white shelled eggs), respectively.

Statistical analysis

The distribution of the various colours, yellow shelled eggs (Y), light yellow shelled eggs (L) and milky white shelled eggs (M) were expressed in percentages (Table 2). The frequencies of the recessive alleles (Y_a for recessive yellow colour, L_a for recessive light yellow colour and M_a for recessive milky white colour) were estimated using Hardy-Weinberg equilibrium (Falconer and Mackay, 1996) as follows:

$$q = \sqrt{N/M}$$

That is $q = \sqrt{M - d/M}$, where $N = M - d$

The frequency of the dominant allele (Y_p for dominant yellow colour, L_p for dominant light yellow colour and M_p for dominant milky white colour) were calculated as follows:

$$p = q - 1$$

Where q is the frequency of the recessive gene, N is the observed number of eggs exhibiting the particular recessive trait, d is the dominancy or dominant gene observed, M is the total number of eggs collected, and p is the frequency of a particular dominant allele.

The observed frequencies were then tested against the Mendelian ratio of 3:1 corresponding to the value of 0.75 for dominant allele and 0.25 for the recessive allele using Pearson's Chi- square test. Pearson's Chi-square test for goodness of fit is as follows:

$$X^2 = \sum (\text{Observed} - \text{Expected})^2 / \text{Expected}$$

The level of significance of the test was examined at $P<0.05$.

Genetic distance between the black skinned and black skinned ectotypes were estimated at the different egg colour locus using their respective gene frequencies. The method of Bodmer and Cavalli-Sforza (1976) was adopted as follows:

$$d^2 = (p_1 - p_2)^2$$

Where d^2 is the genetic distance estimate between the two populations, p_1 and p_2 are genetic frequencies of population one and population two, respectively.

Table 1. Distribution of eggs shell colours in percentages.

Traits	Mating groups							
	Black skinned × Black skinned				White skinned × White skinned			
	No. of eggs	Alleles	No. of eggs	%	No. of eggs	Alleles	No. of eggs	%
Yellow eggs shell (Y)	157	Y_P	73	46.50	106	Y_P	4	3.77
		Y_a	84	53.50		Y_a	102	96.23
Light yellow eggs shell (L)	157	L_P	67	42.68	106	L_P	51	48.11
		L_a	90	57.32		L_a	55	51.98
Milky white eggs shell (M)	157	M_P	17	10.83	106	M_P	51	48.11
		M_a	140	89.17		M_a	55	51.98

Table 2. Gene frequencies and Chi-Square (χ^2).

Traits	Alleles	Mating groups							
		Black skinned × Black skinned				White skinned × White skinned			
		Expected	Observed	G. Freq.	X^2 Test	Expected	Observed	G. Freq.	X^2 Test
Yellow eggs shell (Y)	Y_P	117.75	73	0.26	17.01^c	79.50	4	0.02	71.70^a
	Y_a	39.25	84	0.74		26.50	102	0.98	
Light yellow eggs shell (L)	L_P	117.75	67	0.24	21.57^b	79.50	51	0.31	10.22^b
	L_a	39.25	90	0.76		26.50	55	0.69	
Milky white eggs shell (M)	M_P	117.75	17	0.06	86.20^a	79.50	51	0.31	10.22^b
	M_a	39.25	140	0.94		26.50	55	0.69	

Y_P, L_P and M_P = Dominant allele for yellow, light yellow and milky white egg shell colours, respectively. Y_a, L_a and M_a= Recessive allele for yellow, light yellow and milky white egg shell colours, respectively. ^{abc}Means with different superscript along the same column are significantly different ($P<0.05$).

RESULTS AND DISCUSSION

Table 1 shows the distribution of the shell colour in percentages. The black skinned × black skinned mating group recorded 46.50% over white skinned × white skinned mating group with 3.77% for yellow egg shell colour. The same percentages were recorded by black skinned × black skinned mating group for both light yellow egg shell colour (48.11%) and milky white egg shell colour (48.11%). The black skinned × black skinned mating group recorded lower percentages in light yellow egg shell colour (42.68%) and milky white egg shell colour (10.83%). This apparent wide variation in egg shell colour is an indication that the population has not been purified through impeccable selective breeding (Yakubu et al., 2010).

The gene frequencies of the two ectotypes are presented in Table 2. The frequencies of the dominant alleles for both black skinned and white skinned ectotypes were 0.26 (Y_P,) vs. 0.02 (Y_P,), 0.24 (L_P) vs. 0.31 (L_P) and 0.06 (M_P) vs. 0.31 (M_P) (Table 2). These values were quite lower than the expected Mendelian value of 0.75. At the recessive allele, higher frequencies were observed for both black skinned and white skinned ectotypes; 0.74 (Y_a) vs. 0.98 (Y_a), 0.76 (L_a) vs. 0.69 (L_a) and 0.94 (M_a) vs. 0.69 (M_a), respectively.

Table 3. Genetic distances between Black Skinned and White Skinned Snail Ectotypes.

Traits	White Skinned vs. Black skinned
Yellow egg shell colour	0.060
Light yellow egg shell colour	0.005
Milky white egg shell colour	0.063

Table 3 shows the genetic distance between the black skinned and the white skinned ectotypes. The genetic distance between the black skinned and the white skinned ectotypes of *A. marginata var. saturalis* studied were 0.06, 0.005 and 0.063 at the yellow, light yellow and

milky white egg shell colour loci, respectively. Genetic distance makes it possible to evaluate the degree of genetic similarity between two populations by measuring the probability of one or more characters appearing in one population but not in the other (Sournia, 1991). The smaller value (0.005) obtained at the light yellow locus is an indication of phylogenetic relationship between the two ectotypes, while the higher values at the yellow and milky white loci are indicative of genetic differentiation which could be used to classify the two ectotypes into distinct population (Yakubu et al., 2010).

Conclusion

This study has shown that the ectotypes of snail influenced the gene frequencies of yellow, light yellow and milky white egg shell colours. The dominant alleles in both ectotypes were found to segregate at lower frequencies. Estimate of genetic distance showed the relatedness of white skinned and black skinned ectotypes. Efforts should be geared towards constructing genetic study that will find the genes associated with these morphological differences.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 16(31), pp. 1650-1659, 2 August, 2017
DOI: 10.5897/AJB2016.15846
Article Number: 8D1977265593
ISSN 1684-5315

http://www.academicjournals.org/AJB

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Full Length Research Paper

Exploring genetic diversity and structure of *Acacia senegal* (L.) Willd. to improve its conservation in Niger

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Received 20 December, 2016; Accepted 26 July, 2017

The genetic diversity of African forest resources is poorly documented while it can be the basis for adapting these resources to climatic variations. This study aimed to characterize the genetic diversity of the *Acacia senegal* (L.) Willd in its natural range in Niger. 252 individuals from 10 populations of the species, across three gum basins were analyzed with 9 nuclear microsatellite markers. Genetic diversity indexes are high in all the populations studied: number of allele (Na) varies from 4.00 to 5.44; allele richness (R) varies from 3.42 to 4.49; observed heterozygosity (Ho) and expected heterozygosity (He) range from 0.44 to 0.56 and from 0.46 to 0.63, respectively. The values of differentiation index (Fst) per pair of population range from 0.0057 to 0.110 and 20% of these values are not significant indicating a low differentiation between populations. In addition, Molecular Variance Analysis shows that 93% of total variation is within populations. Through Bayesian model, a structure of population into three groups is observed. These results could form the basis for building sustainable management and conservation strategies of genetic resources of *A. senegal* in Niger.

Key words: Genetic diversity, microsatellites, *Acacia senegal*, climate change, Niger.

INTRODUCTION

The major challenge of biological conservation is the preservation of genetic diversity in the maintenance of the evolutionary process of species (Assogbadjo et al., 2006). Using genetic data to determine evolutionary relationships between species and populations significantly contributes to their conservation (Chiveu et al., 2008). Genetic variation in several trees species on earth is a trans-generational resource of great social, economic and environmental importance. Commonly referred to as "forest genetic resources", these variations are expressed by differences between species, populations, and individuals or chromosomes and have an actual or

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potential value (Young et al., 2001).

The management of forest genetic resources has caused serious national and international concern over the past few years due to overexploitation and unfavorable climatic conditions which have led to severe degradation of ecosystems. This phenomenon affects particularly the Sahelian region including Niger.

Among African forest species, *Acacia senegal* (L.) Willd. has a wide distribution in the arid and semi-arid regions of Africa, from South Africa northwards to Sudan (Maydell, 1983), because it tolerates aridity and eroded soils (Raddad et al., 2005; Chiveu et al., 2008). *A. senegal* (L.) Willd is a leguminous multipurpose species belonging to the subgenus *Aculeiferum* (Arce and Blanks, 2001). The international botanical community accepts the retypifying *Acacia* with a new type which would place most species ascribed to the present subgenus *Aculeiferum* into the genus *Senegalia* (Boatwright et al., 2014; Kyalangalilwa et al., 2013; Haddad, 2011).

In Niger, *A. senegal* is distributed along a climatic gradient between the isohyets 620 and 250 mm (Elhadji et al., 2012, 2016), forming a band from west to east and of which three gum basins are distinct (western basin, central basin and eastern basin) (FAO, 2003). However, since the droughts of 1974 and 1982, natural stands of *A. senegal* are constantly degrading under combined effects of anthropogenic actions and climatic variability (Amani, 2010). The deforestation of the natural stand of the gum groves for installation of crop fields, the exploitation of wood for domestic energy and mutilation of trees to feed animals constitute some factors of deterioration (Ichaou, 2008). In addition to the aforementioned factors, aging of trees, their high mortality and low capacity of regeneration are added (Maisharou and Nourou, 2004). Indeed, the natural stand regeneration, which is mainly done by seeds coming from the trees *in situ*, would depend on the maintenance of genetic diversity of the populations. The genetic diversity would also help to cope with unpredictable climatic changes or to establish breeding programs (Blanc et al., 1999). It is important to know the structure of genetic diversity which constitutes the partition of variation between different populations and which could be their capacity to adapt to environmental changes.

While the importance of *A. senegal* in rural economy (Guinko et al., 1996) and soil stabilization and fertility (Abdou et al., 2013; Wickens et al., 1995) is undeniable, the importance of genetic diversity of natural populations in Niger is still very poorly studied. The knowledge of the partitioning of genetic variation within species is a prerequisite for defining strategies for conservation, management and sustainable use of the species' resources. The main objective of the study is to analyze the genetic diversity and structure of the natural stands of *A. senegal* in Niger through (i) the quantification of genetic diversity within each population, (ii) the determination of genetic structure of populations and (iii) the estimation of gene flow between these populations.

MATERIALS AND METHODS

Presentation of study sites

The study is conducted in the three basins of gum arabic production in Niger, namely western basin, central basin and eastern basin (Figure 1). The sites of western basin (Kiki, Kokoyé, Tempena and Bégorou) are characterized by a Sahelian climate with two seasons (a rainy season from June to October and a dry season from November to May) with rainfall varying between 620 and 400 mm. For the sites of the central basin (Aseye, Dakoro and Bader), the annual rainfall varies between 400 and 350 mm and the climate is Sahelo-Saharan with a long dry season (9 months) and a short rainy season. In the sites of the eastern basin (Malam Mainari, Gouré and Nguel kolo), annual rainfall varies between 300 and 250 mm and this site is characterized by a dry climate (a long dry season and rainy season hardly reaching 3 months in the year). In all the sites, the vegetation consists of woody species: *Acacia tortilis, Acacia seyal, Acacia laeta, Acacia dudgeoni, Balanites aegyptiaca, Boscia senegalensis, Combretun glutinosum, Combretum micratum, Calotropis procera, Leptadenia pyrotechnica, Maerua crassifolia, Ziziphus mauritiana, Tamarandis indica* and Herbaceous: *Alysicarpus avalifolius, Cyperus indica, Eragrostris tremula, Acanthospermum hispidum, Cenchrus biflorus.* But *A. senegal* is dominant. The highest average daily temperature (38.50°C) is observed to the east and north center of Niger, corresponding to the sites of the eastern and central basins in April and May of the year. While in the west, it varies between 35 and 36.50°C (western basin).

Sampling

Ten natural *A. senegal* populations scattered in the three gum basins were sampled (Figure 1 and Table 1). The number of sample per population and basin is consigned in Table 1. A total of 252 trees (young and adult) distant at least 50 m fresh leaves were collected to avoid sampling related to trees. Such a sampling plan makes it possible to capture the maximum of variability within a population. Leaf samples were dried using silica gel before shipment to the genetics laboratory of the IRD (Niamey) representation in Niger.

DNA extraction and SSR marker analysis

DNA was extracted from 0.5 g of dried leaves using the protocol described by Ky et al. (2000). The quality of DNA was assessed by electrophoresis on a 1% agarose gel and quantified using a nano-drop spectrophotometer (ND1000, Thermo Scientific, USA) at Center for Medical Research and Sanitary (CERMES) in Niamey, Niger. Nine nuclear SSR markers (mAsCIRB10, mAsCIRC07, mAsCIRE06, mAsCIRE07, mAsCIRE10, mAsCIRF02, mAsCIRF03, mAsCIRH01 and mAsCIRH09) developed in *A. senegal* var. *senegal* by Assoumane et al. (2009) were used for diversity analysis. The 252 samples were genotyped at the ICRISAT Plant Genetics Laboratory in India.

