African Journal of Biotechnology

Volume 15 Number 22, 1 June 2016
ISSN 1684-5315

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Vol. 15(22), pp. 995-1001, 1 June, 2016
DOI: 10.5897/AJB2015.14946
Article Number: 0AA813458665
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Comparative assessment of different poultry manures and inorganic fertilizer on soil properties and nutrient uptake of maize (*Zea mays* L.)

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Received 26 August 2015; Accepted 23 March, 2016

This study investigated soil physicochemical properties and nutrient uptake of a drought-tolerant maize variety (DT-SR-WC_2) in a derived savanna zone of Southwestern Nigeria when different composted poultry manures (CPM) and inorganic fertilizer (IF) were applied. This was with a view to determining the essential trace elements and nutritional quality of the test crop. The experiment was a randomized complete block design with six treatments [cockerel manure (CM), broiler manure (BM), layers manure (LM), equal proportions of cockerel + broiler + layers manures (CBLM), IF)] and zero manure/fertilizer application served as control. Each treatment was replicated three times. Three months after sowing, the maize ears were manually harvested and threshed for analyses. The results show that addition of CPM increased the soil properties, but only significantly ($p < 0.05$) with the soil organic carbon and cation exchangeable capacity when BM was applied. There were no significant ($p > 0.05$) differences among the treatments considered in the proximate compositions of the harvested maize grains. The concentrations of mineral and trace elements in the harvested maize were generally higher during the wet than dry seasons, but with only Cu showing significant ($p < 0.05$) difference among the elements. It was concluded that BM was the best choice among the CPM considered for enhanced soil health and nutrient uptake of maize.

Key words: Maize, cockerel manure, broiler manure, layers manure, quality maize, soil health.

INTRODUCTION

Maize (*Zea mays* L.) is an annual cereal plant of the Poaceae family and native to Mexico (Hugar and Palled, 2008). Maize was introduced into Nigeria in the 16th century by the Portuguese (Osagie and Eka, 1998) and based on the area cropped and quantity produced; maize was the country's first most important crop among other prominent cereals such as rice, wheat, sorghum and millet (Macauley, 2015). In developing countries, where many of the farmers are resource-poor, maize is a good source of income as the crop could be cultivated on the same piece of land two or more times in one year. It is an important cereal crop that is not only rich in carbohydrate,

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but also a potential source of protein and essential minerals. A typical maize variety contains 65% carbohydrate, 10 to 2% protein and 4 to 8% fat (Iken and Amusa, 2004). Maize grain also contains mineral salts and essential trace element containing-compounds (Adeleye and Ayeni, 2010).

The problem of declining soil fertility was already mentioned by Stoorvogel and Smaling (1998) as becoming one of the major challenges for sustainable agricultural production in sub-Sahara African countries. Improving soil fertility management among smallholder farmers is widely recognized as a critical approach to addressing enhanced crop yields and poverty alleviation, especially in sub-Sahara Africa, where the majority of the populations earn their livelihood as smallholder farmers (Donovan and Casey, 1998). Sustained soil fertility management is a requirement for enhanced crop productivity and income in agri-business.

The use of inorganic fertilizer has not been helpful under intensive agriculture, because it is often associated with increased soil acidity, reduced soil organic matter with consequent deterioration of soil physical and chemical properties, soil nutrient imbalance, and reduced crop yield (Ojeniyi, 2000; Adeniyan and Ojeniyi, 2003; Awodun et al., 2007). In Nigeria, the rising cost of inorganic fertilizers coupled with their inability to recondition the soil, has directed attention to organic manures usage in recent times. Makinde et al. (2001) observed that soil degradation is brought about by loss of organic matter accompanied by continuous cropping. This becomes aggravated when inorganic fertilizers are applied repeatedly because crop response to applied fertilizer depends majorly on soil organic matter.

In Nigeria, arbitrary use of poultry manures as soil additives in crop production is on the increase because of availability, nutritive value and inexpensiveness. Poultry manure, in contrast to chemical fertilizer, adds organic matter to soil which improves soil structures, nutrient retention, aeration, soil moisture holding capacity and water infiltration (Boateng et al., 2006). Optimum use of poultry manure requires knowledge of their composition, not only in relation to enhanced crop yield but also for good crop quality (Ayeni and Adetunji, 2010), particularly in developing countries like Nigeria where maize is largely consumed when roasted, boiled or as supplement in many food diets. Dipeolu (2009) observed preference for organic vegetables due to their enhanced nutritional quality as against vegetables from inorganic fertilization.

Droppings of cockerel, broilers and layer birds have historically been composted into manures to serve as rich sources of plant nutrients and soil amendments (Otitoju, 2014). However, there is dearth of information on the specific type of the poultry manure that could give the best result in terms of quality of product when drought-tolerant maize is the test crop. One of the strategies that are currently being implemented to enhance global poverty reduction due to the impact of climate change on the agro-system is the introduction of drought-tolerant crop varieties to farming systems. This study therefore determined the essential trace elements and nutritional quality of a drought-tolerant maize variety under different poultry manures and inorganic fertilizer applications.

MATERIALS AND METHODS

Location of the study, experimental design and agronomic details

The field experiment was carried out during the dry season (October 2012-January 2013) in the Teaching and Research Farm of the Institute of Agricultural Research and Training, Ilora Substation, Ilora, Afijio Local Government of Oyo State, Southwest Nigeria with latitude 07°48´50.6´´ N and longitude 003°48´55.6´´ E. The experimental plot was cleared manually two times and pre-cropping soil samples (3), within 0 to 15 cm soil depth were taken for soil analysis. The experiment consisted of three, 23.0 × 2.5 m^2 blocks; each block was divided into six plots of 3.0 × 2.5 m^2 with an alley of 1.0 m between blocks and 1.0 m within plots. The experiment was laid out in a randomized complete block design (RCBD) with six treatments, namely: four manures [cockerel only, broiler only, layers only, cockerel + broiler + layers in equal proportion] each applied at 6.0 t ha^{-1}, one inorganic fertilizer (NPK 20-10-10) applied at 0.4 t ha^{-1} and zero manure/inorganic fertilizer application served as control. Each of the treatments was replicated three times to give a total of 18 plots.

The test crop, maize (DT-SR-WC_2), a drought-tolerant and streak resistant variety was planted at three grains per hole using 75 × 50 cm planting distance, and was rain-fed. The maize seedlings were later thinned to two grains per hole at two weeks after planting (WAP) to give a total of 53,333 maize plants population per hectare. A repeat experiment was carried out on the same treatment plots in the subsequent wet season (April-July 2013), but without fertilizer or compost application. Three manual weeding of the plots was carried out at 2, 5 and 7 WAP using hand hoe.

Soil and poultry droppings samples collections, preparation and analyses

Three composite surface soil (0 to 15 cm depth) samples were collected using simple random sampling technique before sowing; air-dried and sieved through a 2-mm mesh to remove debris and stones prior to analyses. Fresh droppings of cockerel, broiler and layer birds from a private poultry farm were separately collected, heaped under a shed and allowed to compost aerobically. The poultry heaps were stirred once in two weeks to enhance aeration and composting. As the decomposing organisms work on the heaped materials, heat was generated which helped to destroy associated fungal diseases and parasites with composts. The heat produced (peak temperature range 62 to 65°C) and moisture provided with addition of water periodically speed up the rate of decomposition. Fully cured dark manures with temperature range of 32 to 34°C were obtained at three months of composting. The poultry composts were air-dried, ground and analyzed for their chemical properties.

The properties of soil and manure samples were analyzed using standard methods (Page et al., 1982). The soil pH was determined in 1:1 soil-1 M KCl suspension using a glass electrode pH meter. Total nitrogen of the soil and manure were determined by the macro-Kjeldahl method. Available phosphorus in the soil and manure were extracted using Bray P1 method and P in the extractants was determined by colorimeter. The organic carbon in

Table 1. Properties of soil and composted poultry manures used for the experiment.

Property	Soil	Cockerel manure	Broiler manure	Layers manure
pH 1: 1 soil-1 M KCl	6.45	nd	nd	nd
Total Nitrogen (g kg^{-1})	0.70	22.40	34.30	24.00
Organic Carbon (g kg^{-1})	9.81	222.50	381.30	248.00
Available P (mg kg^{-1})	9.44	15.50	18.30	16.30
Ca (cmol kg^{-1})	0.75	26.60	31.60	29.30
Mg (cmol kg^{-1})	0.28	34.02	65.10	139.16
Na (cmol kg^{-1})	0.42	15.85	18,25	16.88
K (cmol kg^{-1})	0.35	12.10	13.90	12.50
Zn (mg kg^{-1})	0.14	448.55	475.00	462.85
Fe (mg kg^{-1})	0.20	4,627.65	5,125.70	4,920.50
Mn (mg kg^{-1})	0.17	625.55	643.35	620.95
Cu (mg kg^{-1})	0.27	68.30	75.20	72.09
Co (mg kg^{-1})	0.01	0.10	0.12	0.10
As (mg kg^{-1})	0.01	0.29	0.39	0.32
Pb (mg kg^{-1})	0.09	1.60	1.95	1.83
Cd (mg kg^{-1})	0.02	2.15	2.47	2.16
Textural class	Loamy sand	nd	nd	nd

Nd: Not determined.

soil and manure were determined using Walkley-Black wet oxidation method. Calcium ion, Mg^{2+} and K^+ concentrations in the soil and manure were extracted using 1 M ammonium acetate buffered at pH 7.0 and their concentrations in the extracts were measured using Buck Scientific Model 200 (East Norwalk, Connecticut, USA) Atomic Absorption Spectrophotometer. Also, post-cropping soil analysis was carried out on 54 surface soil samples (3 per plot of 18) to determine the influence of added soil amendments on soil quality after maize harvest.

Maize grains harvesting and analysis

Maize ears were manually harvested at 12 WAP, air-dried inside the maize crib and the field experiment was terminated. The maize grains were manually threshed and weighed. One kilogram of maize grain sample per plot was ground using a stainless-steel milling machine prior to analyses. Nutrient elements and proximate composition of the maize grain were determined using the methods of Association of Official Analytical Chemists (1990).