Data analysis and statistical test

Genetic diversity

The following five parameters were used to quantify genetic

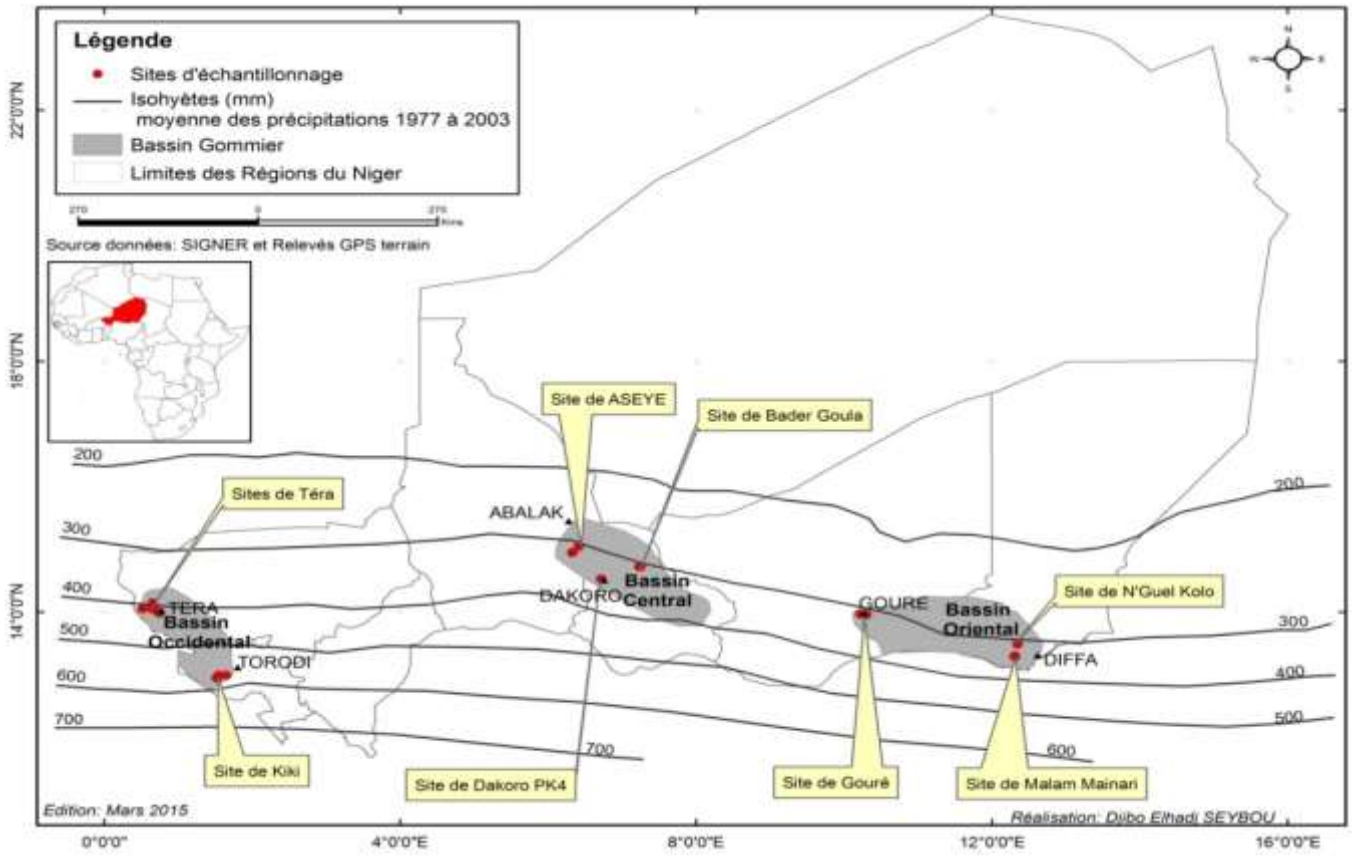

Figure 1. Location map of the study sites.

Table 1. Samples per site and localisation of the sites.

Basin	Site	Sample	Localization
Occidental	Tempena	17	N12°57'04,1" E01°31'14,6"
	Kiki	22	N12°59'35,8" E 01°32'59"
	Begorou	23	N14°03'3,9" E00°38'58,1"
	Kokoyé	32	N13°59'10,30" E 00°44'35"
Central	Aseye	36	N15°02'46,2" E06°24'53,7"
	Bader	25	N14°43'19,7" E07°14'2,6"
	Dakoro	12	N14°31'42,9" E06°42'53,1"
Oriental	Gouré	22	N13°58'06,8" E10°13'33,3"
	Malan Mainari	32	N13°17'26,2" E12°18'29,2"
	N'guel kolo	31	N13°29'24,2" E12°20'51,9"
Total	10	252	-

diversity in the studied populations: Number of alleles per locus (Na), Allelic richness (R); this parameter determines the mean number of alleles taking into account sample size, which conditions the number of alleles observed in the population, observed heterozygosity (Ho), expected heterozygosity (He), and Wright (1965) fixation index (Fis). Fstat software 2.9.3 (Goudet, 2001) was used to calculate these diversity parameters.

Population's genetic structure

Four methods of analysis were used: (1) Fst calculation using Fstat 2.9.3 software (Goudet, 2001); (2) genetic distance tree using Darwin 5.0.85 software (Perrier and Jacquemond-Collet, 2006); (3) Bayesian model using STRUCTURE 2.3.1 software (Pritchard et al., 2000) and (4) Analysis of Molecular Variance Analysis)

Table 2. Parameters of genetic diversity in 10 natural populations of *Acacia senegal* distributed in the three gum basins in Niger.

Gum basins	Population	Average rainfall (mm)	Na	R	Ho	He	Fis (W&C)	N
Western basin	Kokoye	414.2	5.22	4.17	0.50	0.58	0.143**	32
	Begorou	414.2	4.67	3.93	0.49	0.54	0.093^{ns}	23
	Tempena	621.6	5.00	4.49	0.56	0.63	0.123*	17
	Kiki	621.6	4.33	3.80	0.49	0.56	0.118*	22
Eastern basin	Malan Mainari	309.2	4.56	3.53	0.44	0.49	0.102*	32
	N'guel kolo	309.2	4.33	3.48	0.46	0.48	0.033^{ns}	31
	Goure	-	4.00	3.42	0.46	0.46	-0.008^{ns}	22
Central basin	Dakoro	436.5	4.22	4.15	0.52	0.57	0.076^{ns}	12
	Bader	375.7	5.22	4.41	0.48	0.55	0.126**	25
	Aseye	350	5.44	4.07	0.48	0.55	0.131**	36

Na: Average number of alleles; R: allelic richness; Ho: observed heterozygosis; He: expected heterozygosis; Fis: fixing index estimated by Weir and Cockerham (1984); N: number of samples. Level of significance: *P <0.05; **P <0.01 and ns not significant.

(AMOVA) using the GenAlEx 6.5 program (Peakall and Smouse, 2012).

Fst calculation: The significance of Fst values were tested at the 5% threshold after 1000 permutations of alleles within populations.

Gene flow between populations was estimated by the indirect method based on the assumption of mutation-drift equilibrium. The number of migrants per generation (Nm) is calculated from the average of Shannon diversity index. The calculation was done with the GenAlEx version 6.5 software (Peakall and Smouse, 2012) according to the following classical formula:

$$Nm = \frac{1 - Fst}{4Fst}$$

Fst: index of population differentiation.

Genetic distance tree: Jaccard's distance was used to calculate the matrix of genetic distances because some individuals had more than two alleles for a locus. Jaccard's distance makes it possible to obtain an unbiased estimate of the genetic distance between individuals, as it does not consider the absence of the allele (0) as a similarity. It is calculated by the following formula:

$$d_{ij} = \frac{b + c}{a + (b + c))}$$

where dij = dissimilarity between two units i and j; a = number of variables for which xi = presence and xj = presence; b = number of variables for which xi = presence and xj = absence; c = number of variables for which xi = absence and xj = presence; xi and xj being the values of the variables for units i and j.

A Neighbour-Joining genetic distance tree (Saitou and Nei, 1987) was constructed from the genetic distance matrix for all samples.

Bayesian model: The genetic structure was also described by a Bayesian model using the software STRUCTURE 2.3.1 (Pritchard et al., 2000). In the STRUCTURE program, the admixture correlated allele model was used. This model assumes that all individuals share common ancestors and that alleles are correlated within populations. Thirteen "runs" were made to test independent K-values using 400,000 MCMC (Markov Chain Monte Carlo) and 40,000 burn-in periods.

Analysis of Molecular Variance (AMOVA): The distribution of genetic variation was evaluated specifying that the population groups constituting the three basins. AMOVA applied formula is:

$$F_{rt} = AR/(WI + AI + AP + AR) = AR/TOT$$

where AR = Estimated variance among basins; AP = Estimated variance among populations; AI = Estimated variance among individuals; and WI = Estimated variance within individuals.

RESULTS

Genetic diversity

The diversity parameters data are shown in Table 2. The number of alleles per locus (Na) varies from 4 (Gouré) to 5.44 (Aseye). The allelic richness (R) varies from 3.42 (Gouré) to 4.49 (Tempena). The heterozygosis is also higher in Tempena (He = 0.63, Ho = 0.56) and lower in Goure (He = 0.46; Ho = 0.46). These parameters (Na, R, Ho and He) reveal a fairly high genetic diversity in the natural populations studied. The fixation index (Fis) is different from zero and positive in all populations except in Bégorou, N'guelKolo, Dakoro and Gouré populations, where Fis is not significantly different from zero. The overall value of Fis is 0.104 and is significantly different from zero.

Genetic structure

Differentiation detected by Fst

Table 3 presents the matrix of differentiation index (Fst) calculated by population pair. The populations of same gum basin have low Fst values that are not often

Table 3. Fst matrix per population pair.

Population air	Western basin			Eastern basin			Central basin		
	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8	Pop9	Pop10
Pop1	0.0057^{ns}	0.0206^{ns}	0.0145^{ns}	0.1063*	0.0926*	0.0808*	0.0081^{ns}	0.0409*	0.0282*
Pop2		0.0349^{ns}	0.0067^{ns}	0.0962*	0.0817*	0.0538*	0.0269^{ns}	0.0618*	0.0376*
Pop3			0.0036^{ns}	0.0803*	0.0944*	0.0786*	0.0529*	0.0513*	0.0565*
Pop4				0.1103*	0.1107*	0.0892*	0.0536*	0.0784*	0.0644*
Pop5					0.0075^{ns}	0.0451*	0.0944*	0.0531*	0.0885*
Pop6						0.0641*	0.0971*	0.0640*	0.0917*
Pop7							0.0716^{ns}	0.0371*	0.0553*
Pop8								0.0220^{ns}	-0.0064^{ns}
Pop9									0.0212^{ns}

ns: Non significant; *Significant at 5% threshold ; Pop1: Kokoye; Pop2: Begorou; Pop3: Tempena; Pop4: Kiki; Pop5: Malan Mainari; Pop6: N'guel kolo; Pop7: Goure; Pop8: Dakoro; Pop9: Bader ; Pop10: Aseye.

Table 4. Matrix of mean gene flux values (Nm) between populations calculated from Shannon Diversity Index means over 9 loci.

Western basin				Eastern basin			Central basin			Population
Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8	Pop9	Pop10	
0.000										Pop1
15.635	0.000									Pop2
5.271	3.169	0.000								Pop3
8.179	7.788	8.267	0.000							Pop4
0.993	1.618	1.076	1.016	0.000						Pop5
1.126	1.863	0.857	1.006	18.543	0.000					Pop6
1.517	2.900	1.045	1.320	5.506	4.724	0.000				Pop7
5.546	3.177	1.032	1.344	1.768	1.603	2.119	0.000			Pop8
2.420	2.069	1.393	1.130	1.871	1.494	2.673	3.448	0.000		Pop9
2.960	2.479	1.393	1.357	1.408	1.179	2.771	5.743	8.557	0.000	Pop10

Pop1: Kokoye; Pop2: Begorou; Pop3: Tempena; Pop4: Kiki; Pop5: Malan Mainari; Pop6: N'guel kolo; Pop7: Goure; Pop8: Dakoro; Pop9: Bader ; Pop10: Aseye.

significant at the 5% threshold. The overall Fst is 0.059; this reflects moderate differentiation according to Wright's ranking. In addition, overall average number of migrants (Nm) per generation is 3.288. It is high between populations in same basin and moderately low between populations of different basins (Table 4).

Structure detected by genetic distance tree

The individual genetic distance tree of the Neighbour-Joining type (Saitou and Nei, 1987) presents a low individualization of branches. However, almost 3 distinct groups can be observed corresponding to 3 gum basins and two pools gathering individuals from all the basins (Figure 2).

Structure detected by Bayesian model

For K = 3, the model highlights three groups corresponding exactly to 3 gum basins (Figure 3). Nevertheless, in each group, individuals with a genetic proportion coming from the neighbouring groups were observed.

When using a K = 2 value to evaluate population structure, populations of western basin and central basin formed a single group and eastern basin populations form a second group (Figure 4).

Genetic variability partitioning by AMOVA

The AMOVA showed that 83% of variation is within individuals, 10% between individuals, 2% between populations and 5% between basins (Table 5).

DISCUSSION

The distribution of forest tree species has a major impact on the extent of their genetic variability and structure. *A. senegal* is widely distributed in Niger, from west to east

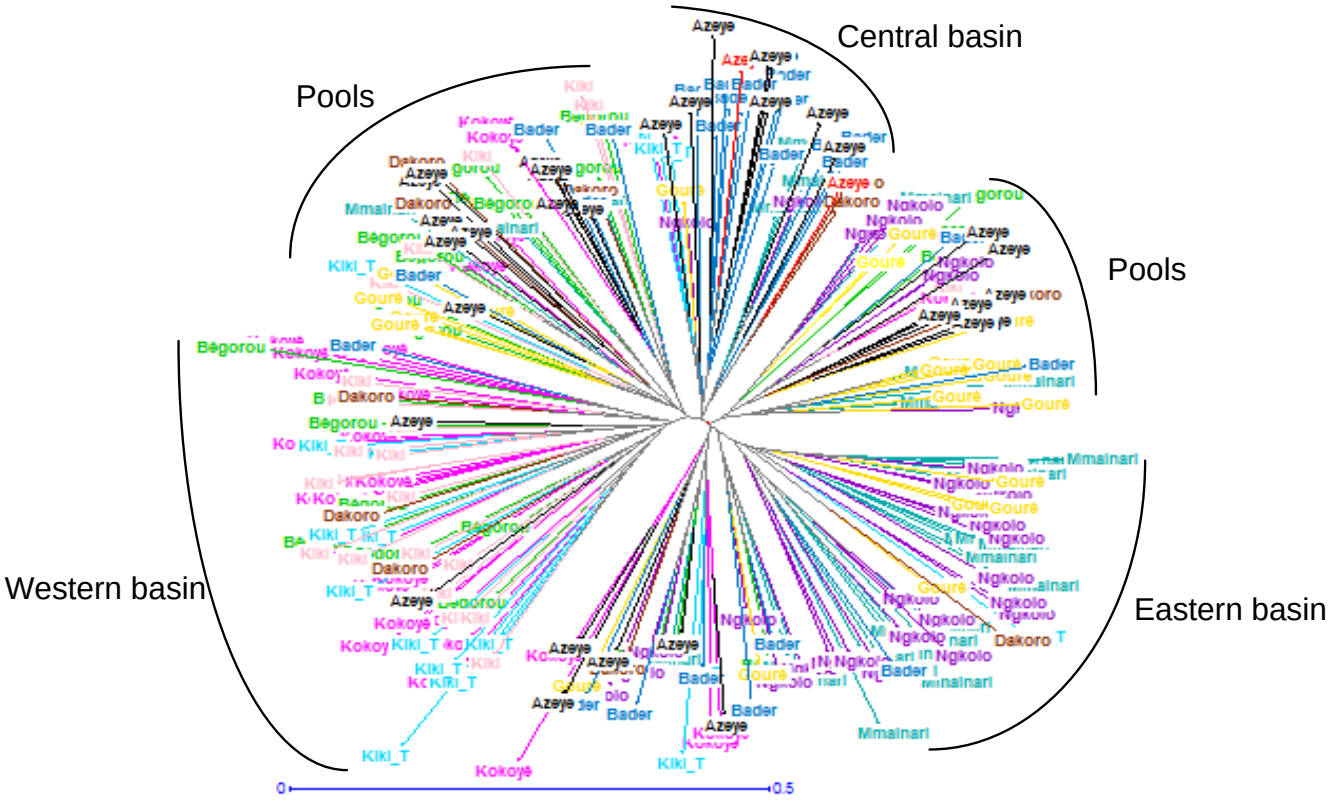

Figure 2. Neighbour-joining tree realized according to Jaccard distance. Individuals in the same color belong to the same population.