Statistical analysis

Data collected on the post-cropping soil properties, minerals and trace elements composition, and proximate composition of the harvested maize grains were subjected to analysis of variance at $p < 0.05$ following the procedure of Gomez and Gomez (1984). Test of significance for differences in means was compared using new Duncan's multiple range tests (SAS version 9.1 at $p < 0.05$).

RESULTS

Properties of soil and composted poultry manures

The pre-cropping soil properties and chemical composition of different poultry composts used in the experiment are shown in Table 1. The mean soil pH was 6.45, a slightly acidic soil condition. The soil texture was loamy sand with particle size distribution of 720, 230 and 50 g kg^{-1} for sand, silt and clay proportions, respectively. Soil organic carbon and total N were 9.81 and 0.70 g kg^{-1}, respectively, while available P was 9.44 mg kg^{-1}. However, the organic carbon and total N for the poultry composts ranged from 222.50 to 381.30 g kg^{-1} and from 22.40 to 34.30 g kg^{-1}, respectively, while available P ranged from 15.50 to 18.30 mg kg^{-1}.

Effects of poultry manures and inorganic fertilizer applications on soil properties

Soil properties of the experimental sites after the first and second cultivations of maize are shown in Table 2. Soil properties increased in all treated plots with application of composted poultry manures and inorganic fertilizer, but only significantly ($p < 0.05$) with the soil organic carbon and cation exchangeable capacity when BM was applied. The post-cropping soil pH ranged from 6.20 to 6.33 and from 6.15 to 6.38 in the dry and wet seasons, respectively.

The minerals and trace elements composition in maize

Influence of different poultry manure and inorganic fertilizer applications on the minerals and trace elements uptake by the drought-tolerant maize is shown in Table 3. The concentrations of minerals and trace elements increased when composted poultry manures and inorganic

Table 2. Post-cropping soil properties of the experimental site for the two seasons.

Treatment	pH	OC (g kg^{-1})	N (g kg^{-1})	P (g kg^{-1})	K (cmol kg^{-1})	CEC (cmol kg^{-1})	As (mg kg^{-1})	Zn (mg kg^{-1})
Dry season								
CM	6.20	10.59^b	1.35	11.70	0.61	4.24^b	0.02	1.30
BM	6.27	13.89^a	1.48	11.48	0.58	5.49^a	0.01	1.20
LM	6.20	11.62^b	1.40	11.85	0.81	4.37^b	0.02	1.36
CBLM	6.27	12.10^b	1.45	11.65	0.62	3.89^b	0.02	1.18
IF	6.20	11.15^b	1.37	11.40	0.61	4.06^b	Bdl	1.32
CT	6.33	10.80^b	1.26	11.47	0.54	3.06^b	Bdl	1.17
Wet season								
CM	6.15	10.22^b	1.30	12.00	0.75	3.31^b	0.02	1.42
BM	6.27	12.97^a	1.39	12.02	0.66	4.84^a	0.02	1.48
LM	6.27	11.10^b	1.36	12.03	0.86	3.30^b	0.02	1.46
CBLM	6.20	10.49^b	1.31	12.17	0.65	3.44^b	0.02	1.31
IF	6.28	10.05^b	1.26	11.90	0.72	3.08^b	Bdl	1.41
CT	6.37	9.73^b	1.20	11.35	0.61	2.83^b	Bdl	1.20
Interaction								
CM × BM × LM × CBLM × IF × CT	ns	*	ns	ns	ns	*	ns	ns

Mean with the same superscript within the same column do not differ significantly at $p < 0.05$ by new Duncan multiple range test. CM, Cockerel manure; BM, broiler manure; LM, layers manure; CBLM, cockerel + broiler + layers manure; IF, inorganic fertilizer; CT, control; ns, no significant interaction; Bdl, below detection level.

Table 3: Influence of soil amendments on the minerals and trace elements composition (mg kg^{-1}) of a drought-tolerant maize

Treatment	Ca	Mg	K	Mn	Zn	Fe	Co	Cu	As	Pb
Dry season										
CM	5.88	155.90	404.00	5.09	1.75	2.39	0.01	1.13^a	0.01	0.01
BM	5.70	153.70	371.20	5.50	2.21	2.60	0.01	1.20^a	0.01	0.01
LM	5.87	156.40	379.70	5.33	2.06	2.40	0.01	1.10^a	0.01	0.01
CBLM	5.62	157.30	411.20	5.28	1.93	2.39	0.01	1.07^a	0.01	0.01
IF	6.05	155.20	419.50	4.63	1.89	2.53	0.01	0.97^a	Bdl	Bdl
CT	5.24	147.20	400.00	5.28	1.78	2.44	0.01	0.43^b	Bdl	Bdl
Wet season										
CM	6.46	162.93	428.10	4.43	2.48	2.81	0.01	1.39^a	0.01	0.01
BM	6.70	167.05	419.30	4.77	2.31	2.77	0.01	1.48^a	0.01	0.01
LM	6.50	168.13	431.10	4.79	2.29	2.75	0.01	1.30^a	0.01	0.01
CBLM	6.13	162.90	438.90	4.52	2.36	2.52	0.01	1.64^a	0.01	0.01
IF	6.15	163.80	425.30	4.10	2.37	2.55	0.01	0.96^a	Bdl	Bdl
CT	6.20	159.08	410.30	4.34	2.43	2.60	0.01	0.47^b	Bdl	Bdl
Interaction										
CM × BM × LM × CBLM × IF × CT	ns	ns	ns	ns	ns	ns	ns	*	ns	ns

Mean with the same superscript within the same column do not differ significantly at $p < 0.05$ by new Duncan multiple range test. CM, Cockerel manure; BM, broiler manure; LM, layers manure; CBLM, cockerel + broiler + layers manure; IF, inorganic fertilizer; CT, control; ns, no significant interaction.

fertilizer were applied, though with no significant ($p > 0.05$) difference, except for Cu. The minerals in the maize grains ranged from 5.24 to 6.70 mg kg^{-1} for Ca, 147.20 to 168.13 mg kg^{-1} for Mg and 371.20 to 438.90 mg kg^{-1} for K.

Table 4. Influence of soil amendments on the proximate composition (g 100 g^{-1}) of a drought-tolerant maize.

Treatment	CPT	CFB	CFT	TAH	CHO	TSG	RSG	VitC
Dry season								
CM	10.33	1.50	1.60	2.75	73.82	5.63	3.63	1.22
BM	10.49	1.53	1.59	2.66	74.30	5.10	2.98	1.24
LM	10.16	1.52	1.61	2.71	74.00	4.86	3.46	1.24
CBLM	9.90	1.40	1.45	2.69	75.04	5.37	3.40	1.24
IF	9.93	1.49	1.60	3.07	73.35	5.72	3.53	1.33
CT	9.90	1.31	1.36	2.55	74.88	5.32	3.95	1.23
Wet season								
CM	11.20	1.52	1.64	2.90	72.08	6.32	4.70	1.30
BM	10.90	1.55	1.60	2.90	73.18	5.80	4.01	1.28
LM	10.47	1.54	1.65	2.87	73.47	5.72	4.01	1.49
CBLM	10.71	1.49	1.51	2.87	73.43	6.32	3.99	1.32
IF	10.90	1.54	1.65	3.28	72.46	6.60	4.21	1.36
CT	10.15	1.35	1.47	2.21	73.56	6.06	4.38	1.33
Interaction								
CM × BM × LM × CBLM	ns	ns	ns	ns	ns	ns	ns	ns
IF × CT	-	-	-	-	-	-	-	-

Mean within the same column do not differ significantly at $p < 0.05$ by new Duncan multiple range test. CM: Cockerel manure; BM, broiler manure; LM, layers manure; CBLM, cockerel + broiler + layers manure; IF, inorganic fertilizer; CT, control; CPT, crude protein; CFB, crude fibre; CFT, crude fat; TAH, total ash; CHO, carbohydrate; TSG, total sugar; RSG, reducing sugar; VitC, vitamin C; ns, no significant interaction.

Proximate composition of a drought-tolerant maize variety

The effects of different poultry manure and inorganic fertilizer as soil amendments on the proximate composition of drought-tolerant maize are presented in Table 4. Except for the carbohydrate, other proximate compositions (crude protein, crude fiber, crude fat, total ash, reducing sugar, vitamin C) of the harvested maize grains when no soil amendment was applied were the least and with no significant ($p > 0.05$) difference among the treatments. The carbohydrate content ranged from 73.35 to 75.04 g 100 g^{-1}, in the dry season and from 72.46 to 73.56 g 100 g^{-1}, in the wet season. Zero manure/fertilizer application had least values of 9.90 g 100 g^{-1} crude protein, 1.31 g 100 g^{-1} crude fiber, 1.36 g 100 g^{-1} crude fat and 2.55 g 100 g^{-1} total ash.

DISCUSSION

Sobulo and Osiname (1987) earlier recommended 17.40 g kg^{-1} organic carbon, 1.50 g kg^{-1} total nitrogen, 8 to 10 g kg^{-1} available phosphorus and 0.20 cmol kg^{-1} exchangeable K as nutrient critical level for maize production in Southwestern Nigeria. Therefore, for the soil organic carbon (9.81 g kg^{-1}) and total nitrogen (0.70 g kg^{-1}) obtained; each value was approximately half of the recommendation, hence were considered very low while the available phosphorus (9.44 g kg^{-1}) obtained was adequate for maize production within the same agro-ecological western zone of Nigeria. In this study, composted broiler manure gave the highest N, P and K concentrations of 34.3, 18.3 and 13.9 g kg^{-1}, respectively while composted cockerel manure had the least values. A previous study by Farhad et al. (2009) gave the N, P and K concentrations in poultry manure as 20.4, 20.6 and 18.6 g kg^{-1}, respectively. The birds are raised for different purposes; hence, quality of their feeds varied. Broilers were primarily raised for meats; fast-growing formulated feeds rich in protein formed bulk of their food. The chemical compositions of poultry manures therefore depended very much on the quality and quantity of the feeds the birds ate. This fact agreed with the observation of Oyedeji et al. (2014) that the protein constituent in the poultry feeds had a direct relationship with manure nitrogen.

Studies by Hirzel et al. (2007), Farhad et al. (2009) and Ayeni and Adetunji (2010) had similar results when poultry manure was added to soil as amendment and maize was the test crop; although poultry manures from different birds were not considered by these authors. There was slight increase in soil acidity of the post-cropped soil which must have been brought about by the addition of composted poultry manures and inorganic fertilizer when compared with the pre-cropped soil pH value (6.85). Comparable results of increase in soil acidity as a result of addition of poultry manure were

obtained by Gupta and Charles (1999). The treatments, though with single application, had positive influence in the post-cropped soil values of N, P and K. In a similar study by Boateng et al. (2006), enhanced soil physical properties and increased values of these macro-nutrients (N, P and K) were obtained. The values obtained after the second (wet season) harvest of maize were relatively lower than the first (dry season) and with no significant ($p > 0.05$) difference, except for soil organic carbon and cation exchangeable capacity. The treatments residual effect and maize biomass from the first cropping which was incorporated into the soil during land preparation for the second cropping could be attributable to these relatively comparable values. The residual effect of added organic materials and incorporation of crop residues into soil have been well documented in many studies (Nottidge et al., 2005; Ayeni et al., 2009).

Lower values of these minerals were obtained by Hirzel et al. (2007), while Mg and K values obtained by Iken et al. (2002) were higher. This disparity in the results could be attributed to differences in the treatments considered and maize varietal differences. Also, the trace element concentrations in the maize grains were in the order: Mn > Fe > Zn > Cu with greater values in the wet than dry seasons. The influence of composted poultry manures treatments on the concentration of Co, As or Pb in the maize grains did not exceed 0.01 mg kg^{-1} and their concentrations were below detection levels for IF and control treatments. The available compound forms of Co, As and Pb that could enhance their mobility from either the soil or added treatments might be presumably not very mobile. The ability to form insoluble carbonate by Pb and arsenate by As in the soil medium would reduce their mobility (Khan et al., 1982). Generally, relatively higher values of these minerals and trace elements were obtained in the wet than dry seasons. Generally, wet season favored enhanced proximate compositions of maize grains, except for carbohydrate that increased marginally across all the treatments in the dry season. Wet season values of other properties (crude protein, crude fiber, crude fat, sugar and vitamin C) were also marginally higher.

Availability of moisture in the form of rain water during the wet season favored soil nutrients mobility and nutrient uptake by the maize plants. Enhanced nutrients uptake by crops has direct relationship with the crop quality (Warren et al., 2006).

Conclusion

Composted poultry manures, particularly broilers manure had positive and superior influence on soil organic carbon and cation exchangeable capacity when compared with inorganic NPK fertilizer, composted cockerel or layers manures. The proximate composition of maize was enhanced by poultry manures as soil amendments than with chemical or zero manure/fertilizer application. It could be concluded that composted broiler manure had superior effect on nutrient uptake of maize, and particularly in the wet season.

Conflict of Interests

The authors declared that there was no conflict of interests in respect of this paper.

ACKNOWLEDGEMENT

The authors are grateful to Prof. S. A. Olakojo, Head, Cereals Improvement Program, Institute of Agricultural Research and Training, Obafemi Awolowo University, Moor Plantation, Ibadan, Nigeria for providing them with the viable maize variety (DT-SR-WC_2) seeds and space for the research work in the Institute Teaching and Research Farm at Ilora Substation, Ilora, Nigeria.

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Vol. 15(22), pp. 1002-1007, 1 June, 2016
DOI: 10.5897/AJB2015.14894
Article Number: 32CCDDE58667
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Effects of 1.84 GHz radio-frequency electromagnetic field on sperm maturation in epididymis micro-environment

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Received 1 August, 2015; Accepted 19 November, 2015

In recent years, intense interest has been drawn to the effects of radio-frequency electromagnetic field (RF-EMF) on reproduction. To explore the effects of RF-EMF on sperm maturation in epididymis microenvironment, 24 male Sprague Dawley (SD) rats were randomly divided into three exposed groups (SAR 1, 2 and 4 W/kg) and one sham group. The rats in exposed group were exposed to 1.84 GHz RF-EMF for 5 days (1 h/day). After that, the rats were anaesthetized immediately and epididymis were taken out. Half of them were fixed in 4% formalin and the others were placed in tissue homogenate. The morphology of sperm and microstructure of epididymis were observed under microscope after hematoxylin-eosin (HE) staining. Expression of Bin1b protein was detected by immunohistochemistry; the level of glutathione (GSH) and enzymes including superoxide dismutase (SOD), acid phosphatase (ACP), alkaline phosphatase (ALP) and disaccharidase were determined by commercial kits. It was found that, compared with sham group, the sperm morphology and microstructure of epididymis did not change obviously; similarly, there was no significant change in Bin1b protein expression and the levels of GSH, SOD, ACP and ALP in exposure group. These results suggest that 1.84 GHz RF-EMF under this experimental condition could not affect the sperm maturation in epididymis micro-environment of SD rats.

Key words: 1.84 GHz, radio-frequency electromagnetic field (RF-EMF), epididymis, sperm maturation, Bin1b.

INTRODUCTION

As human society and technology develops fast, electrical products are applied widely, and the potential harmful effects of electromagnetic fields emitted from these products, especially communication devices are

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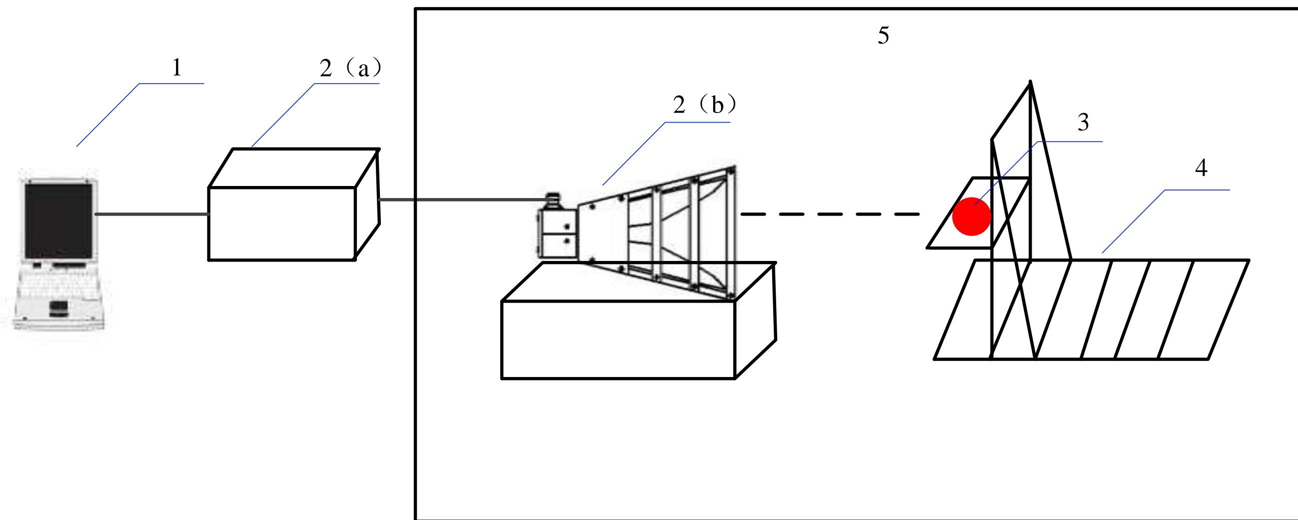

Figure 1. Schematic diagram of the irradiation platform. 1, Computer; 2, microwave irradiator; 2(a), microwave source; 2(b), antenna; 3, animal; 4, 2D objective table; 5, microwave anechoic chamber.

already given high attention. It was found that the RF-EMF (spectral bandwidth ranging from 300KHz-300 GHz) used in communication devices had various biological effects (Megha et al., 2015; Wang et al., 2015; Trivino et al., 2012). However, the biological effects of 1.84 GHz RF-EMF, which are widely selected for mobile communication in China are rarely reported.

As we know, epididymis provide an important micro-environment for sperm maturation. Epididymis consisting of many tortuous tiny ducts can be divided into three main regions (caput, corpus and tail); the caput is connected to ductus deferens and the tail is connected to testis. Once sperm leave testis, they enter epididymis for maturation and then stay here. Epididymis micro-environment includes the length and patency of ductus, epididymis secretion ability, and immune barrier function. Some important enzymes in epididymal fluid such as ACP could promote sperm maturation and motility (Li et al., 2005; Wang et al., 2006). Once the epididymis micro-environment is damaged, sperm maturation could be harmed and result in infertility.

Bin1b is a small peptide specially expressed and secreted from the caput region of rat epididymis (Li et al., 2001). Bin1b not only kills bacteria as the epididymis-specific β-defensin, but also mediates the induction of sperm motility by inducing uptake of Ca^{2+}; therefore, it can help sperm maturation through initiating sperm motility (Zhou et al., 2004). If the expression of Bin1b is interfered, the sperm motility may be impaired and finally result in infertility and sub-fertility.

It was reported that RF-EMF influenced reproductive system (Gutschi et al., 2011; Kesari et al., 2011), but at present, few studies have been performed to investigate the effects of RF-EMF on sperm maturation in epididymis microenvironment. In this study, the expression of Bin1b protein and some other important components playing an important role in maintaining the stability of microenvironment of epididymis have been examined.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the ethical committee and conformed to internationally accepted ethical standards. The experimental 24 male SD rats of 6 weeks were supplied by the Laboratory Animal Centre, the Fourth Military Medical University, Xi'an, China. Rats were randomly divided into three exposed groups by specific SAR value (SAR 1, 2 or 4 W/kg) and one sham group. The rats in exposed group were continuously exposed to 1.84 GHz RF-EMF for 5 days (1 h/day, continuous wave). When 5 days exposure finished, rats were anaesthetized immediately and all epididymis were taken out; half of them were for fixed in 4% formalin and the others were involved in tissue homogenate and protein extraction.