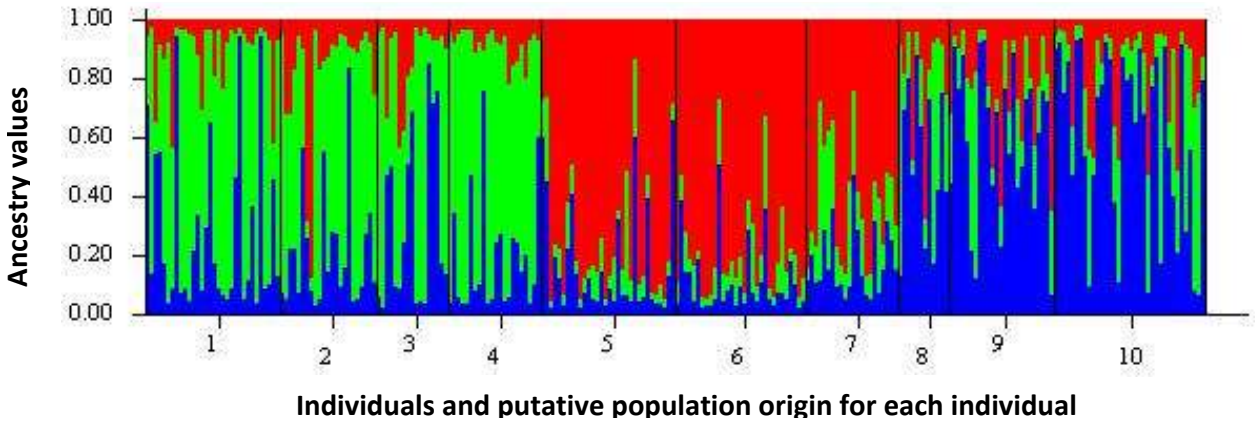

Figure 3. The grouping of individuals and populations realized using the model "admixture alleles correlated" and K = 3. The groups are represented by different colors (green, red and blue) and individuals are represented by vertical lines. The same color in different individuals indicates that these individuals belong to the same group. Different colors in the same individual group indicate the posterior probability of belonging to different groups. Populations 1, 2, 3 and 4 belong to the western basin; populations 5, 6, and 7 belong to the eastern basin and populations 8, 9 and 10 belong to the central basin.

forming a broad band in which three large gum basins were singled. In this study, the populations of these gum basins have a fairly high mean genetic diversity (Ho = 0.48, He = 0.54) compared to values found for other forest species analyzed with the same types of markers (Table 6). Genetic diversity in *A. senegal* species is

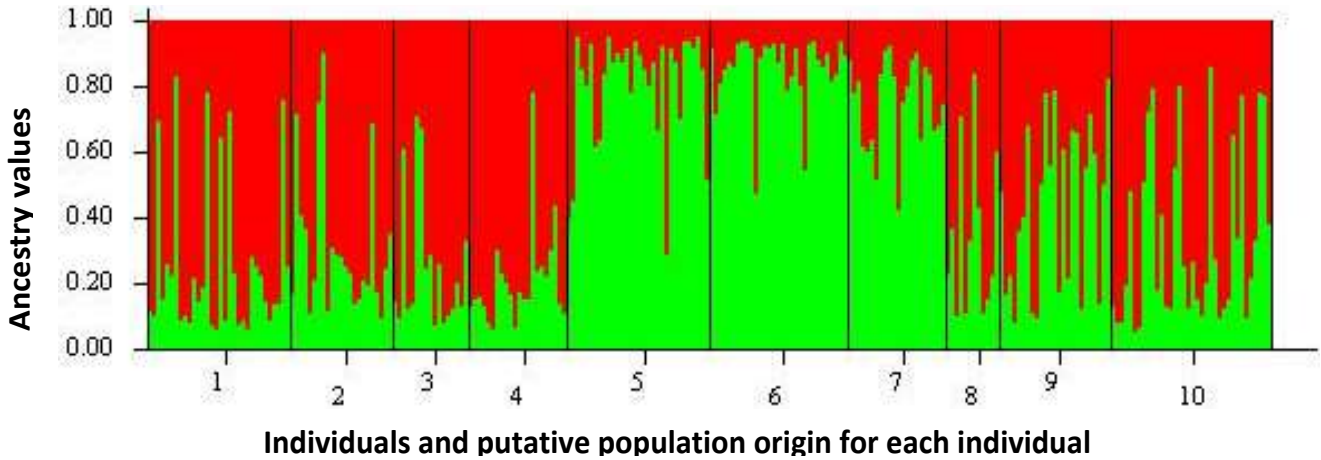

Figure 4. The grouping of individuals and populations realized the model "admixture alleles correlated" and K = 2. The groups are represented by different colors (green, red) and individuals are represented by vertical lines. The same color in different individuals indicates that these individuals belong to same group. Different colors in the same individuals indicate the posterior probability of belonging to different groups. Populations 1, 2, 3 and 4 belong to the western basin; populations 5, 6, and 7 belong to the eastern basin and populations 8, 9 and 10 belong to the central basin.

Table 5. AMOVA of 252 individuals belonging to 10 populations of *Acacia senegal* distributed in the three gum basins in Niger.

Source of variation	df	SS	MS	Est. Var.	%/total	P-value
Among-basins	2	55.51	27.75	0.13	5	<0.001
Among-populations	7	35.92	5.13	0.05	2	<0.001
Among-individuals	242	647.14	2.67	0.25	10	<0.001
Within-individuals	252	546.50	2.17	2.17	83	-
Total	503	1285.07	-	2.60	100	-

higher than average genetic diversity of tropical trees (He = 0.211) and conifers (He = 0.207) (Borgel et al., 2003). However, the genetic diversity observed in *A. senegal* is lower than that found in *Acacia mangium* (He = 0.70) by Butcher et al. (2000) or in *A. senegal var. kerensi (*He =0.63-0.70) by Omondi et al. (2009).

The fixation index (Fis) indicated a deficiency in heterozygosity from expected results under the assumptions of Hardy Weinberg. A positive value of Fis indicates a heterozygosity deficiency relative to the panmictic balance. A deficiency in heterozygosity can be explained mainly by 3 factors (Assoumane, 2011; Mohamed et al., 2010; Jordana, 2003): breeding regime that does not favor cross-breeding, existence of null alleles and effect of Wahlund. Indeed, for crossbreeding between relatives, it is well known that inbreeding (mating between an individual and his ascendants, his collaterals and/or his descendants) modifies the genotypic frequencies resulting in loss of genetic variability over generations. The second factor may be inherent to the existence of null alleles for some loci. Null alleles are alleles which do not amplify during polymerase chain reaction (PCR) due to mutation in the binding of the primer region. Then, individuals are considered homozygous for these loci, while they are heterozygous and this phenomenon would reduce the number of heterozygous. The last factor refers to the presence of subpopulations within populations considered (substructure) can induce Wahlund effect. The presence of null alleles was not tested in our data. Nevertheless, a Wahlund effect can be assumed because at Aseye population where two groups distant about 10 km were sampled, a Fis value (Fis = 0.131) was found which is significant at the 1% threshold. Then the two groups can be considered as different populations. The Fis (0.104) is comparable to that Fis (0.08) measured by Assoumane et al. (2012) and significantly different from zero in a population of *A. dudgeoni* (Dogona) in the western areas of Niger.

The Fst calculated among all populations (Fst = 0.059) indicates moderate differentiation among populations. In addition, Fst observed in the study is comparable to those found in Table 6 in forest species with same markers. The genetic differentiation level is due to the existence of gene flow among populations.

The importance of gene flow between populations could be explained by the mode of seed dispersal and

Table 6. Parameters of diversity and genetic differentiation among populations of some forest species, estimated with nuclear microsatellites.

Species	Distribution	Dispersion mode	N	He	Fis	Fst	Reference
A. senegal (L) Willd.	Continental	Anemochore/Zoochore	252	0.46-0.63	-0.01-0.143	0.059	This study
Dacryodes buettneri	Continental	Barochore/Anthropochore	170	0.35	0.25	0.08	Todou et al. (2013)
Dacryodes edulis	Continental	Barochore/Anthropochore	524	0.47	0.12	0.03	Todou et al. (2013)
A. senegal (L) Willd.	Continental	Anemochore/Zoochore	469	0.41-0.60	-0.21-0.08	0.09	Assoumane (2011)
A. senegal var. kerensi	Continental	Anemochore/Zoochore	300	0.63-0.70	-0.20-0.04	0.04	Omondi et al. (2009)
Pterocapus officinalis Jacq.	Insular	Barochore/Hydrochor	202	0.25-0.59	-0.04-0.37	0.29	Muller et al. (2008)
Santalum insulare	Insular	Zoochore	162	027-056	-0.10-0.14	0.23	Lhuillier et al. (2006)
Vitellaria paradoxa	Continental	Barochore/Zoochore	169	0.25-0.42	-0.22-0.18	0.05	Sanou et al. (2005)
Vouacapoua americana	Continental	Zoochore	408	0.34-0.52	0.09-0.22	0.08	Dutech et al. (2004)
Swietenia macrophylla	Continental	Barochore/Zoochore	194	0.78-0.81	0.06-0.10	0.1	Lemes et al. (2003)
Swietenia macrophylla	Continental	Barochore/Zoochore	284	0.59-0.80	0.07-0.24	0.11	Novick et al. (2003)
Grevillea macleayana	Continental	Barochore	130	0.42-0.53	0.07-0.31	0.22	England et al. (2002)
Caryocar brasiliense	Continental	Barochore/Zoochore	314	0.58-0.85	0.07-0.05	0.11	Collevatti et al. (2001)
Melaleuca alternifolia	Continental	Barochore/Zoochore	500	0.13-0.92	0.07-0.29	0.07	Rossetto et al. 1999
Symphonia globulifera	Continental	Barochore/Zoochore	914	0.72-0.85	0.2	-	Aldrich et al. (1998)

N: Number of samples; He: expected heterozygosity; Fis: fixation index; Fst: differentiation index.

the presence of pollinators. Seed dispersal of *A. senegal* can be ensured by wind (anemophilic mode) after dehiscence of the pods. The socio-economic role of *A. senegal* in rural households (gum arabic) could also contribute to the exchange of plant from region to another and village to another (Ichaou, 2008). In addition to human action (anthropozoic mode), animals through grazing also contribute significantly to gene flows among *A. senegal* populations. Indeed, most of the studied populations constitute grazing areas for transhumant herders moving from one point to another and facilitating seed dissemination.

According to Kremer et al. (2012) and Buckley et al. (2010), improved forest species adaptation to changes in environmental conditions is better when there is more gene flow between populations up. For Slatkin (1987), a low gene flow (Nm<1) results from local differentiation that leads to genetic drift and a higher gene flow (Nm>1) makes population adjustment for survival. Values of 1 to 10 migrants per generation are essential for restoration and resistance to genetic drift and prevention of natural selection (Blanquart and Gandon, 2011; Lopez et al., 2009). In ours study, the average number of migrants per generation (Nm) is 3.288 and high Nm are observed between populations in the same gum basin. Low Nm is recorded between the most geographically distant populations: Kokoyé-Malan Mainari with Nm = 0.993 and Tempena-N'guelkolo with Nm = 0.857. Indeed, populations of low Nm are spaced further by 1500 km, which would probably reduce gene flow between them.

The total variability is explained by intra-population variation (93%) and 2 and 5% of variability are attributed, respectively to variations among populations and among gum basins. This result, which confirms that of Fst, is in agreement with Chavallier and Borgel (unpublished results), who observed that most genetic variability in higher plants and particularly in woody plants is within Populations (Fst = 8.45%). However, Rathore et al. (2016) observed in 11 populations of *Gymnema sylvestre* contrary results. These authors found a strong genetic differentiation of populations of the species (Fst = 70%) and low gene flow (Nm = 0.21) through SSR markers. Their results are explained by the great geographical distance between populations studied, which created a geographic isolation limiting the possibilities of gene flow among populations.

With STRUCTURE program, individuals group by basin mainly due to small difference between populations that constitute the gum basin.

Geographical proximity promotes gene flow

between populations of the same basin. This is confirmed for k = 2, where we got the grouping of populations of western basin and those of central basin in the same group. The assignment of populations of two gum basins in a group is probably due to gene flow favored by low geographical distance between them.

Implications for sustainable management and conservation of the species

The assessment of genetic variability among and within forest tree populations is a prerequisite for any restoration or renewal of these species (William and Hamrick, 1996). From results of this study, we can say that conservation of the species in Niger could be done easily through the method of *in situ* conservation. However, the species is threatened by human actions related to abuse of natural resources and degradation of its habitat. Effective and rigorous measures are needed to reverse current trends, because if nothing is done, it will inevitably lead to the disappearance of several populations of the species and these resources. The organization of genetic variability in the species revealed in this study is an important parameter to include in any future sustainable management strategy. Two aspects are of primary importance for the implementation of *in situ* conservation programs: local pedoclimatic conditions of origin and genetic diversity within the species. Indeed, the inclusion of these aspects would avoid the lack of seed germination, seedling mortality and/or long term loss. The effective management of genetic resources requires the identification of priority areas where conservation efforts can be better focused. Structuring by gum basin identified in our study helps to retain basin scale as an *in situ* conservation unit. Any time, if needed, seeds from western basin can be used to repopulate populations of central basin and vice versa, because there is a similarity (low differentiation) between the two gum basins.