Exposure system

The schematic diagram of the animal irradiation platform with controllable electromagnetic irradiation dose is shown in Figure 1. The platform consists of a microwave irradiating subsystem and a control subsystem of irradiation dose in animal organ. In detail, the microwave irradiating subsystem consists of a microwave irradiator and a 2D objective table. The microwave irradiator could operate with the frequency of 1.84 GHz and the maximum power of 200 W. Moreover, the control subsystem could realize two functions. One displays the irradiation dose in the important organs in real time. The other controls the operation of the microwave irradiation source.

In this experiment, 24 male SD rats were randomly divided into three exposed groups (SAR 1 g, 2 and 4 W/kg) and one sham group. The rats in exposed group were whole-body exposed to be 1.84 GHz RF-EMF for 5 days (1 h/day, continuous wave).

HE staining

The rats were anaesthetized and given a limited gross necropsy with a focus on the reproductive organs. The epididymis were removed from the scrotum, freed from adherent tissues, weighed on analytical scales (Sartorius Co., Ltd, Gottingen, Germany), and fixed in 4% formalin solution. Each portion was dehydrated in a graded series of ethanol, saturated in benzene, benzene-paraffin, embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin-eosin (Toman et al., 2012). Six sections of each rat were randomly chosen for light microscopy evaluation (Leica Microsystems, Wetzlar, Germany) at 400 fold magnification to detect qualitative histological changes induced by being exposed to 1.84 GHz RF-EMF.

Immunohistochemistry staining

The epididymis samples fixed in 4% formalin solution were dehydrated and embedded in paraffin as described in HE staining. After sectioned at 5 μm, paraffin was removed. Then sections were immersed in distilled water following routine methods. Afterwards, all operations were conducted according to SABC immuno-histochemical staining kit (BosterCompany, Wuhan, China) instructions. In short, after sectioning, endogenous enzymes were inactivated, thermal antigen repair was done; they were incubated with Bin1b antibody (1:500 dilution, Bioss Company, Beijing, China) and biotinylated goat antibody (Boster Company) in that order. The sections were incubated with SABC for 20 min at 37□C followed by PBS rinsing (pH 7.2-7.6) four times. After that, sections were stained using DAB kit (Boster Company). At last, sections were mildly stained with hematoxylin, and after dehydration, transparency, neutral gum mounting, photos were taken using light microscope (Leica Microsystems).

Detection of GSH, SOD, ACP, ALP and disaccharidase

The epididymis samples were homogenized by a Polytron homogenizer followed by centrifugation at 3000 rpm/min for 15 min; supernatant was collected for GSH or enzyme level measurement.

The superoxide dismutase detection kit (A001; Jiancheng Bioengineering Institute, Nanjing, China) was selected for SOD measurement; A GSH Detection Kit (A006-1; Jiancheng Bioengineering Institute) was selected to determine the GSH level; An ACP detection kit (A060; Jiancheng Bioengineering Institute) was selected to determine the ACP level; A disaccharidase detection kit (A082-1; Jiancheng Bioengineering Institute) was selected to determine the disaccharidase level; AnALP Detection Kit (A059-1; Jiancheng Bioengineering Institute) was selected to determine the ALP level.

All assays were conducted according to the manufacturer's instruction.

Statistical analysis

All experiments were conducted at least in triplicate, and data analysis was performed using SPSS software (SPSS 16.0, SPSS Inc.,Chicago, USA). Data were analyzed by ANOVA with $P<0.05$ as the criterion for significance in all statistical comparisons.

RESULTS

The morphology of epididymis in SD rats after being exposed to RF-EMF

Compared with sham group, the epididymal tubules as well as the morphology of mature sperm in their lumen showed no differences in exposed group (Figure 2).

The protein level of Bin1b in epididymis after being exposed to RF-EMF

Compared with sham group, the protein levels of Bin1b in different SAR groups did not change significantly. These results suggest that 1.84 GHz RF-EMF could not affect Bin1b protein expression in epididymis (Figure 3).

The Level of GSH, SOD, ACP, ALP and disaccharidase after being exposed to RF-EMF

Compared with sham group, the level of GSH, SOD, ACP, ALP and disaccharidase in different SAR groups did not show significant difference ($p>0.05$).These results suggest that 1.84 GHz RF-EMF could not affect the levels of these enzymes (Figure 4).

DISCUSSION

The integrity of epididymis structure is essential for maintaining the stability of sperm mature micro-environment. From HE staining results, we found 1.84 GHz RF-EMF did not affect epididymis structure under this experimental condition.

Epididymis microenvironment is vital for sperm maturation. Some representative components have been examined in this study, such as Bin1b, GSH and some enzymes. It was reported that Bin1b protein, ACP and disaccharidase could promote sperm maturation or motility (Li et al., 2005; Wang et al., 2006; Zhou et al., 2004). SOD and GSH belong to the anti-oxidant system that can antagonize oxidant system to maintain the balance between the two sides, and once the balance was disrupted, the epididymis microenvironment would be disturbed and further influence sperm maturation.

Based on RF-EMF exposure limit (2 W/Kg) in China, three doses (<2 W/Kg, =2 W/Kg, >2 W/Kg) were designed in corresponding exposed group to compare the biological effects caused by different doses. From the results, we did not find any significant change in Bin1b protein, GSH, SOD, ACP and ALP levels between exposure group and sham group. In conclusion, 1.84 GHzRF-EMF under these experimental conditions has no influence on sperm maturation in SD rats.

Our results are consistent with those reports that did not find the positive effects of RF-EMF on reproduction or sperm maturation (Adams et al., 2014; Falzone et al., 2010; Lee et al., 2010) but there are some reports that found positive effects of RF-EMF on reproduction. For example, Mortazavi reported that RF-EMF from mobile jammers significantly decreased sperm motility (Mortazavi et al., 2013). Kesari et al. (2011) exposed the

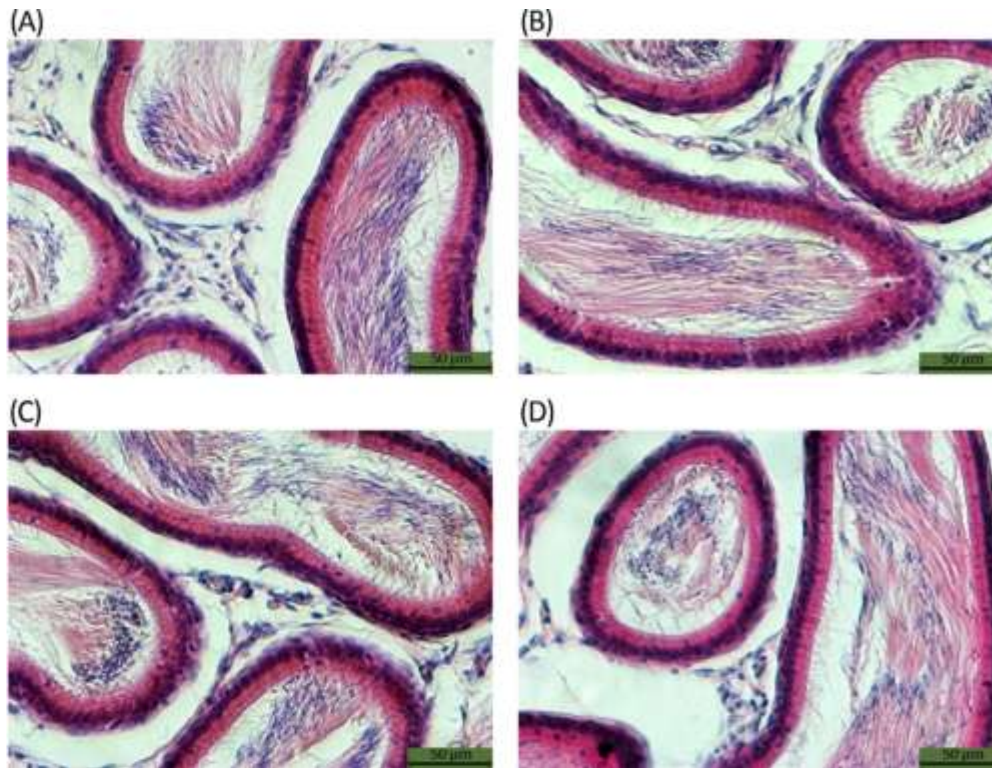

Figure 2. The epididymis morphology under microscope in different groups (images, ×400 magnification). **(A)** sham group. **(B)** 1 W/kg group. **(C)** 2 W/kg group. **(D)** 4 W/kg group.

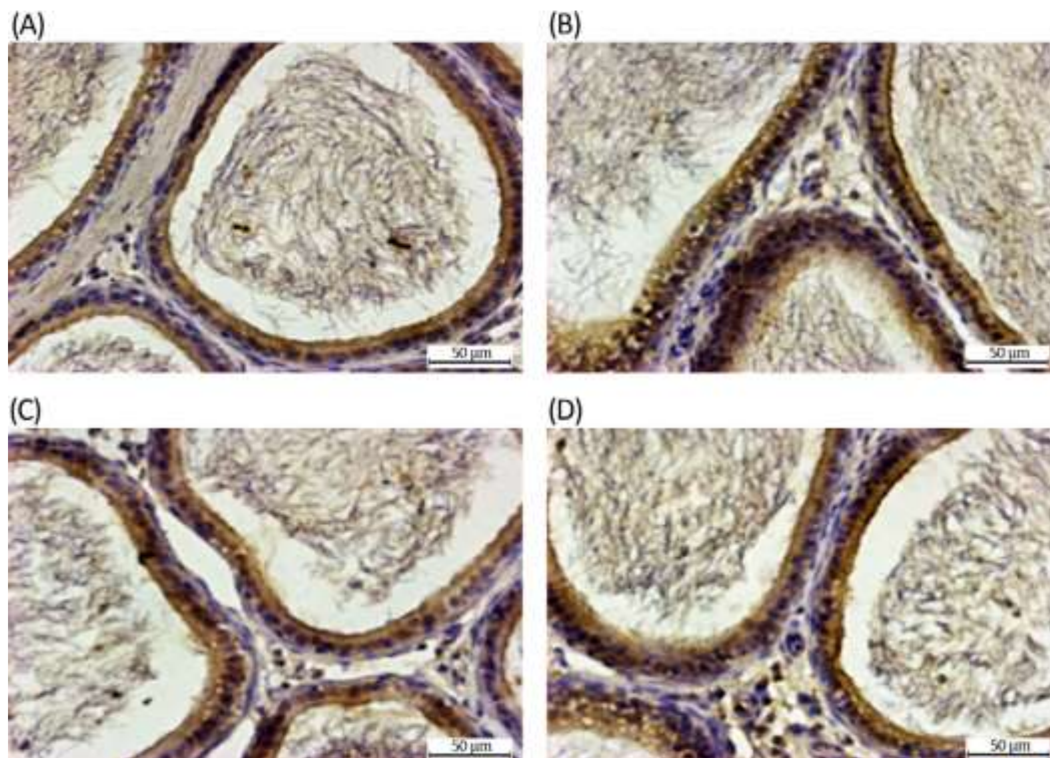

Figure 3. The Bin1b protein expression level in different groups (images, ×400 magnification). (A) sham group (B) 1 W/kg group (C) 2 W/kg group (D) 4 W/kg group.

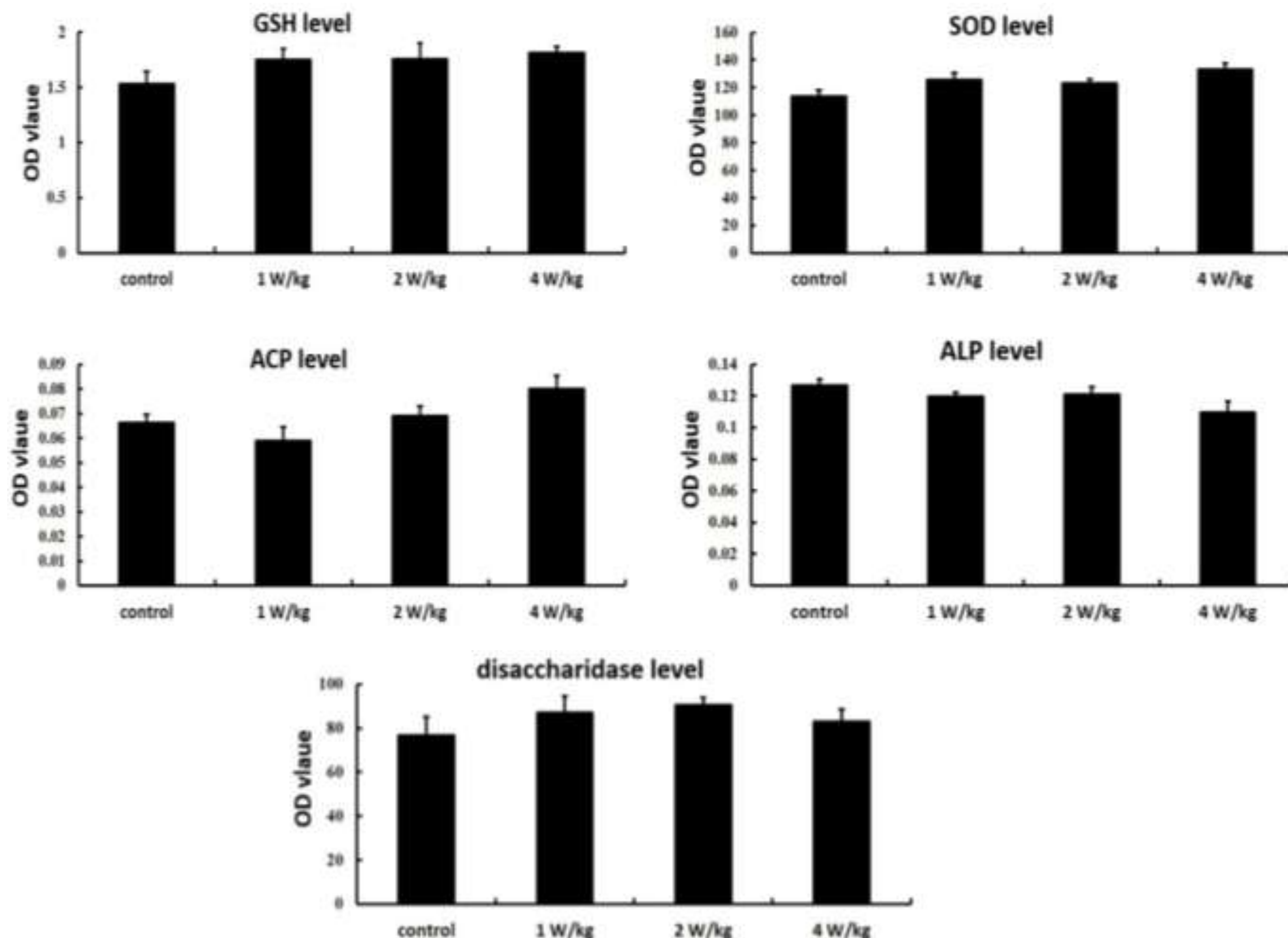

Figure 4. The GSH, SOD, ACP, ALP and disaccharidase level in different groups.

rats for 2 h a day in 35 days to mobile phone frequency electromagnetic field. They found a significant decrease in GSH peroxidase and SOD dismutase in the exposed rats compared to control group (Kesari et al., 2011). Gorpinchenko et al. (2014) found a correlation exists between mobile phone exposure, DNA-fragmentation level and decreased sperm motility. The contradiction among these reports may be ascribed to differences in exposure parameters, experimental animals and experimental conditions. For example, under the same experimental conditions, the transcription level of HSP70 increased after intermittent RF-EMF exposure, but not continuous RF-EMF exposure (Valbonesi et al., 2014). In addition, the accumulated exposure time is another factor that influences RF-EMF biological effects.

Some limitations of the current study are relatively short exposure time and small sample size.

Although the results were negative in this study, it provides some information for further study. Moreover, this is the first study to report the effects of RF-EMF on Bin1b protein in this filed. Since the exposure time chosen in this study was limited compared with that of mobile phone users in our daily life, it is too early to conclude that RF-EMF exposure is safe or not for male reproduction. Whether higher SAR value and longer exposure duration could influence epididymis microenvironment needs further study.

Conflict of Interests

The authors have not declared any conflict of interests.

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Vol. 15(22), pp. 1008-1014, 1 June, 2016
DOI: 10.5897/AJB2016.15208
Article Number: D0F12C158669
ISSN 1684-5315
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http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Investigation of yeast genes possibly involved in mtDNA stability using the nematode *Caenorhabditis elegans*

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Received 5 January, 2016; Accepted 6 May, 2016

Screening of *Caenorhabditis elegans* genes possibly involved in the mitochondrial genome maintenance was performed using our previous validated method of RNAi combined with ethidium bromide. This was to knock down *C. elegans* genes homologous to yeast genes known to be involved in mtDNA stability but of unknown molecular function or to identify transient components that could play important role on the stability of mtDNA in a temporal and/or spatial manner. *C. elegans* homologs for 11 genes among 27 yeast genes for which deletion leads to a rho0 state were found, however, only 5 genes were present in the RNAi library. Out of these 5 genes, 1 gene (homolog of *GEM1*) gave a clear L3 arrest on RNAi and ethidium bromide indicating its involvement on mtDNA stability. Four other genes homologs of *MTG2, YER087W, AV*L9 and *RRG3* did not lead to L3 arrest even though their deletion in *Saccharomyces cerevisiae* leads to rho0 state. Although *MTG2* has been reported to be important in the function and structure on mtDNA stability in yeast, our results did not support those findings in *C. elegans*. The human homolog of this gene (MIRO1) can be considered as a candidate gene involved in mtDNA stability and sequenced in patients with mtDNA depletion diseases.

Key words: mtDNA, *Caenorhabditis elegans*, nucleoid, RNAi, candidate genes, homolog, MIRO1.

INTRODUCTION

The clinical and biochemical heterogeneity of mitochondrial diseases is one of the major problems for identifying the disease genes. Genetic defects of oxidative phosphorylation (OXPHOS) account for a large variety of clinical symptoms in childhood. The mitochondrial respiratory chain (RC) is made up of about 100 different

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proteins (Hatefi, 1985). It is hypothesized that several hundreds of nuclear genes are also needed for various functions of the RC. Mutations in any of these genes will result in mitochondrial RC disorders. Since the discovery of mutations in mitochondrial DN (mtDNA), the organization and segregation of mtDNA and nucleoid proteins has become a topic of active investigation and significant advances have been made in understanding the composition and dynamics of human mitochondrial nucleoids (Gerhold et al., 2015). Various approaches have been developed by different groups which resulted in the identification of disease genes. Yet, owing to the large number of possible candidate genes, these approaches most often remains laborious (De Lonlay et al., 2002). Again, considering the large number of genes involved in mitochondrial functions, using a systematic study to identify candidate genes for mitochondrial disorders is unrealistic.

The extensive conservation of mitochondrial structure, composition, and function across evolution offers a unique opportunity to expand the understanding of human mitochondrial biology and disease. By investigating the biology of much simpler model organisms, it is often possible to answer questions that are unreachable at the clinical level. Model organisms have an essential role in this process and might help to make these genes and their products amenable to pharmacological intervention. They are invaluable for understanding and elucidating the molecular bases of the pathophysiologies resulting from mitochondrial dysfunction. The discovery in the late 1980s of the first association of mtDNA mutations with human disorders (Holt et al., 1988; Wallace et al., 1988) has given an enormous boost to mitochondrial genetic research.

Qualitative or quantitative anomalies of this genome result in various types of mitochondrial diseases. Mutations in nuclear genes encoding proteins involved in mtDNA maintenance can result in large-scale mtDNA rearrangements and abnormal copy number of the mitochondrial genome.