Conclusion

Knowledge of genetic diversity and its partitioning within the species is essential to build any strategy for management and sustainable conservation of natural resources. In this study, a relatively high genetic diversity was found in natural populations of *A. senegal*. The bulk of genetic variability in the species is within populations. The populations belonging to the same gum basin are similar, resulting from gene flows between them. The interpretation of individual's differentiation within populations assumed a Whalund effect. Further studies need to be conducted to confirm this factor or to explore other factors explaining the deficit in heterozygote's observed in more than half of the studied populations.
These results could be used as part of the restoration of degraded lands and rangelands through the plantation of *A. senegal* performed annually by the environmental services, development programs and projects and national, international non-governmental organizations (NGOs). It is recommended to use the seeds from the populations of the catchment area of the intervention zone to set up the nurseries.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful to AIRD for funding the research program on the Agriculture Adaptation to Climate Change (AVACLI), which supported the realization of the study. They are also grateful to Dr Salako Kalawolé Valere from Laboratory of Bioinformatics and Forestry resource Estimation (LABEF) at Abomey Calavi University for English grammatical arrangement.

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Vol. 16(31), pp. 1660-1669, 2 August, 2017
DOI: 10.5897/AJB2017.15889
Article Number: 5AB3F8565597
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Evaluating auto-detoxification of *Jatropha curcas* Linnaeus, 1753 kernel cake with brine shrimp *Artemia salina* (Linnaeus, 1758) lethality test

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Receieved 12 January, 2017; Accepted 6 July, 2017

***Jatropha curcas* Linnaeus, 1753 is a plant with several uses for communities in Cameroon and other African countries. However, present methods of detoxifying *J. curcas* kernel cake (JKC) to add value to its further utilization are too sophisticated for cottage operations in rural areas. This study sought to evaluate two auto-detoxification methods using the shellfish brine shrimp, *Artemia salina* (Linnaeus, 1758). Two identical *J. curcas* auto-detoxification apparatuses (JADA) were developed: one simulating diffuse daylight (DJADA) and the other solar irradiation (SJADA) conditions. JKC was pretreated by either soaking or boiling in water or lye. The pretreated samples were then placed in the JADA and either re-moistened to 66% dry matter (DM) or left un-moistened. Among the 14 pretreated samples, water soaked solar without re-moistening (WS1), lye soaked solar without re-moistening (LS1), and water soaked solar with re-moistening (WS2) had LC_{50} values above 1000 ppm after four weeks. On the other hand, un-moistened diffuse daylight spread (UDC), boiled in water and exposed to diffused daylight without remoistening (WD3), and un-moistened solar spread (USS) were the least detoxified within the same period of four weeks with LC_{50} values less than 60 ppm. Irrespective of the pretreatment, extracts from the SJADA had higher LC_{50} values when compared to that of DJADA. This finding suggests that sunlight is an important factor in auto-detoxification. The top three most efficient detoxified treatments: WS1, LS1 and WS2 are recommended for further development and testing with livestock and fish models. This study confirms that JKC is toxic, but under natural conditions is exposed to auto-detoxifying factors which are both endogenous and environmental. When these conditions are optimally manipulated, the detoxification rate is enhanced. Farmers in rural communities can use this strategy to detoxify JKC, transform it into livestock and fish dietary ingredient and consequently enhance its contribution to climate change mitigation.**

Key words: *Jatropha curcas*, auto-detoxification, solar irradiation, *Artemia salina*.

INTRODUCTION

The reality of climate change has encouraged a search for renewable energy sources. However, the cultivation of energy crops for biofuel production has resulted in an increased competition between food and fuel. Therefore,

renewable energy crops such as *J. curcas* L. which are not consumed by humans have gained interest (Devappa, 2012). As an endemic plant of tropical America, *J. curcas* is widely distributed throughout the tropics as an ornamental and medicinal plant. It produces high-quality biodiesel fuel after the appropriate processing of oil extracted from its seeds. The nutrient profile of the resultant *J. curcas* seed cake (JSC) or *J. curcas* kernel cake (JKC) surpasses the Food and Agriculture Organization of the United Nations (FAO) reference protein apart from lysine (Makkar et al., 1998). JSC is toxic to humans and animals even after its further processing. *J. curcas* can provide opportunities for additional income and therefore improve food security (Lyimo, 2010). To enhance the value of JKC, several methods have been proposed for its detoxification, such as: (1) solvent extraction methods (Chivandi et al., 2004; Martınez-Herrera et al., 2006; Makkar et al. 2008; Rachadaporn et al., 2012); (2) chemical treatment methods (Makkar and Becker, 1998; Haas and Mittelbach, 2000; Aregheore et al., 2003; Devappa and Swamylingappa, 2008; Azza and Ferial, 2010); (3) bio-detoxification methods such as fungal detoxification (Belewu and Akande, 2010; Belewu et al., 2010 Azhar et al., 2014; Sulaiman et al., 2014), bacterial detoxification (El-Zelaky et al., 2011; Phengnuam and Suntornsuk, 2013; Widiyastuti et al., 2013; Chin-Feng et al., 2014) and ensilage (Oliveira et al., 2012); physical methods such as ionizing radiation (Runumi et al., 2014), ozonation and solar irradiation (Susan et al., 2015), heat (Workagegn et al., 2013), roasting (Azza and Ferial, 2010), germination (Azza and Ferial, 2010), soaking (Azza and Ferial, 2010), and boiling (Annongu et al., 2010; Fakunle et al., 2013; Alatise et al., 2014). All these methods are characterized by different levels of success. An improved efficiency is observed after the combination of two or more approaches. The challenge today therefore is not only the JKC detoxification to levels safe enough for animal consumption, but also whether the detoxification method can stimulate local interest by ensuring its biological, chemical, technical and economic feasibility for large scale industrial as well as cottage operations in rural communities of resource-poor countries like Cameroon.

The majority of the current successful detoxification methods is sophisticated and cannot be applied at a cottage industry level in rural communities where *J. curcas,* which has already several uses, is additionally expected to contribute in energy security and poverty alleviation. This fact poses limitations on its potential contribution to sustainable agriculture strategies, since climate activists suggest that 80% of current crude oil reserves should remain in the subsoil in order to avoid the critical 2°C rise in global temperature. Easily applied methods such as boiling need further investigation, because phobol esthers (PE), the main toxic component in JKC, are heat stable. They remain in the JKC even when autoclaved at 121°C for 30 min (Aregheore et al., 2003). However, the naturally occurring phobol esters are unstable and are susceptible to oxidation, hydrolysis, transesterificaion and epimerization during isolation procedures (Runumi et al., 2014). By taking into consideration this background, an effort needs to be made to find an easy and cheaper method to detoxify *J. curcas* for rural communities in countries like Cameroon. The present study was therefore aimed to develop two *J. curcas* auto-detoxification apparatuses (JADA) and test their efficacy using brine shrimp lethality test.

MATERIALS AND METHODS

Design and construction of *J. curcas* auto- detoxification apparatuses (JADA)

A natural convection greenhouse solar drier with chimney (Ekechukwu, 1999) was modified to a *J. curcas* auto detoxification apparatus (JADA) by compartmentalizing the access door air inlet in such a way that various levels of air access could be achieved. This modification allowed the adjustment of the apparatus from full detoxification (when air access is completely closed) to full drying mode (the access is fully open). Between these two extreme modes, partial drying and detoxification was also possible. The JADA was constructed to have a triangular drying chamber with a height of 2.7 m, fitted on a rectangular base measuring 2.3 m wide and 5 m long. This chamber had an attached 2.3 m high vertical cylindrical chimney at one end, while at the other end a door existed for air inlet and access to the chamber. Thus, the total height of the chimney from the base of the apparatus was 5 m and had a north-south orientation. In Buea, located at 4.1667° N, and 9.2333° E, the chimney was located in the south and the door in the north. The floor was insulated with 30 cm thick compacted wood shaving covered with black plastic to keep it in place and absorb light. On the top of the floor were two rock piles gathered to retain any trapped heat during the day. The rock piles were covered externally by black corrugated sheets which trapped heat from the sun.

Two identical *J. curcas* auto-detoxification apparatuses were constructed at the Faculty of Agriculture and Veterinary Medicine Farm in the University of Buea. In the first, the north was covered with transparent plastic, and the rest of the apparatus covered with black plastic to simulate diffuse daylight conditions and was named diffuse daylight *J. curcas* auto-detoxification apparatus (DJADA). In the second, the northern end with the door was covered with black plastic sheets, while the rest of the apparatus was covered with transparent plastics to maximize the effects of solar irradiation and was called solar *J. curcas* auto-detoxification apparatus (SJADA).

Preparation and pre-treatment of *J. curcas* kernel cake (JKC)

Jatropha curcas seeds were harvested from farms, live fences and plantations within six regions of Cameroon

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(South West, North West, Littoral, Adamawa, North and Extreme North). The seeds were transported to the University of Buea where they were manually de-shelled to produce kernels. The kernels were de-oiled using a hydraulic press and *J. curcas* kernel cake (JKC) was produced. The JKC was ground into powder using a plate mill, and the powder was homogenized by hand mixing. The JKC samples were either physically or chemically pre-treated before being placed in the two types of JADA for detoxification.

Physical pretreatment was performed by soaking or boiling the JKC samples in water. In the physical pretreatment by soaking, homogenized JKC was soaked overnight in water at the ratio of 1:2 w/v by adding fixed weight of JKC to fixed volume of water and stirring the mixture to ensure complete dispersion in water. This procedure resulted in a pretreatment additional moisture content of 66.67%. This material was thereafter divided into 4 treatments (WS1, WS2, WD1 and WD2) with 4 replicates per treatment. In the boiling method, homogenised JKC was poured into boiling water in an aluminium pot at the ratio of 1:2 w/v and stirred continuously while still on fire for 30 min to produce dough. The resultant dough was sub-divided into two treatments (WS3 and WD3) each with 4 replicates.

Chemical pretreatment was achieved by soaking or boiling the JKC samples in lye. The lye used was produced locally by modifying the procedure described by Kent et al. (2014). About 50 kg of wood ash from a local palm oil processing plant was leached for three days in a 120 L plastic tank fitted with a tap. In the chemical pretreatment by soaking, homogenized JKC was over soaked overnight in lye at the ratio of 1:2 w/v by adding fixed weight of JKC to fixed volume of lye and stirring the mixture to ensure complete dispersion in lye. This procedure offered a pretreatment additional moisture content of 66.67%. This material was thereafter divided into 4 treatments (LS1, LS2, LD1 and LD2) with 4 replicates per treatment. In the boiling method, homogenised JKC was poured into boiling lye at the ratio of 1:2 w/v and stirred continuously while still on fire for 30 min to produce dough. The resultant dough was sub-divided into two treatments (LS3 and LD3) each having 4 replicates.

Auto-detoxification of the JKC

The 12 pretreated JKC samples (WS1, WS2, WS3, WD1, WD2, WD3, LS1, LS2, LS3, LD1, LD2 and LD3) plus two untreated or unmoistened samples (USS and UDC) were subjected to auto-detoxification using the JADA. The two JADA modifications were operated at full detoxification mode. The samples were placed in steel plates that were arranged on two tables within the JADA; each treatment had four replicates. The WS1, WS2, WS3, LS1, LS2, LS3 and USS were placed in the SJADA while WD1, WD2, WD3, LD1 LD2, LD3 and UDC were placed in DJADA (Table 1). During the detoxification process, WS2 and WD2 and LS2 and LD2 were remoistened daily with water and lye, respectively, to obtain a moisture level of 66% every morning. However, there was no remoistening on the day proceeding the day of sample collection for evaluation (Table 1).

Assessment of the level of detoxification of JKC by performing the brine shrimp lethality test

Preparation of crude methanol auto-detoxified J. curcas kernel cake extracts (CMJKCE)

Forty grams of each treatment sample (10 g per replicate) were collected weekly to assess the level of auto-detoxification. The four replicates per treatment were pooled and further dried within the JADA for another week. Each sample was homogenised, powdered and 20 g of it were mixed with 200 ml of methanol for 72 h with regular agitation. Filtration using Whatman No. 541 filter papers followed and the CMJKCE were obtained by complete evaporation of the solvent using a rotary evaporator.

Brine shrimp bioassay

The brine shrimp lethality test (BSLT) as described by Meyer et al. (1982) was used to test toxicity of CMJKCE after some modifications. The toxicity is reported as LC_{50} values in mg/ml (ppm). Brine shrimps [*Artemia salina* (Linnaeus, 1758)] were hatched from eggs in rectangular dishes divided by a perforated barrier, under constant aeration using natural seawater. The perforated barrier divided the dish into two chambers. The bigger chamber receiving the eggs was in the dark while the smaller chamber receiving the hatched nauplii in anticipation was under constant light. Forty eight hours were allowed for the eggs to hatch and the photropic nauplii to mature. Ten nauplii were collected using a pipette and placed in marked vials, each containing 4 ml of natural seawater. Two hundred miligrams of each examined CMJKCE were dissolved in 2 ml of pure dimethyl sulfoxide (DMSO) to get a solution of 100,000 ppm (100,000 mg/l). A unit volume of this solution was diluted in one mililitre of natural seawater to give a concentration of 50,000 ppm. Ten-fold serial dilutions were further performed in sea water to produce concentrations of 5000, 500, 50 and 5 ppm. One milliliter of each of these dilutions was delivered into pre-marked vials containing 4 ml of natural sea water and 10 nauplii. As a result, the final concentrations of CMJKCE were 10,000, 1000, 100, 10 and 1 ppm. Three replicates of each concentration were prepared for CMJKCE samples. The negative controls were dilutions of DMSO in seawater without CMJKCE. Un-detoxified whole *J. curcas* kernel cake served as the positive control. The vials were kept incubated under light for 24 h after which the surviving nauplii were counted. The nauplii were counted against a lighted background using a 3× magnifying hand lens. They were considered dead if they lay immobile at the bottom of the vial. The mortality percentage was then calculated. The mortality data was corrected using Abbott's (1925) formula (Apu et al., 2013), since a mortality percentage more than 10% was recorded in the controls. Subsequently there were corrections for 0 and 100% as proposed by Ghosh (1984). The formulae used in calculating and correcting brine shrimp mortality are presented in Table 2: The surviving nauplii were killed by addition of 5 ml of 5% v/v phenol to each vial. The vials were crosschecked to ensure that all the nauplii were dead before discarding.

Determination of 50% lethal concentration (LC_{50})

The lethal concentration of CMJSCE resulting in 50% mortality of brine shrimp (LC_{50}) was determined from the 24 h counts by a plot of percentage of the shrimps killed against the logarithm of the CMJKCE concentration. The best-fit line was obtained from the curve data by means of regression analysis (MS Excel version 7) and the LC_{50} was derived from the slope of the best-fit line obtained.

Statistical analysis

Levene's test for equality of variances and t-test for equality of means were performed on LC_{50} using the IBM SPSS Statistics version 22 (IBM Corp. Released 2013). The mean (±SEM) were quarreled.

Selection of the most promising auto-detoxified JKC (ADJKC) treatments for further development

Selection of the most promising ADJKC treatments was achieved

Table 1. Summary of JKC auto-detoxification treatment groups.

Form of treatment of JKC	Lighting exposure (JADA)	
	Solar spread (SJADA)	**Diffuse daylight spread (DJADA)**
Un-moistened	USS (un-moistened solar spread)	UDC (un-moistened diffuse daylight spread)
Soak in water. No remoistening	WS1 (water soaked, solar spread without remoistening)	WD1(water soaked, exposed to diffuse daylight without remoistening)
Soak in lye. No remoistening	LS1 (lye soaked, solar spread without remoistening)	LD1 (lye soaked exposed to diffuse daylight without remoistening)
Soak in water. Remoistening to 66% DM	WS2 (water soaked, solar spread and remoistened to 66 % DM)	WD2 (water soaked exposed to diffuse daylight and remoistened to 66 % DM)
Soak in lye. Remoistening to 66% DM	LS2 (lye soaked, solar spread and remoistened to 66 % DM)	LD2 (lye soaked exposed to diffuse daylight and remoistened to 66 % DM)
Boil in water. No remoistening	WS3 (boil in water and solar spread, without remoistening)	WD3 (boil in water, exposed to diffuse daylight without remoistening)
Boil in lye. No remoistening	LS3 (boil in lye, solar spread without remoistening)	LD3 (boil in lye exposed to diffuse daylight without remoistening)

Table 2. Formulae used in calculating and correcting brine shrimp mortality.

Method	Formulae
Percentage mortality	% of mortality = (number of dead nauplii / total number) × 100
Abbott (1925) formula	Corrected % mortality = {(Mobs - Mcontrol) / (100 - Mcontrol)} × 100. Where, Mobs = observed % mortality; Mcontrol = control % mortality
Ghosh (1984) 0 and 100% correction	For 0% mortality: 100 × (0.25 × n). For 100% mortality: 100 × (n - 0.25/n); Where, n= no. of test animal in each group

by ranking the LC_{50} from the largest to the smallest values with the largest values indicating the least toxicity. A t- test was used to compare the LC_{50} of ADJKC from the SJADA and DJADA to determine the significance of solar radiation in auto-detoxification of JKC at an experimental level.

RESULTS

The level of auto detoxification of the different experimental groups has been ranked using the mean of the LC_{50} values for all the treatments from weeks 1 to 4 (Table 3). From the results presented in Table 3, WS1, LS1 and WS2 treatments all had LC_{50} values above 1000 ppm after four weeks of exposure in the JADA, indicating that they were the most detoxified. On the other hand UDC, WD3 and USS were the least detoxified within the same period of 4 weeks with LC_{50} of less than 60 ppm.

The extent of lethality was found to be proportional to the concentration of the CMJKCE. High mortality rates were recorded at 1000 and 10,000 ppm, while lower values were recorded at 1 and 10 ppm. Mortality rates of brine shrimp exposed to whole *J. curcas* kernel cake just after oil extraction are presented in Figure 1. Thereafter, corrected brine shrimp mortality rates in the 4^{th} week for UDC, WS1, LS1 and WS2 are presented in Figures 2 to 5, respectively.

The means (and standard error of the means) of the LC_{50} of the extracts of solar and diffused daylight detoxified JKC extracts over the four week detoxification period are presented in Table 4. Irrespective of the pretreatment, SJADA treated extracts had higher LC_{50} values compared to the DJADA ones, with the WS1, LS1 and WS3 having significantly higher values ($P<0.05$) compared to WD1, LD1 and WD3 groups, respectively (Table 4).

DISCUSSION

The JADAs were easy to construct onsite because of their simple design and low cost which is characteristic of natural convection greenhouse solar driers with chimney. These characteristics attributed to natural convection greenhouse driers (Weiss and Buchinger, http://www.aee-intec.at/0uploads/dateien553.pdf) make development of the JADA a suitable tool for *J. curcas* detoxification in remote rural communities. Other solar dryer designs may not be appropriate for re-design into a

Table 3. LC_{50} of crude methanol auto detoxified *J. curcas* kernel cake extracts (CMJKCE) ranked from highest to least detoxified (mean of 4 weeks).

Treatment	Week 1	Week 2	Week 3	Week 4	Mean LD_{50}
WS1	776.24712	986.12330	1,050.32265	1,483.32158	1,074.00366
LS1	873.32616	913.38623	981.89826	1,087.76440	964.09376
WS2	92.08173	419.98449	1,057.21209	1,836.53834	851.45416
WS3	58.42075	120.43669	280.94207	395.32296	213.78062
WD2	63.90805	80.78246	183.36201	383.08736	177.78497
LS3	30.07266	74.42734	121.45608	304.24754	132.55091
WD1	38.74078	50.79456	123.68662	204.03416	104.31403
LD1	38.22643	51.12171	131.46350	154.46779	93.81986
LS2	9.32810	17.22766	127.26505	201.52612	88.83673
LD2	0.88057	1.91720	92.08173	63.89076	39.69256
USS	15.79273	16.66887	49.32035	58.28871	35.01766
LD3	3.02178	5.56577	26.00650	98.35801	33.23802
WD3	0.37125	1.08390	5.12075	21.06534	6.91031
UDC	0.09094	0.35058	2.90193	5.21849	2.14049
Whole *J. curcas* kernel cake	0.00035				0.00009

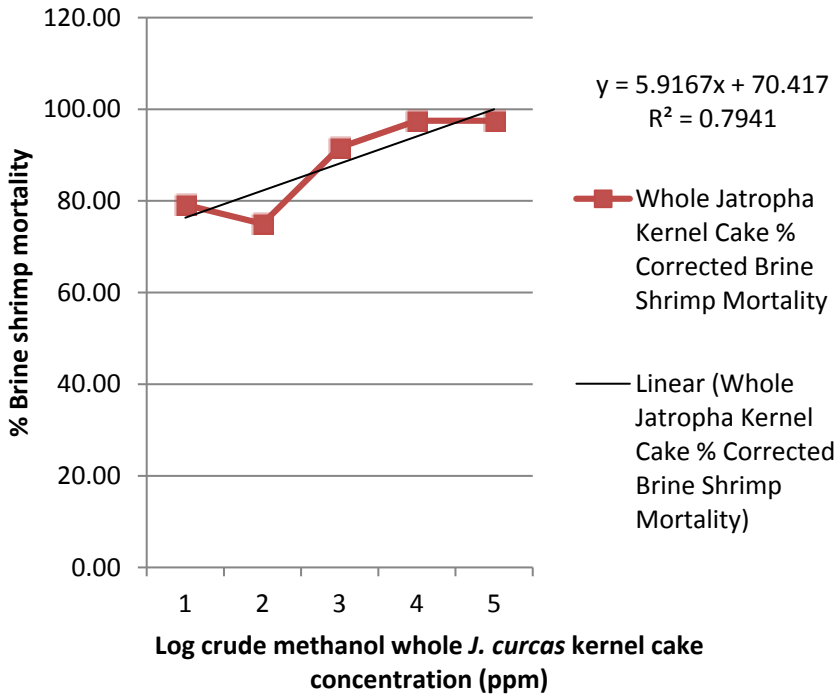

Figure 1. Mortality rates (%) of brine shrimp exposed to whole *J. curcas* kernel cake just after oil extraction.

JADA. This is because in the JADA, there is need for the crop to have optimised access to environmental conditions such as light, temperature, moisture, wind, oxygen and microbes that contribute to its auto-detoxification. Besides, *J. curcas* is a tropical and sub-tropical crop, grown in areas with ubiquitous solar radiation.

The proportional response of brine shrimp to the increased concentration of the CMJKCE confirms that auto-detoxification was effective in reducing JKC deleterious effects. The present study also supports the earlier findings of Chikpah and Demuyakor (2012), who found that approximately 60% reduction in crude phorbol ester levels can be achieved within 21 days of

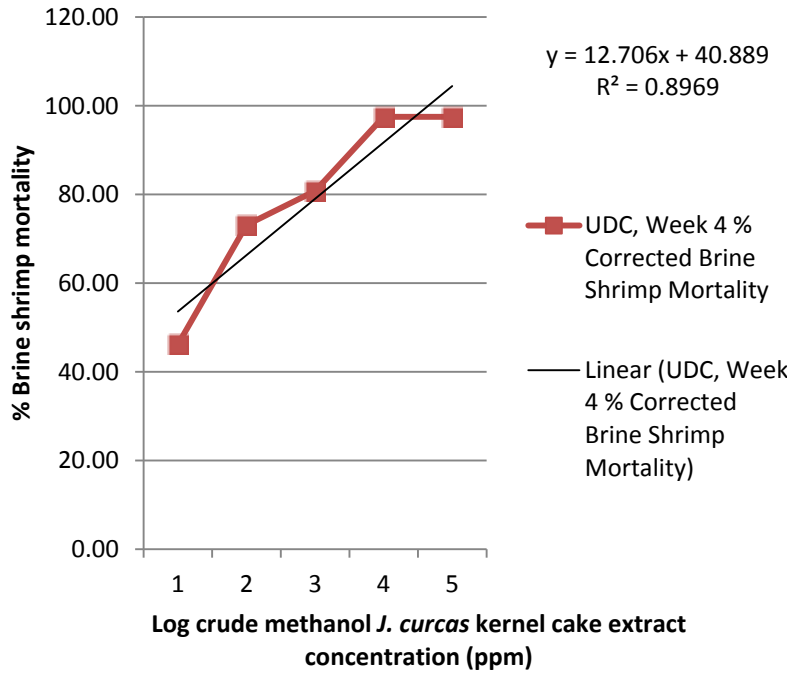

Figure 2. Corrected brine shrimp mortality rates (%) for UDC (Un-moistened diffused daylight spread) JKC treatment after 4 weeks of auto detoxification.

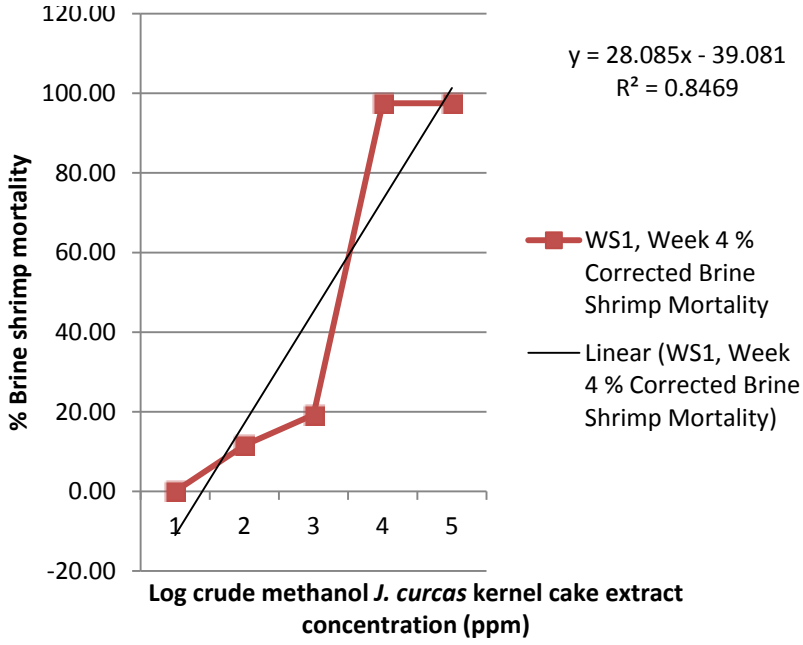

Figure 3. Corrected brine shrimp mortality rates (%) for WS1 (water soaked, solar spread, without remoistening) JKC treatment after 4 weeks of auto detoxification.

spontaneous fermentation of *J. curcas* kernel meal.

According to Meyer et al. (1982), several extracts derived from natural products which had LC_{50} ≤ 1000 µg/ml using BSLT were known to contain physiological active principles while those with LC_{50} values > 1000 ppm are considered inactive. Thus, exposure of the JKC in the JADA for four weeks rendered them inactive and therefore the detoxified cake extract was less toxic to

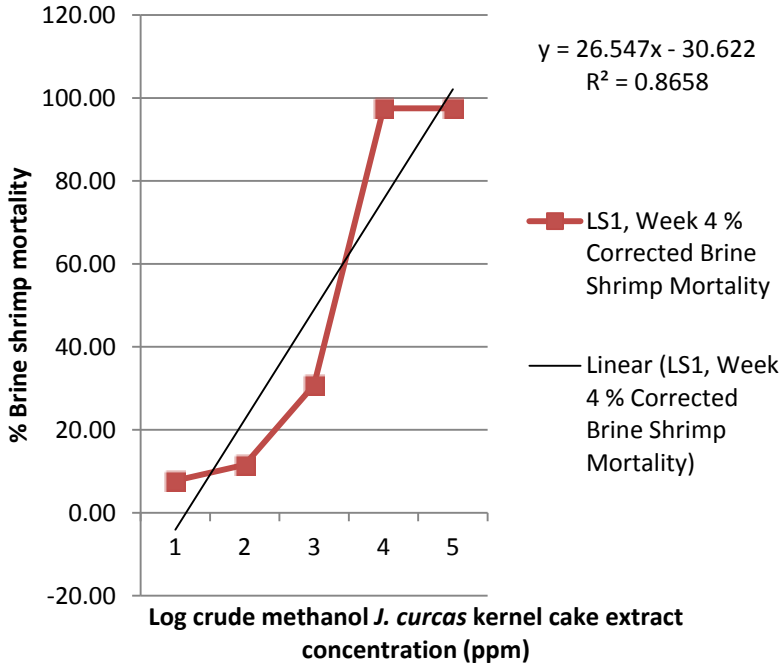

Figure 4. Corrected brine shrimp mortality rates (%) for LS1 (Lye soaked, solar spread, without remoistening) JKC treatment after 4 weeks of auto detoxification.

brine shrimps. However, working with phorbol ester enriched fraction (PEEF) of *J. curcas* seeds, Devappa et 72%, which was achieved at 47 mg L^{-1}, further increase in concentration was not effective in increasing mortality rates of *A. salina*. 100% mortality was only achieved at a high concentration of 6000 mg L^{-1}. This observation prompted the testing of CMJKCE at a concentration of 10,000 ppm in the present study.