The yeast *Saccharomyces cerevisiae* is probably the most studied model organism for acquisition of basic knowledge on mitochondrial function and biogenesis. Its suitability as a model for human mitochondrial disease studies has been well demonstrated (Bolotin-Fukuhara et al., 2010; Rinaldi et al., 2010). About 40% of human genes whose mutations lead to diseases have an ortholog in yeast (Bassett et al., 1996) and genomic screens have been extended to mitochondrial diseases (Steinmetz et al., 2002). The study of mitochondrial functions and dysfunctions is of special interest in the yeast *S. cerevisiae* because it can survive without its mtDNA (also called rho factor) if it is supplied with fermentative substrate. Thus, all mutations of the mitochondrial genome can be studied without cell lethality. Also, a clear advantage in the use of *S. cerevisiae* is the ease with which to introduce nuclear- gene mutations, mitochondrial gene mutations and screens for complementation or synthetic lethality. It is therefore an ideal organism for dissecting the molecular processes required for maintenance of respiratory competent mitochondria (Dimmer et al*.,* 2002; Steinmetz et al., 2002).

However, notable differences in mtDNA structure and dynamics between yeast and human did not make the use of *S. cerevisiae* a perfect tool to study the mtDNA maintenance. Indeed, human cells contain 10^2–10^4 mtDNA copies, whereas yeast cells contain only 20 to 100 copies. The mitochondrial genome, 16.6 kb in human and 85.8 kb in yeast, is predominantly linear in yeast but is circular in human (Burger et al., 2003; Legros et al., 2004; Williamson, 2002). Finally, heteroplasmy is very frequently observed for mtDNA mutations in human, whereas yeast cannot normally maintain stably heteroplasmy (Shoubridge, 1998; Zeng et al., 2007). Furthermore, because this yeast can grow robustly by fermentation in the absence of mtDNA, it loses its mitochondrial genome very rapidly upon inactivation of a large class of genes encoding mitochondrial proteins involved in almost all the mitochondrial biogenesis pathways (mitochondrial translation, adenosine triphosphate (ATP) synthesis, iron homeostasis, mitochondrial import, and morphology). As such, it cannot be used easily to address the question of mtDNA transmission control (Contamine and Picard, 2000).

The characteristics of *Caenorhabditis elegans* make it a perfect complement to the yeast system. The success of *C. elegans* as a model organism in biological research is attributed to a number of biological and easy handling properties of the worm, such as short generation time, fixed cell lineage, transparent body, ease of maintenance and cryopreservation, sequenced genome, life synchronization, power of its genetics and a wide spectrum of tools for genome manipulation.

Previous studies have shown that, down expression by RNA interference of genes involved in mtDNA replication such as polg-1, encoding the mitochondrial DNA polymerase, results in reduced mtDNA copy number but in a normal phenotype of the F1 worms. By combining RNAi of genes involved in mtDNA maintenance and EtBr exposure, Addo et al. (2010) were able to reveal a strong and specific phenotype (L3-developmental larval arrest) associated to a severe decrease of mtDNA copy number.

Genome-scale approaches and various computational predictions of mitochondrial proteins (Small et al., 2004) have catalyzed the identification of a large number of mitochondrial proteins. From all these data, a human mitochondrial proteome has been proposed as MitoP2 (Andreoli et al., 2004). Ryohei et al. (2008) used the information available for human mitochondrial proteins in the MitoP2 to search for *C. elegans* genes encoding mitochondrial proteins. In total, 1009 putative genes were identified by a BLAST search using 719 human proteins (Ryohei et al., 2008). The availability of RNAi library, which is used to produce efficiently loss-of-function RNAi

phenocopies by feeding the worms with bacteria expressing specific double-stranded RNAs, includes 86% of the putative genes in *C. elegans* (Kamath and Arhinger, 2003).

In a recent genome-wide study in *S. cerevisiae*, Merz and Westermann (2009) found that out of 319 respiratory deficient deletion strains, 51% (162 strains) displayed the irreversible phenotype corresponding to the loss of intact wild-type mitochondrial genome. It is therefore important to know if these genes are also involved in mtDNA stability in *C. elegans.* To address this question, this study aims at investigating in *C. elegans* the role of the homologs of yeast genes of unknown or poorly assigned function, by knocking-down by RNAi combined with ethidium bromide (EtBr), the *C. elegans* genes homologous to the yeast genes, which upon deletion lead to a rho0 state.

MATERIALS AND METHODS

Media and cultivation of *E. coli*

Luria-Bertani-Media (LB) (Sambrook et al., 2001): 1% (w/v) Tryptone; 0.5% (w/v) yeast extract; 1% (w/v) NaCl pH 7.5. For solid media, 1.5% (w/v) agar was added before sterilization. For plasmid selection, the antibiotic Ampicillin was used at a concentration of 0.1 mg/ml. *E. coli* was cultivated in LB-medium over night at 37°C. Liquid cultures were incubated with shaking (150 rpm). To select transformants, the antibiotic Ampicillin (100 mg/L) was added to the media.

Yeasts strain, media and cultivation

Yeast deletion mutant strain used in this study was BY4741 (Openbiosystem). Cells were grown in complete liquid medium at 28°C overnight or for selection in minimal medium complemented with the necessary requirements at 28°C. Liquid cultures were incubated with shaking (150 rpm). Complete medium: 1% (w/v) yeast extract; 1% (w/v) bactopeptone; 2% (w/v) of carbon source Glucose (YPD) and Glycerol (YPG). For solid medium, 2% agar (Difco) was added before sterilization.

C. elegans strain and growth conditions

The *C. elegans* wild type worm N2 Bristol and *unc*-119 (ed3)III were used in this work and these were provided by the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota. The worms were maintained at 20°C on NGM plates seeded with *Escherichia coli* strains OP50 or HT115(DE3) following standard protocols (Brenner, 1974).

Bacterial preparation and induction

Nematode Growth Medium (NGM) agar (Epstein and Shakes, 1995) was prepared including 25 µg/ml carbenicillin (carb) and 1 mM IPTG (Kamath and Ahringer, 2003). 4.0 ml of agar was dispensed into 5 cm plates (Nunc). Plates were allowed to dry inverted at room temperature before use. Bacteria (HT115 strain) expressing the gene of interest were streaked onto LB–agar plates including 50 µg/ml Ampicillin (LBA) and 15 µg/ml Tetracycline and incubated over-night. Large innocula of bacteria were picked, inoculated into LB broth with 50 µg/ml Ampicillin and grown for 6 to 8 h with shaking at 37°C. Twenty (20) microliters of 0.1 M IPTG was added to 2 ml of the incubated LBA (final concentration of 1 mM) to induce expression of dsRNA gene of interest. About 200 µl of this culture was seeded/spread unto each of the aforementioned NGM derivative plates. The plates were dried thoroughly before being incubated overnight (~12 to 24 h) at room temperature in the dark to allow the bacteria to grow and to continue induction of the dsRNA gene of interest. *E. coli* transformation was performed with the $CaCl_2$ method.

RNA interference and EtBr assay

RNAi experiments were performed using the feeding procedure described by Kamath and Ahringer (2003) with slight modifications (Addo et al., 2010). For ethidium bromide (EtBr) plates, stock solution of 10 mg/ml was used to prepare different concentrations of 0, 50 and 125 µg/ml as required and added before dispensing into the 5 cm plates. Plates were then incubated at room temperature in a dark container for 48 h to allow the expression of the double-stranded RNA (dsRNA). Worms feeding on HT115 bacteria carrying the empty vector (L4440) were used as controls in all the experiments. Synchronized L1-stage N2 worms were placed onto NGM (RNAi) plates seeded with bacteria expressing the dsRNA gene of interest and were incubated for 72 h at 20°C. Four adult worms were independently picked up and transferred to fresh RNAi plates with or without different concentrations of ethidium bromide (EtBr). Worms were allowed to lay between 80 and 100 eggs before being removed. Eggs were immediately counted and the F1 progeny produced was analyzed after 3 and 4 days. At day 4, evaluation of the F1 progeny arrested at the L3 stage was compared to the number of adults on the same plate. The phenotype was scored as sensitive to RNAi and EtBr if more than 80% of worms were arrested at the L3-stage on plates containing 50 µg/ml of EtBr. A gene was considered as positive for a given phenotype if the same result was observed in at least two independent feeding experiments.

Bioinformatics

BLAST information available in the *Saccharomyces* Genome Database was used to explore the *C. elegans* homologs for yeast genes at: http://www.yeastgenome.org/cgi-bin/blast-sgd.pl. MITOP2 was used to find the *C. elegans* homologs to yeast genes that are completely devoid of mtDNA (rho0) upon their inactivation: http://ihg.gsf.de/mitop2.

Staining

MtDNA presence was also estimated by DAPI staining and confocal fluorescence microscopy.

RESULTS AND DISCUSSION

A systematic functional screen using the pre-existing whole genome pool of yeast deletion mutants (BY4741, *Openbiosystem*) was performed to expand the list of genes involved in respiratory competence at different temperatures. Using the Blast algorithm available in the SGD database (*Saccharomyces* Genome Database), potential human homologs for 108 yeast genes for which no function has yet firmly been assigned were found.

Among them, 75 encode proteins present in the yeast mitochondrial proteome (Sickmann et al., 2003; Pflieger et al. 2002). Twenty seven mutant strains completely devoid of mtDNA (rho0) were found by using DAPI staining and deconvolution fluorescence microscopy (Merz and Westermann, 2009). *C. elegans* homologs for 11 genes among 27 yeast genes for which deletion lead to a rho0 state were also identified. The genes identified as required for mtDNA stability in *C. elegans* as reported in earlier work (Addo et al., 2010) do not only mirror those already known in human to be implicated in human diseases but also support predictions of the importance of the nucleoid dynamics for mtDNA stability. Out of these 11 genes, only 5 were present in our RNAi library (Kamath and Ahringer, 2003). Screening of these 5 genes by combining RNAi and EtBr was performed and found that RNAi of 4 genes (*M01E5.2, T27F6.5, T26A5.6* and *C45G3.3* with the *S. cerevisiae* ortholog as MTG2*, YER087W (AIM10), AVL9* and *AIM22 respectively*) did not lead to L3 arrest whereas 1 of these genes *K08F11.5* (*GEM1*) gave a clear L3 arrest on EtBr (Table 1 and Plate 1, respectively).