The *J. curcas* plant contains many biologically active phytochemicals including proteins, peptides and diterpenes which exhibit a wide range of biological activities (Devappa et al., 2010, 2011a). However, the seeds contain anti-nutritional and toxic factors such as phytate, trypsin inhibitor, lectin, curcin and phorbol esters (PEs) (Makkar et al., 1997). In the majority of the cases, al. (2011b) observed that after a mortality "plateau" of toxicity of seeds and seed derivatives is attributed to the presence of PEs (Devappa et al., 2011b). In a previous study, Kinghorn et al. (1977) had demonstrated that there are differences in the toxicity of specific purified phorbol esters. While purified PEs such as phorbol 12-tetradconoate 13-acetate, phorbol 12, 13-didecanoate, and phorbol 12, 13-dibenzoate induced toxicity with an effective mortality dose (ED_{50}) of 3.8, 6.8 and 11.8 mg L^{-1} respectively, the phorbol and 4a-phorbol 12, 13-didecanoate were relatively nontoxic (ED_{50} > 1000 mg L^{-1}). Referring to the above study, Devappa et al. (2011b) concluded that bioactivity of PEs depends on their chemical structure/configuration and purity.

The BSLT only indicates the level of toxicity of an extract or a substance and does not identify the toxic component. However, it can be used anywhere in the world as a general toxicity test because the eggs are readily available. It is a rapid, inexpensive and simple bioassay for testing plant extract lethality which in most cases correlates reasonably well with cytotoxic and anti-tumour properties (McLaughlin et al., 1991). This assay is considered as a useful tool for preliminary assessment of toxicity and it has been used for the detection of fungal toxins, plant extract toxicity, heavy metals, pesticides and the cytotoxicity testing of dental materials (Harwing and Scott, 1971; McLaughlin et al., 1991; Martinez et al., 1998; Barahona and Sanchez-Fortun, 1999; Pelka et al., 2000). Comparing the BSLT with the respective snail bioassay, Devappa et al. (2011b) concluded that the BSLT is less sensitive towards *J. curcas* PEs with respect to toxicity screening. Nonetheless, the use of *A. salina* and *Daphnia magna* Straus, 1820 is preferred for assaying a large number of PE samples because the test can be performed in 96-well plates (Ruebhart et al., 2008). At the same time, BSLT is the only feasible choice in countries like Cameroon, where the common bladder snail [*Physa fontinalis* (Linnaeus, 1758)], un-adapted to tropical environments, is not readily available and the response of local snails to PEs has not yet been studied. In addition, the preliminary testing of ADJKC for toxicity should not only be focused on PEs but should detect additional toxins developed during the process. Makkar et al. (2012) recognized that no information is available on the nature of the phorbol ester degraded products and

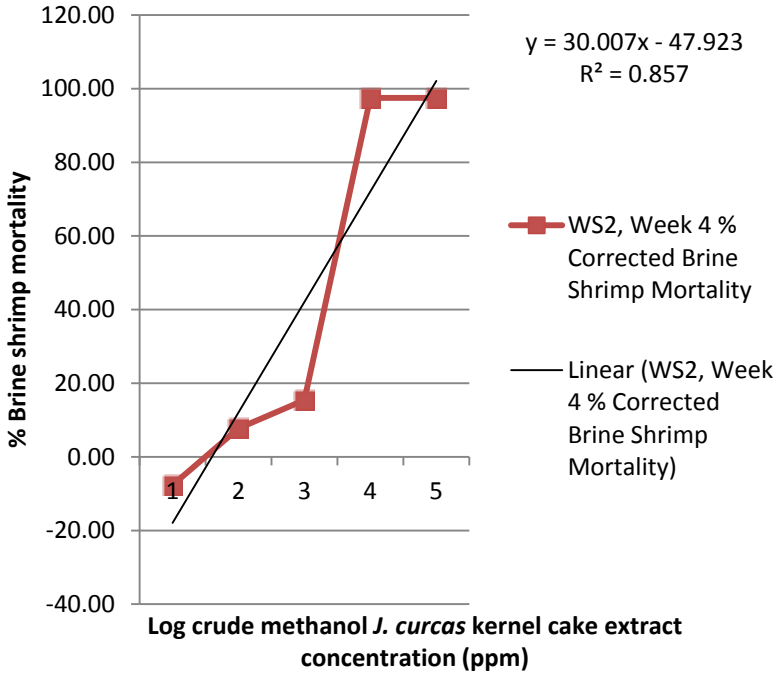

Figure 5. Corrected brine shrimp mortality rates (%) for WS2 (water soaked, solar spread and remoistened to 66% DM) JKC treatment after 4 weeks of auto detoxification.

Table 4. Comparism of mean (± sem) LD_{50} for solar JADA and diffused daylight JADA.

Pre-treatment	Mean (± sem) LC_{50} SJADA	Mean (± sem) LC_{50} DJADA	P-value
WS1/WD1	1,074.004 ± 148.56	104.314 ± 38.170	0.001
LS1/LD1	964.094 ± 46.923	93.820 ± 28.881	0.000
WS2/WD2	851.454 ± 384.658	177.785 ± 73.347	0.136
WS3/WD3	213.781 ± 76.550	6.910 ± 4.833	0.036
LS3/LD3	132.551 ± 60.196	33.238 ± 22.310	0.173
LS2/LD2	88.837 ± 46.211	39.693 ± 22.846	0.377
USS/UDC	35.018 ± 11.001	2.140 ± 1.206	0.025

Sem = Standard error of the mean.

their possible toxicity. The naturally occurring PEs are unstable and are susceptible to oxidation, hydrolysis, trans-esterification and epimerization during isolation procedures (Runumi et al., 2014). Auto detoxification could exploit the opportunities presented by these PEs properties to render JKC innocuous.

Irrespective of the pretreatment, SJADA extracts had higher LC_{50} values compared to the DJADA ones. This observation indicates that exposure of JKC to sun-light has a significant effect on the auto-detoxification process. Auto-detoxification is a self-detoxification process induced by endogenous and environmental factors including enzymes, microbes, sunlight, temperature, humidity and wind. It is the natural way to transform the toxic *J. curcas* seeds into an innocuous material. These processes take considerable time under natural conditions but their duration can be shortened by human manipulation. Materials are pre-treated through physical, chemical or both ways, in order to induce the necessary changes that contribute in their detoxification. One of the known changes is a process called autoxidation. Autoxidation is any oxidation that occurs in open air or in presence of oxygen (and sometimes UV radiation) and forms peroxides and hydroperoxides. In a wider sense, any oxidation process which produces peroxy radicals, irrespective of their origin, is also considered as an autoxidation process (Michael, 1981). The main toxin of *J. curcas* seeds is represented by the phorbol esters that are highly susceptible to autoxidation. Schmidt and Hecker (1975) investigated the various products arising from two PEs - 2-O-tetradecanoylphorbol-13-acetate (TPA) and 4a-phorbol-12, 13-diacetate – derived by

autoxidation under different conditions of storage. It was found that in addition to the traces of polar products that were not isolated, the aldehyde, 0-epoxide, hydroperoxide, hydroxylated A5-6 isomer, ketone and 6, 7-seco hemiacetal are formed from TPA. If TPA is stored as a powder at 25°C in diffuse daylight for 3 months, it is converted preferentially into the hydroperoxide, with smaller amounts of the other products. If spread on a large surface as a thin film (on glass plates, beads, or silica gel thin layers) and kept in diffuse daylight at 25°C, TPA is oxidized very rapidly. In this case, the 6, 7-seco hemiacetal is formed preferentially, in addition to smaller amounts of the other products. This rapid conversion is dependent on light and the reaction is slowed down in the dark. Moreover, among the various autoxidation products that could be obtained from TPA, a sample of 4a-phorbol-12, 13-diacetate yielded (~30%) the corresponding 20-aldehyde as the only conversion product after storage for 1 year at room temperature in the dark. Storage in the dark is mandatory to avoid the known "lumi reaction" of 4a-phorbol, which is independent of the presence of oxygen (Schmidt and Hecker, 1975). It can be concluded from the above study that all phobol esters do not follow the same path of autoxidation in the presence of light. This may explain the differences in auto-detoxification observed between the SJADA and DJADA. The challenge for auto-detoxification of *J. curcas* seed materials is to maintain an adequate post-detoxification value for their utilization as feed ingredients for livestock and fish.

The top three most detoxified treatments: WS1, LS1 and WS2 are recommended for further development and testing as livestock and fish dietary ingredients by using the respective farm animal models. WS3 could also be considered for further development. It could be possible that pre-treatment heating influenced some factors that subsequently affected the level of auto-detoxification of WS3. Aregheore et al. (2003), found that autoclaving at 121°C for 30 min did not change the PE content in *J. curcas* kernel meal. However, the concentration of lectins was significantly ($P<0.05$) reduced by the heat. Makkar et al. (2012) recognize that the main category of toxic compounds, the phorbol esters, is to a large extent heat stable. Therefore, other strategies must be applied for their removal. Alatise et al. (2014), Fakunle et al. (2013) and Annongu et al. (2010) concluded that *Clarias gariepinus* (Burchell, 1822) could tolerate up to 30% of boiled *J. curcas* kernel meal in the diet in place of soybean meal. One of the challenges concerning the future development of WS3 treatment is that it uses heat as a pre-treatment prior to auto-detoxification. This may increase its carbon footprint particularly in countries like Cameroon where 98.7% of rural households rely on wood as their only fuel source (Ego, 2009). Moreover, Cameroon's wood biomass demand grows nearly at the same pace with total energy demand; approximately 2.4% per year (AEEP, 2013).

Conclusion

This study has confirmed that JKC is quite toxic. However, under natural conditions, it is exposed to auto-detoxifying factors which are both endogenous and environmental. When these conditions are optimally manipulated as in the SJADA, the detoxification rate is enhanced. Solar radiation plays an important role in auto detoxification process of JKC. Farmers in rural communities can use this strategy to detoxify JKC, transform it into animal feed and consequently enhance its contribution to climate change mitigation. The role of microbial succession in auto-detoxification needs to be further investigated. Although the BSLT is rather inadequate in identifying the toxic ingredients in ADJKC, it is useful to determine the level of detoxification. The top three most detoxified treatments: WS1, LS1 and WS2 are recommended for further development and testing as livestock and fish dietary ingredient.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 16(31), pp. 1670-1677, 2 August, 2017
DOI: 10.5897/AJB2017.16044
Article Number: 099A99665601
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Purification and characterization of thermostable and alcohol tolerant lipase from *Pseudoxanthomonas* sp.

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Received 23 April, 2017; Accepted 25 July, 2017

An extracellular lipase from *Psedoxanthomonas* sp. was purified 47.3 folds with an overall yield of 27% through purification procedure of acetone precipitation, ion exchange and gel filtration chromatography. Protein precipitation using acetone fractionation showed that the enzyme was precipitated at the fraction of 0 to 40%. Further purification of the enzyme by ion exchange followed by gel filtration chromatography showed that there were two types of lipases with similar molecular size at around 50 kDa. Characterization of optimum pH, temperature, and substrate specificity were carried out against the isolated lipase. Lip1 showed specific substrate preferences towards *p*-nitro phenyl myristate meanwhile Lip2 exhibited hydrolytic activity towards short and medium acyl chain of the substrate. Further analysis of both enzyme activities on variation of pH and temperature showed that the optimum pH and temperature for Lip1 were at pH 10.0 and 70°C, respectively while pH 8.0 and 50°C were the optimum pH and temperature for Lip2 respectively. Lyophilized lipase isolated from acetone fraction showed lipolytic activity under variation of methanol concentration up to 30%.

Key words: *Pseudoxanthomonas* lipase, ion exchange chromatography, gel filtration chromatography.

INTRODUCTION

Lipase is one of the hydrolase enzymes used as biocatalysts in industry. The ability of lipase in catalyzing reactions in nonpolar environments makes lipase as the preferable catalyst in organic reactions. Lipase is generally used as biocatalyst in synthesis of flavor compounds, food industry, modification of the physicochemical properties of triglycerides in the oil and fat industry, and the synthesis of biopolymers and biodiesel (Gupta et al., 2014; Houde et al., 2004; Jaeger and Eggert, 2002; Salihu and Alam, 2014). Thermostable and solvent tolerant lipases play important roles in industrial processes, since the enzymes are applicable in the enzymatic processing of lipids, even at high temperature and alkaline condition (Lotti et al., 2015). The enzyme has been used in many fields of industries such as lipid and oil hydrolysis, detergent, peptide synthesis, and pharmacy (Hasan et al., 2006).

Application of lipases for chemical synthesis has

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numerous advantages such as general ease of handling, broad substrate tolerance, high stability towards temperatures and convenient commercial availability. Most of the synthetic reactions on industrial scale are carried out in organic solvents due to the easy solubility of non-polar compounds (Kumar et al., 2016). This approach requires an enzyme which is stable in the organic solvents. General enzymes show very low rate of reactions or inactive in non-aqueous media; therefore, search for solvent stable enzymes has been an extensive area of research (Yilmaz and Sayar, 2015; Bisht et al., 2013; Rahman et al., 2005).

Application of lipase as biocatalyst in the biotechnological industries like production of biodiesel, preparation of food emulsifiers, personal care and cosmetic products, flavours and pharmaceuticals require alcohol stable enzyme (Uphues et al., 2001; Ozyilmaz and Gezer, 2010). Methanol however, is reported to hamper the activity of several lipases when used at concentrations that would be optimal for the alcoholysis reaction (Lotti et al., 2015). Enzyme stability against methanol may be improved by many techniques, like directed evolution or random mutagenesis. Korman et al. (2013) applied directed evolution to a *Proteus mirabilis* lipase that is relatively tolerant to short chain alcohols but is irreversibly inactivated when incubated at over 50% methanol. Random mutagenesis by error prone PCR was used to increased methanol stability in the lipase from *Geobacillus stearothermophilus* T6, an enzyme able to resist high temperature but which is poorly stable in polar organic solvents (Dror et al., 2014). Other researches also focused in search of novel lipase from new bacterial isolate. Enzymes secreted from organic solvent tolerant microorganisms tend to be stable under solvent rich environment. A few solvent stable lipases have been reported from solvent tolerant *Pseudomonas* and *Bacillus* sp. (Isken and de Bont, 1998; Baharum et al., 2003). Madayanti et al. (2008) cultivated and collected 10 isolates from thermogenic phases (50-70°C) during composting process which showed lipolytic activity.

In previous report (Syihab et al., 2015), a methanol tolerant *Pseudoxanthomonas* strain was isolated from domestic compost. The isolate able to survive in medium containing 3% of methanol and showed better lipolytic activity among other isolates tested. The objective of this research is to purify and characterized lipase from compost isolate. This present paper describes purification of lipase from *Pseudoxanthomonas taiwanensis* using ion exchange chromatography, followed by gel filtration chromatography. The purified lipases were characterized to determine optimum pH, temperature and substrate specificity.

MATERIALS AND METHODS

The purification of protein employed two types of resin, that is, DEAE Sepharose FF (GE Life Science) and Sephadex G-75 (Sigma). Hydrolytic activity of the lipase was tested using substrates of p-nitrophenyl acetate (pNPA), p-nitrophenyl butyrate (pNPB), p-nitrophenyl capric (pNPC), p-nitrophenyl lauric (pNPL), p-nitrophenyl myristate (pNPM), and p-nitrophenyl palmitate (pNPP) from Sigma Chemicals, USA. All the other chemicals used were of analytical grade.

Inoculum preparation

Inoculum was prepared by transferring loopful of stock culture to the medium consisting 0.5% meat extract, 0.5% yeast extract, 0.1% NaCl and 0.1% $CaCl_2.2H_2O$. The cultivation was performed at 55°C with shaking at 150 rpm for 20 h.

Cultivation for lipase production

Medium for lipase production (100 mL) was prepared based on the same composition with media using 500 ml Erlenmeyer flask. The media was added by 1 mL of inoculum and incubated at 150 rpm in a shaker maintained at 55°C. After 17 h, cells were harvested by centrifugation at 8,000 g at 4°C for 30 min. The cell-free supernatant was used as crude extract for lipase purification.

Lipase assay

Lipolytic activity was measured by spectrophotometer based on assay with p-nitrophenyl fatty acids dissolved in acetonitrile at concentration of 0.01 M as substrate (Lee et al., 1999). Subsequently, ethanol and sodium phosphate buffer (0.05 M; pH 8.0) were added to final composition of 1:4:95 (v/v/v) of acetonitrile/ ethanol/ and buffer respectively. The enzyme was added to the substrate (1:3, v/v), then incubated at 55°C for 15 min. Enzyme activity was measured by monitoring the absorbance at 405 nm, representing the amount of p-nitrophenol (PNP) released. One unit of lipase activity is defined as the amount of enzyme producing 1 mmol PNP per minutes under the assay conditions.

Protein concentration

The protein concentration was determined based on Bradford dye method, and standard protein was made using bovine serum albumin. The enzyme was mixed with Bradford reagent (1:1, v/v), then incubated at room temperature for 10 min. Protein concentration was measured by monitoring the absorbance at 595 nm wavelength (Nurhasanah et al., 2017).

Methanol tolerance lipase assay

To determine the lipase tolerance against methanol, enzyme activity was assayed at various methanol concentrations (3-30%). The assay was conducted based on lipase activity assay with the replacement of ethanol with methanol (Lee et al., 1999). The varying amount of methanol was prepared under similar conditions.

Lipase purification

The cell free supernatant was fractionated using two types of precipitation, that is, ammonium sulfate and acetone. All subsequent steps were carried out at 4°C. The protein pellet from ammonium sulfate precipitation was resuspended in 0.05 M sodium phosphate buffer pH 8.0 and dialyzed against the same buffer to remove the residue of ammonium sulfate. Lyophilization was conducted against the pellet from acetone fraction to evaporate the

Table 1. Purification of *Pseudoxhantomonas* lipase.

Purification step	Total protein (mg/mL)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	38.989	21.690	0.6	1.0	100
0-40% Acetone fraction	12.424	17.064	1.4	2.5	79
DEAE 1st peak	7.085	12.026	1.7	3.1	55
DEAE 2nd peak	3.463	10.021	2.9	5.2	46
Sephadex G-75 1st peak	0.456	7.130	15.6	28.1	33
Sephadex G-75 2nd peak	0.218	5.752	26.3	47.3	27

remaining solvent, and continued by resuspending the protein using the same buffer.

The precipitated enzyme was loaded onto a column of DEAE-sepharose fast flow (1.2 × 50 cm), previously pre-equilibrated with 0.02 M sodium phosphate buffer pH 6.0. The column equilibrated with 0.02 M sodium phosphate buffer pH 6.0 which contained 0.1 M NaCl. Subsequently, 10 mL of enzyme was loaded into the column. The column was eluted with 0.02 M sodium phosphate buffer pH 6.0. It was subsequently eluted with step gradient of 0.2 to 0.8 M NaCl (0.2, 0.4, 0.6 and 0.8 M NaCl) in 0.02 M sodium phosphate buffer pH 6.0 at flow rate of 0.5 mL/minute. The fractions which showed higher protein concentration (A 280) were collected.

The enzymes collected from DEAE sepharose FF were dialyzed using 0.02 M sodium phosphate buffer pH 6.0 containing 0.15 M NaCl. Subsequently, the enzymes were loaded to a Sephadex G-75 column (0.6 × 5 cm), and elution conducted using the same buffer at flow rate of 0.2 mL/minute. Enzyme activity and protein concentration of pooled fraction was determined. Specific activity of enzyme was calculated in every step of purification.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970), using 12% crosslinked polyacrylamide gel to separate and determine the molecular mass of the purified enzymes.

Zymography analysis was carried out for detection of lipase activity. The enzyme was assayed and visualized on zymograms using 12% polyacrylamide gels after renaturation using 0.05 M phosphate buffer pH 8.0, containing 0.1% Triton X for 4 h at 4°C. The gels were finally incubated for 1 h at 65°C in developing solution consisting of 0.003 M α-naphthyl acetate, 0.001 M Fast Red TR (Sigma), and 0.05 M sodium phosphate buffer, pH 8.0 (Soliman et al., 2007).

Optimum pH and temperature assay

To determine the optimum pH of purified lipase, enzyme activity was assayed at various pH values (5.0-12.0). Lipase activity was examined at 55°C using 0.05 M buffer with pH range from 5.0 to 12.0 were used in this experiment. To determine the effect of temperature on lipase activity, the assay was carried out at various temperatures (30 to 80°C) under standard conditions (0.05 M Tris-HCl, pH 8.0).

Substrate specificity assay

Substrates of p-nitrophenyl fatty acid esters at varying chain length (C2, C4, C10, C12, C14 and C16) were used at 0.001 M concentration and the enzyme activity was measured based on optimum pH and temperature.

RESULTS AND DISCUSSION

Production and purification of lipase

An extracellular lipase was produced on the media consisting of 0.5% meat extract, 0.5% yeast extract, 0.1% NaCl and 0.1% $CaCl_2.2H_2O$. The lipase was produced optimally at 55°C and pH 7.0 on the late of stationary phase. From 2000 mL of culture, extracellular lipase was recovered at 9.62 mg of crude enzyme with specific activity of 0.19 U/ mg enzyme (Table 1).

The crude extract of lipase was purified using 2 methods, that is, ammonium sulfate and acetone precipitation. Either precipitation using ammonium sulfate or acetone was carried out at 0-40, 40-60, and 60-80% saturation, respectively. Specific activity among ammonium sulfate fractions showed no differences, indicating that the lipase was precipitated in all fractions. The acetone fractions showed higher specific activity compared to ammonium sulfate fraction, whereas the 0-40% acetone fraction has the highest specific activity among other fractions (Figure 1).

The SDS-PAGE and zymogram analysis revealed that there were two lipase band with the size of 30 and 50 kDa from 0-40% ammonium sulfate fraction. Meanwhile in 40-60 and 60-80% fraction, the 50 kDa lipase appeared with less intensity (Figure 2A). Another result based on SDS-PAGE and zymogram towards the acetone fractions showed that 0-40% fraction has several lipase bands with the size of 70, 50, and 40 kDa (Figure 2B), while other fraction showed no lipase band. The hydrophobicity of amino acid residue in lipase may lead to more protein precipitated in acetone fractionation. Acetone fractionation method is widely used to precipitate proteins with many hydrophobic amino acids residue such as lipases. Bihst et al. (2013) reported that the maximum lipase activity of *Pseudomonas aeruginosa* was obtained on the addition of acetone with a ratio of 1: 1 to the crude extract of the enzyme. In this study, application of acetone fractionation managed to precipitate lipase in one fraction. Based on the results, the acetone fraction was purified further.

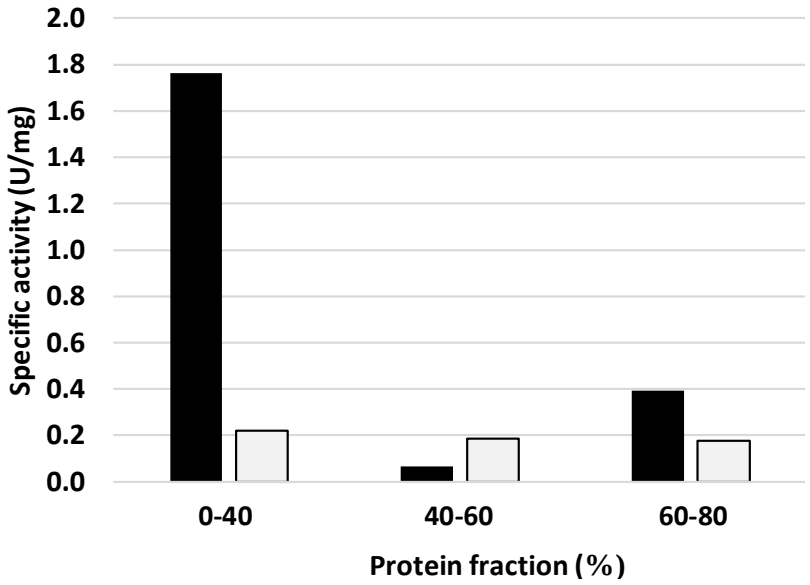

Figure 1. Specific activity of enzyme fraction; specific activity of ammonium sulfate fraction (■) and acetone fraction (□). The ammonium sulfate fractions showed higher specific activity compared to acetone fractions.

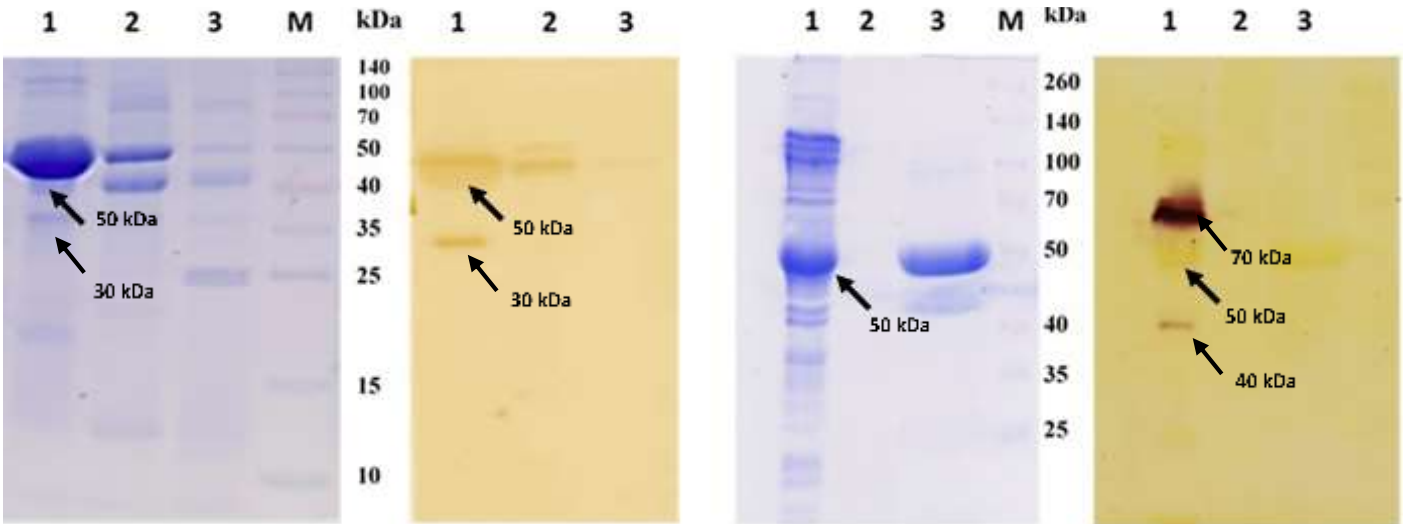

Figure 2. Electrophoregram of lipase fractions. SDS-PAGE (left) and zymography (right) of lipase from ammonium sulfate fraction (A) and acetone fraction (B). Lane 1, 0-40% fraction; lane 2, 40-60% fraction; lane 3, 60-80% fraction; M, molecular mass protein marker. The error mark (→) showed the target of 50 kDa lipase.

Purification of lipase from acetone fractionation was followed by ion exchange chromatography using DEAE Sepharose Fast Flow (FF) as the matrix. The pattern of ion exchange chromatography showed 2 peaks of protein (Figure 3). The 1st peak was eluted with 0.4 M NaCl, meanwhile the 2nd peak was eluted with 0.6 M NaCl. The SDS-PAGE and zymogram analysis showed that both peaks have the same molecular mass of 50 kDa. Further purification using Sephadex G-75 showed that the lipases were successfully purified into one single band with the size at 50 kDa (Figure 4).