RNAi EtBr non-sensitive genes (MTG2 orthologous gene GTPB5_HUMAN)

Mtg2 is a GTPase, member of the Obg family that comprises a group of GTPases acting as translation factors. The Obg subfamily of GTPases has been identified in all organisms sequenced to date. It is a mitochondrial inner membrane protein that is essential for mitochondrial ribosome function in yeast. It associates with the large ribosomal subunit; required for mitochondrial translation, possibly via a role in ribosome assembly. It has been shown that elevated levels of Mtg2p partially suppress the thermosensitive loss of mtDNA in a 21S rRNA methyltransferase mutant, *mrm2* (Datta et al., 2005). It is therefore important to check the plasmid sequence used for the RNAi expressing the dsRNA-*MTG2* in subsequent work, as some errors may have occurred during the construction of the library. The efficiency of the RNAi by RT-q-PCR should also be controlled as well as the measurement of the mtDNA content after RNAi in subsequent work. Inactivation of the three other genes did not seem to affect the mtDNA content in *C. elegans*. *YER087W (AIM10)* is a protein with similarity to tRNA synthetases. This gene encodes a mitochondrial protein with similarity to tRNA synthetases. *AVL9* encodes a conserved protein of unknown function involved in exocytic transport from the Golgi whilst *AIM22* encodes a putative lipoate-protein ligase.

RNAi EtBr sensitive genes (*GEM1* (MIRO-1 or RHOT1))

In yeast, *GEM1* encodes an evolutionary-conserved tail-anchored outer mitochondrial membrane GTPase which regulates mitochondrial morphology. Cells lacking Gem1p contain collapsed, globular, or grape-like mitochondria that are not caused by defects in mitochondrial fission and/or fusion. It has been shown that Gem1p functions to maintain mitochondrial morphology, retain mitochondrial DNA nucleoids, and promote mitochondrial inheritance in yeast (Frederick et al., 2004). Vance (2014) reports that the contact sites between mitochondria and the ER are hubs for lipid translocation and Ca^{2+} traffic between the ER and mitochondria. The endoplasmic reticulum-mitochondria encounter structure (ERMES) forms a junction between mitochondria and the endoplasmic reticulum (ER). Four ERMES proteins are known in yeast, the ER-anchored protein Mmm1 and three mitochondria-associated proteins, Mdm10, Mdm12 and Mdm34, with functions related to mitochondrial morphology and protein biogenesis (Stroud et al., 2011). There is evidence that the metazoan Gem1 ortholog Miro-1 localizes to sites of ER–mitochondrial contact, suggesting that some of the features ascribed to Gem1 may be evolutionarily conserved and that ERMES-mediated ER–mitochondria connections lie at a cross-roads of several biosynthetic pathways (Kornmann et al., 2011). Their study again identified the Ca^{2+}-binding Miro GTPase Gem1 asan ERMES subunit. Michel and Kornmann (2012) also report that the protein complex is physiologically involved in a plethora of mitochondrial processes, suggesting that ER-mitochondria connections play a central co-ordinating role in the regulation of mitochondrial biology. Defects of mitochondrial inheritance were also observed after inactivation of the Miro GTPase (homolog to *GEM1*) in fly and human suggesting a conserved role in mitochondrial distribution (Guo et al., 2005). More recent work suggests that the Miro GTPases form a link between the mitochondria and the trafficking apparatus of the microtubules (Cox and Spradling, 2006; Fransson et al., 2006; Guo et al., 2005; Glater et al., 2006) whereas in yeast Gem1 interacts with actin cables to anchor mitochondria to the cytoskeleton. Our work supports the predictions that the gene homologous to human MIRO-1 (RHOT-1) is involved in the attachment of mitochondria to the cytoskeleton that are required for mtDNA maintenance in *C. elegans* post-mitotic cells. Involvement of RHOT1 in mtDNA stability has never been described for MIRO-1. Mitochondrial RHOT1 (MIRO-1) gene encodes a tail-anchored outer mitochondrial membrane GTPase which has been shown to bind directly to the microtubule-dependent motor KIF5 proteins (Macaskill et al., 2009). These proteins that link Miro to the cytoskeleton, are required for normal distribution of mitochondria and have been shown to often locate in the vicinity of nucleoids (Iborra et al., 2004). Thus, microtubule-mediated mitochondrial transport seems to play an important role on the stability of the mitochondrial genome in *C. elegans*. Recent biochemical characterization of this protein complex has led to the

Table 1. Yeast deletion strains (genes) that were either negative or positive to ethidium bromide and RNAi treatment and may or may not be involved in mtDNA stability.

SGD	Strain with human homolog	SGD comment	RNAi EtBr 50 µg/ml screen 30	Similarity (%)	Worm ortholog	Human ortholog	Similarity (%)
AIM10	YER087W	Protein with similarity to tRNA synthetases; non-tagged protein is detected in purified mitochondria; null mutant is viable and displays elevated frequency of mitochondrial genome loss	Not sensitive 30%	1.50E-40 (78)	T27F6.5	SYPM_HUMAN, Probable prolyl-tRNA	1.9e-68 (78)
MTG2	YHR168W	Putative GTPase, member of the Obg family; peripheral protein of the mitochondrial inner membrane that associate with the large ribosomal subunit; required for mitochondrial translation, possibly via a role in ribosome assembly	Not sensitive 60%	1.50E-48 (78)	M01E5.2	GTPBP5, GTP binding protein 5 (putative)	3.8e-77 (99.2)
AVL9	YLR114C	ORF, uncharacterized, defective in late secretory pathway. Conserved protein involved in exocytic transport from the Golgi; mutation is synthetically lethal with apl2 vps1 double mutation; member of a protein superfamily with orthologs in diverse organisms	Not sensitive 28%	1.20E-26 (78)	T26A5.6	NP_055875.1,	3.4e-36 (93)
AIM22	YJL046W	Putative lipoate-protein ligase, required along with Lip2 and Lip5 for lipoylation of Lat1p and Kgd2p; similar to E. coli LplA; null mutant displays reduced frequency of mitochondrial genome loss	Not sensitive 71%	6.90E-31 (78)	C45G3.3	LIPT1, Lipoyltransferase 1, mitochondrial	3.0e-45 (96.9)
GEM1	YAL048C	Outer mitochondrial membrane GTPase, subunit of the ERMES complex; potential regulatory subunit of the ERMES complex that links the ER to mitochondria and may promote inter-organellar calcium and phospholipid exchange as well as coordinating mitochondrial DNA replication and growth; cells lacking Gem1p contain collapsed, globular, or grape-like mitochondria; ortholog of metazoan Miro GTPases	Yes sensitive 95%	1.50E-77 (78)	K08F11.5, RNAi no abnormalities	RHOT1, calcium ion binding; GTP binding	6.7e-144 (98.6)

SGD: Saccharomyces genome database; *C. elegans* wild type worm N2 Bristol was used as controls.

discovery that GTPases of the Miro family are part of ER-mitochondria connections.

The yeast Miro GTPase Gem1 localizes to ER-mitochondria interface and influences the size and distribution of mitochondria. Thus Miro GTPases may serve as regulators of the ER-mitochondria connection (Michel and Kornmann, 2012).

Apart from yeast, a relationship between MIRO-like proteins and mtDNA copy number has never

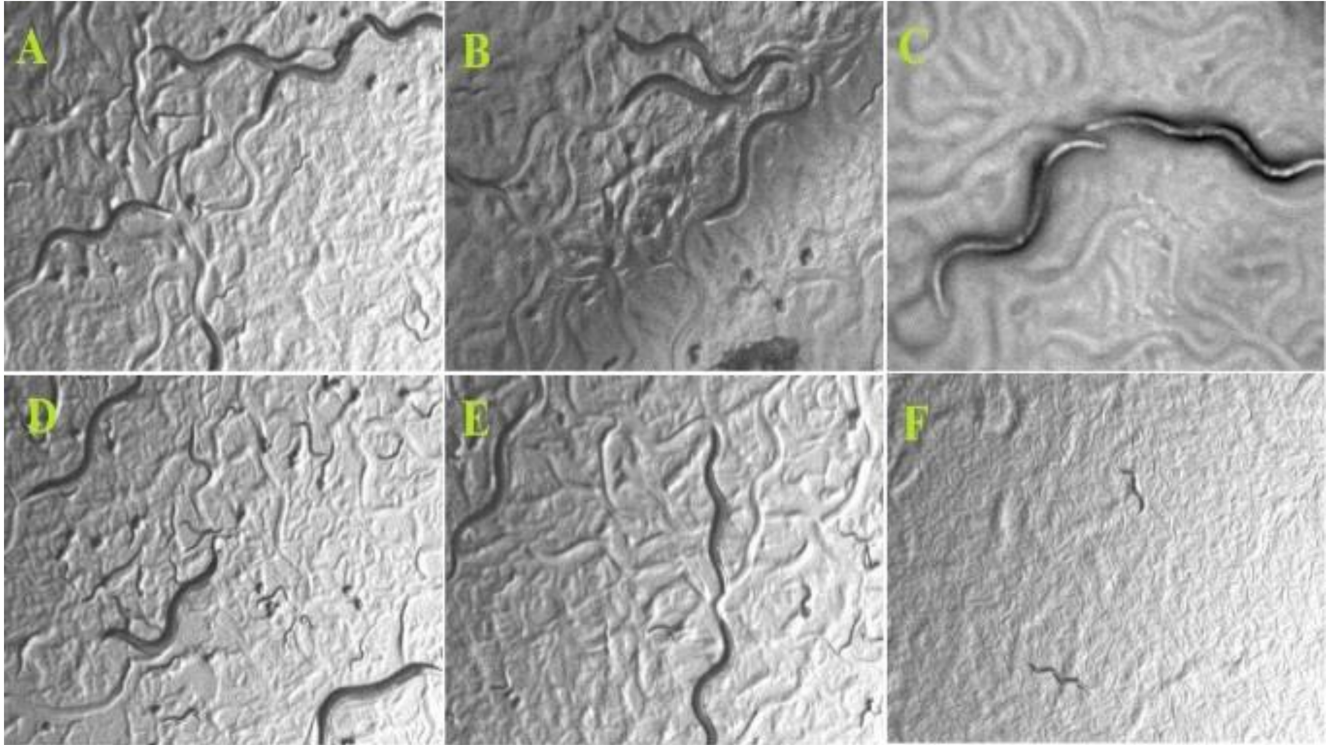

Plate 1. Microscopic images of L3 developmental arrest after RNAi of (A) N2 wild-type control worms, (B) *MTG2* gene, (C) *AIM10* gene, (D) *AVLA9* gene, (E) *AIM22* gene and (F) *GEM1* gene. Inactivation of A – E with 50 µg/ml ethidium bromide did not affect mtDNA stability. *GEM1* mutants however were sensitive to the RNAi knockdown.

been observed presumably because it has not been addressed. Nevertheless, our results do suggest that there is an unexplored interplay between mitochondrial network dynamics and nucleoid dynamics/segregation at least in the yeast *S. cerevisiae* and *C. elegans*.