Purification of lipase from DEAE Sepharose FF and Sephadex G-75 managed to isolate 2 lipases with the same size of 50 kDa. Acetone fraction from AL17 showed 2 peaks from anion exchange chromatography. The 1st peak has protein with the size of 50 kDa with specific activity of 1.7 U/mg, 3.1 purification fold, and 55% yield, while 2nd peak has specific activity of 2.9 U/mg, 5.2

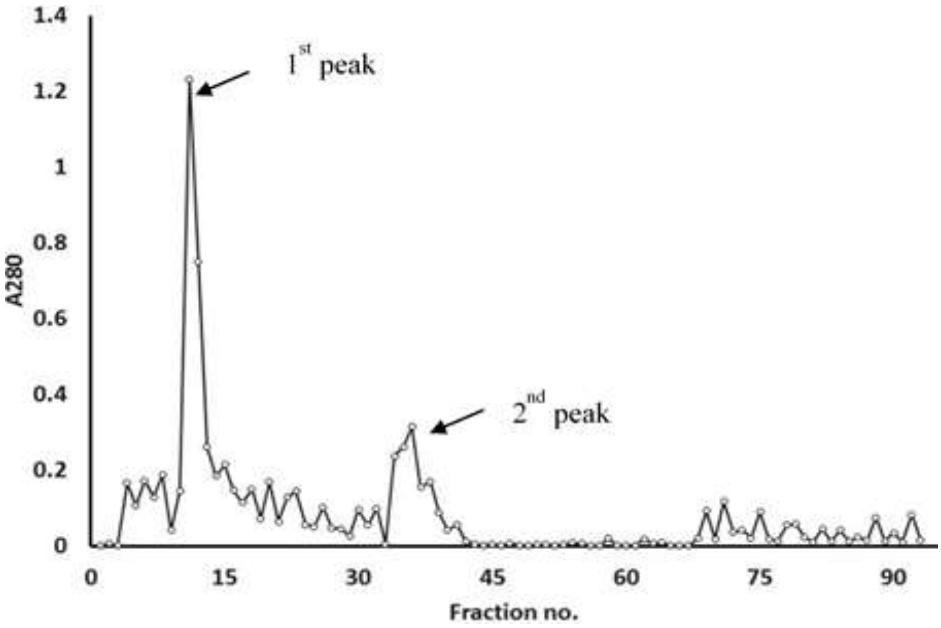

Figure 3. Purification of *Pseudoxanthomonas* lipase from DEAE sepharose fast flow. Protein concentration was measured using spectrophotometer at 280 nm wave length. The first peak (A) was eluted in sodium phosphate buffer containing 0.4 M NaCl, while the second peak (B) eluted in sodium phosphate buffer containing 0.6 M NaCl.

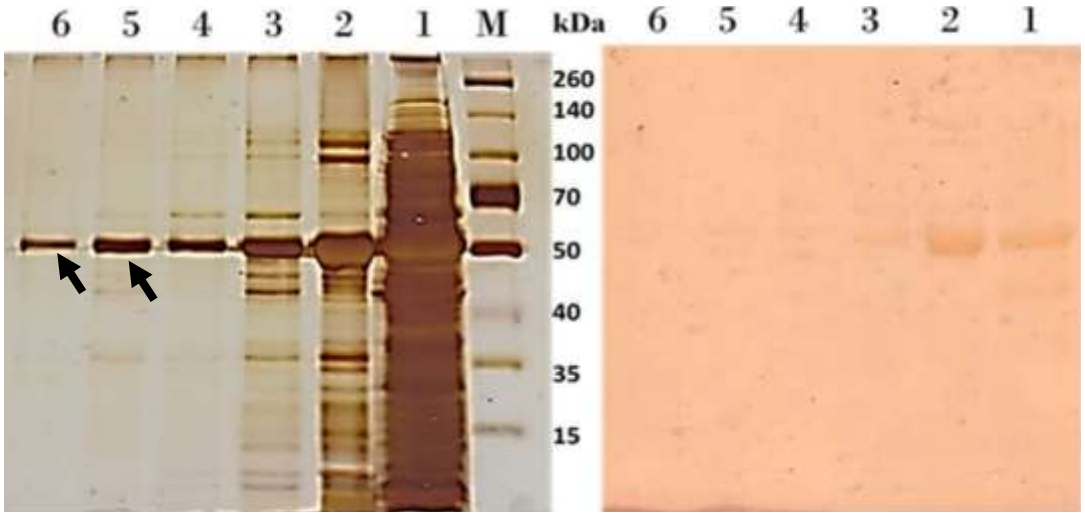

Figure 4. Electrophoregram of purified lipase. SDS-PAGE (left) and zymography (right) pattern of purified lipase. M, Molecular mass protein marker; lane 1, crude extract; lane 2, 0-40% acetone fraction; lane 3, 1st peak from DEAE Sepharose FF; lane 4, 2nd peak from DEAE Sepharose FF; lane 5, 1st peak from Sephadex G-75; lane 6, 2nd peak from Sephadex G-75. The error mark (→) showed the target of 50 kDa lipase.

purification fold, and 46% yield. Further purification towards 1st peak by gel filtration chromatography managed to isolate 50 kDa lipase with specific activity of 15.6 U/mg, 28.1 purification fold, and 33% yield, meanwhile 2nd peak also has 50 kDa lipase with specific activity of 26.3 U/mg, 47.3 purification fold, and 27% yield (Table 1).

Lipase purification from *Pseudomonas* S5 was successfully carried out with high recovery by using affinity chromatography in combination with ion exchange chromatography (Rahman et al., 2005). Other lipase from *Trichoderma viride* was purified using ammonium sulfate

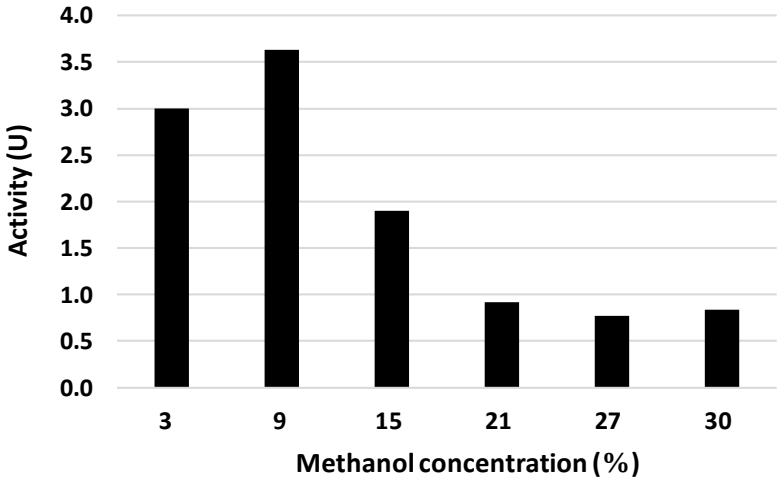

Figure 5. Methanol tolerance of lipase. Activity of lipase in various concentration of methanol range from 3 to 30%.

precipitation, ion exchange and gel permeation chromatography resulting in lipase with 134-folds purification with an overall yield of 46% (Kashmiri et al., 2006). Lipase from local thermophilic microorganism was successfully isolated using DEAE Sepharose FF and Sephacryl S-200, resulting in 4 bands of protein exhibiting lipase activity (Febriani et al., 2010).

Methanol tolerance of the enzyme

Effect of methanol at various concentrations towards lipase activity was determined using lyophilized lipase from acetone fraction. Lipase is capable to maintain its activity in various methanol concentrations after incubation for 15 min at 60°C. The enzyme showed activity of 0.84 U in 30% methanol (Figure 5). Employing lipase as a biocatalyst in biodiesel synthesis requires a stable and active enzyme, hence lipase activity in methanol is considered as an important parameter. Most of microbial lipases rarely showed high stability in hydrophilic solvents (Doukyu and Ogino, 2010; Zhao et al., 2008). The isolated lipase from *P. taiwanensis* showed stability in hydrophilic solvents, therefore appear promising for catalysis in low water medium. This property is present only in several cases viz. *Pseudomonas aeruginosa* (97% after 24 h in methanol), *Bacillus megaterium* CCOC-P263 (97% after 1 h in isopropanol), and *Serratia marcescens* (108% after 24 h at 10% DMSO) (Gaur et al., 2008; Zhao et al., 2008).

Optimum pH, temperature, and substrate specificity

Characterization of lipases from previous purification carried out against optimum pH, temperature and substrate specificity. The activity of lipase in various condition of pH was examined from pH 5.0 to 12.0. The 1st peak exhibited high hydrolytic activity at the pH range of 9 to 12, with the maximum activity at pH 10, suggesting that the enzyme was an alkaline lipase. In contrast, 2nd peak showed high hydrolytic activity in a neutral environment at around pH 7 to 8, with the maximum activity at pH 8 (Figure 6A). The high activity of isolated lipases over a wide alkaline pH, suggests its application in a range of industrial applications, such as synthesis of biodiesel, biopolymers, and other industries such as pharmacy, cosmetics, and flavor (Abdelkafi et al., 2009).

Optimum temperature for 1st and 2nd peak was tested based on their activity on various temperatures ranged from 30 to 75°C. The 1st peak showed high activity at 60 to 75°C, with optimum activity at 65°C (Figure 6B). Activity of 1st peak showed no significant decrease even when it was incubated at 75°C. Optimum temperature of 2nd peak was at 50°C, and significantly dropped at 55°C. Overall, activity of 1st peak was higher than 2nd peak at temperature range of 30 to 60°C. However, 1st peak has higher optimum temperature compared to 2nd peak. Activity of lipase at high temperature could be due to the optimum temperature needed to trigger the lid opening of the lipase (Masomian et al., 2013). The stability of enzyme at high temperature was also found in lipase from *Bacillus thermoleovorans* ID-1 and *Geobacillus* sp. (Dong-Woo et al., 1999; Abdel-Fattah, 2002).

Substrate specificity of 1st peak and 2nd peak was determined using *p*-nitrophenyl esters with varying acyl chain lengths at 60°C and pH 8.0. 1st peak demonstrated a single substrate specificity towards *p*-nitrophenyl myristate while 2nd peak showed broader substrate specificity, from *p*-nitrophenyl butyrate to *p*-nitrophenyl myristate (from C_4 to C_{14}), with preference to *p*- nitrophenyl

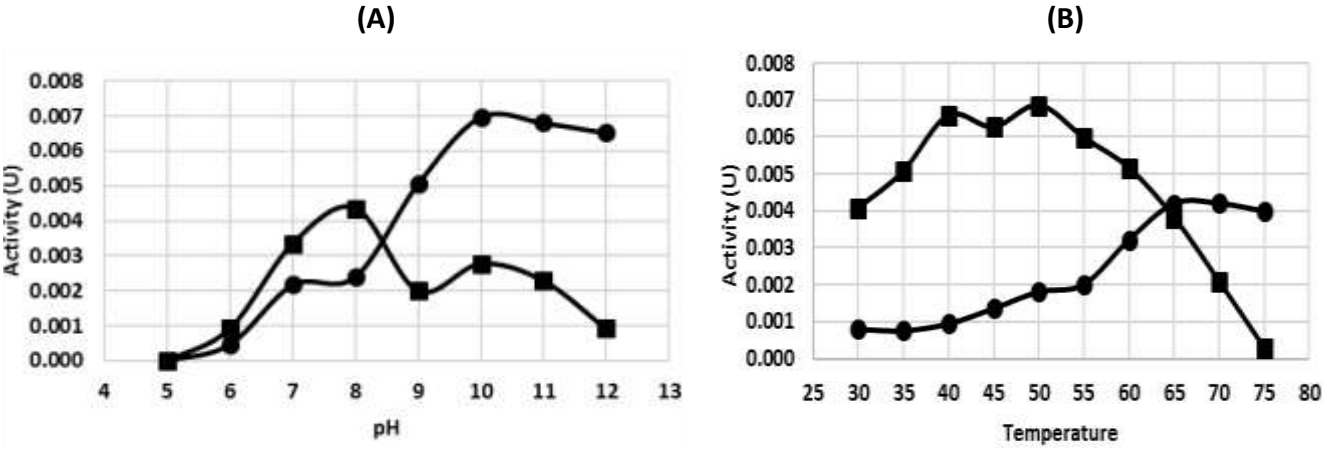

Figure 6. Optimum pH and temperature of purified lipase. A, Activity of first peak (-●-) and second peak (-■-) in various pH condition; B, Activity of first peak (-●-) and second peak (-■-) in various incubation temperature.

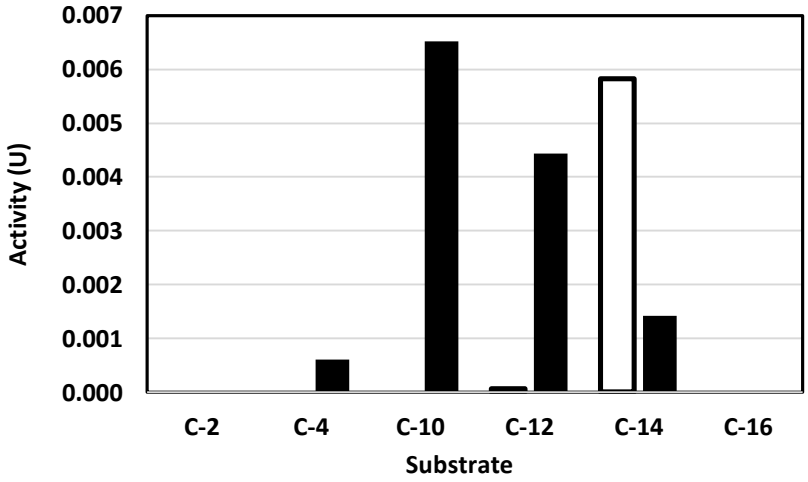

Figure 7. Substrate specificity of purified lipase. The lipase activities of 1st peak (■) and 2nd peak (□) towards various *p*NP-esters were determined based on each optimum pH and temperature for their activities.

caprate (Figure 7).

Based on the results, it was suggested that 1st peak is a different enzyme from 2nd peak because it has different optimum pH, temperature, and substrate specificity. Since, the enzymes were different type of lipase, therefore 1st peak was namely as Lip1 and 2nd peak as Lip2. The specificity of Lip1 towards *p*-nitrophenyl myristate is favorable in enzymatic synthesis of myristyl myristate which is commonly used in cosmetic industries (Garcia et al., 2009). True lipases were defined as enzymes that able to hydrolyze ester substrates with long chain fatty acids (Glogauer et al., 2011; Reyes-Duarte et al., 2005). Lip1 is most likely to be true lipase, since it showed high hydrolytic activity towards long chain fatty acid. Meanwhile, Lip2 were classified as esterase group, since it has activity towards short and medium acyl chain length. Substrate specificity of lipases may be due to differences in the geometry and size of their active sites (Pleiss et al., 1998). This type of lipase is more suitable for short chain ester synthesis such as synthesis of flavour esters (Langrand et al., 1990).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors are grateful for the ITB Research Fund, Ministry of Research Technology and Higher Education

of the Republic of Indonesia.

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