This supports the assumption made by Spelbrink (2010) of a potential conservation of function between yeast and mammalian nucleoid organization and dynamics (Spelbrink, 2010). In yeast, replicating nucleoids have been observed in close vicinity of Mdm10/Mdm34/Mdm12/Mmm1 (ERMES) complex that is at least responsible for ER-mitochondrial tethering (Kornmann et al., 2009; Michel and Kornmann, 2012) and that associates with the actin cytoskeleton involving the Arp2/3 and Puf3 proteins (Boldogh et al*.,* 2003). In Spelbrink's (2010) scenario, mammalian MIRO-1 would anchor the mtDNA nucleoids to the cytoskeleton and potentially also to the ER-mito junctions, thus playing a very important role in mtDNA nucleoid dynamics. Our results therefore give credence to Spelbrink's assumption.

To conclude, our result supports the existence of a membrane scaffold structure, as suggested earlier (Hobbs et al., 2001), that is at least functionally conserved in all respiring eukaryotes and would coordinate mtDNA maintenance with mitochondrial replication, transcription, translation, protein assembly and mitochondrial dynamics. The human homolog (MIRO-1) of the *GEM1* gene can be considered as a candidate gene to be sequenced in patients suffering of mtDNA depletion syndrome.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The author acknowledges the French Government through its Embassy in Ghana for the financial support extended to this study.

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Vol. 15(22), pp. 1015-1025, 1 June, 2016
DOI: 10.5897/AJB11.2656
Article Number: 6866F1B58671
ISSN 1684-5315
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http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Molecular characterization of potato (*Solanum tuberosum* L.) genotypes using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers

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Received 30 August, 2011; Accepted 10 August, 2012

19 random amplified polymorphic DNA (RAPD) and five Inter-simple sequence repeat (ISSR) primers were used to characterize 35 potato accessions originating from Mexico, Europe and U.S.A, with the aim of generating information on the genetic diversity and relationship among the genotypes for better exploitation in breeding programs. A total of 222 and 82 *loci* were generated by the RAPD and ISSR primers, respectively. Both RAPD and ISSR primers revealed 81.45 and 82.98% polymorphism, respectively. Mantel test showed no correlation between RAPD and ISSR (0.27), RAPD and RAPD + ISSR (0.47) similarity matrices. However, a high level of correlation (0.74) was observed between ISSR and RAPD + ISSR similarity matrices. The ISSR marker was found to be more efficient compared to RAPD marker, thereby influencing more the consensus data. The use of consensus data generated more information related to genetic diversity in potato. RAPD and ISSR markers successfully differentiated between the potato accessions and subgrouped the genotypes based on origin. Information on genetic diversity and relationships will be useful in the selection of parents and mapping studies.

Key words: *Solanum tuberosum* L., random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), genetic diversity, relationships.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most economically important crops in the world and is the fourth after rice, wheat and maize (Horton, 1987). In Mexico, potato cultivation is carried out on about 64,000 ha annually with a production of 1.7 Mt and an average yield of 27 t/ha (FAOSTAT, 2007). Potatoes are grown in the following principal states: Chihuahua, Coahuila, Guanajuato, Jalisco, Michoacan, Nuevo Leon, Sinaloa, Sonora and Zacatecas (SAGARPA, 2010). Although Mexico is not a center of origin for potatoes, there exists

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great diversity of potato germplasm. Potato varieties in Mexico can be distinguished into three groups: genotypes from Holland, USA and varieties improved by Instituto Nacional de Investigaciones Forestales Agricolasy Pecuarias (INIFAP) (Ferroni, 1981). The first group represents 50% of cultivated varieties (Alpha), the second 38% and the last about 8%. The two principle centers of diversity and production of native potatoes in Mexico are Nevado de Toluca and Pico de Orizaba (Ugent, 1968). Information on the genetic diversity and relationships existing among the potato collections in Mexico is limited, which impedes their use in breeding programmes (Becerra and Paredes, 2000).

Identification of crop plants, studies in their genetic diversity and relationships is crucial for the development of breeding programmes. Historically, this has been achieved in potato through the use of morphological markers (Hijmans and Spooner, 2000). However, these markers are limited, complex and greatly influenced by the environment (Semagn, 2006). Although biochemical markers are limited and influenced by the condition of the plant, they have also been used to describe potato cultivars (Douches and Ludlam, 1991). To complement these methods and address some of the limitations, molecular markers have been developed, among which there are those based on hybridization and PCR methods (Semagn, 2006). RFLP is a molecular marker system based on hybridization and has been used extensively to construct genetic maps (Bonierbale et al., 1988), conduct phylogenetic and diversity studies in potato (Görg et al., 1992; Ritter, 2000). Nevertheless, it is time consuming, expensive and requiring high level technical support (Karp et al., 1997).

The PCR based markers developed include RAPD and ISSR (Reddy et al., 2002). These markers are simple, rapid, economic, requiring minimum level technical support and do not require prior knowledge of the genome sequence (Karp et al., 1997). RAPDs are a technique based on the amplification of discrete regions of the genome by polymerase chain reaction (PCR) with short oligonucleotide primers of arbitrary sequence (Williams et al., 1990). Although limited in reproducibility, being a dominant marker and showing problems with homology, the technique has been useful in construction of genetic maps (Perez et al., 1999), analyzing genetic diversity (Orona-Castro et al., 2006; Yasmin et al., 2006) and conducting taxonomy and phylogenetic studies in potato (Sun et al., 2003). ISSR amplification uses SSR primers (anchored or nonanchored) to amplify DNA sequences between two inverted SSRs made up of the same sequence. ISSR was first used by Zietkiewicz et al. (1994) to rapidly differentiate between closely related individuals. The technique combines most of the benefits of AFLP and microsatellite analysis with the universality of RAPD (Bornet and Branchard, 2001). ISSR markers have been successfully used in potato for fingerprinting (Bornet and Branchard, 2001) and diversity studies (Bornet et al., 2002).

There is limited use of the great potato genetic diversity existing in Mexico because of available limited genetic information concerning these genetic materials. Genetic information is paramount for the conservation, improvement and use of genetic resources. In this paper, we characterize potato genotypes in Mexico using RAPD and ISSR molecular markers with the aim of generating information on genetic diversity and relationships for better exploitation in potato breeding programs.

MATERIALS AND METHODS

35 accessions of potato were obtained from various states in Mexico and germplasm bank at the Autonomous University of Chapingo (Table 1). Tuber sections were made from five randomly selected tubers for analysis.

Deoxyribonucleic acid (DNA) extraction

The extraction of DNA was conducted according to a protocol by Sambrook et al. (1989) with modifications. Tuber sections weighing 0.3 g were obtained and ground in liquid nitrogen to form fine powder. The sample was then placed in a microfuge tube (Eppendorf) of 1.5 mL with 600 µL of extraction buffer (20 mL Tris-HCL 1 M, pH 8.0; 20 mL EDTA 0.5 M, pH 8.0; 20 mL NaCl 5 M; 35 µL β-mercaptoetanol; 40 mL sodium dodecyl sulfate 20%), then it was incubated at 65°C for 10 min, with occasional inversion of the tubes. After that, 200 µL of potassium acetate 5 M was added, mixed by inversion and incubated in ice for 30 min, then it was centrifuged for 8000 × *g* for 10 min at room temperature, and the supernatant was transferred to another tube containing 700 µL of cold isopropanol (-20°C). It was mixed by inversion and incubated at -20°C for 30 min and centrifuged for 5 min at 8000 × g, at room temperature. The supernatant was eliminated; the precipitate was recovered and dissolved in 200 µL of solution (Tris-HCl 50 mM, EDTA-Na_2 10 mM, pH 8.0).

To eliminate RNA, 2 µL of RNase A (Invitrogen, U.K) were added and incubated at 37°C for 1 h. 20 µL of sodium acetate 3 M and 200 µL of isopropanol were added, mixed by inversion and left to precipitate at -20°C for 2 h. The mixture was centrifuged at 8000 × *g* for 5 min at room temperature. The supernatant was eliminated and the precipitate washed with 300 µL of 70% ethanol. The pellet was dried and dissolved in 100 µL of TE buffer (Tris-HCl 10 mM, EDTA-Na_2 1 mM, pH 8.0) at 4°C.

The concentration of DNA was quantified using a spectrophotometer Genesys 10 uv Scanning® (Thermo Scientific) and the quality was verified by electrophoresis in agarose gel at 0.8% (w/v). The DNA was used in subsequent PCR reactions.

Random amplified polymorphic DNA (RAPD) reaction conditions

A total of 25 RAPD primers of the series A, B and D Operon® were tested (Operon technologies Inc, Alameda, CA, USA). 19 primers that showed polymorphism and clear RAPD band patterns were selected (Table 2). The PCR reactions were realized in a thermocycler Techne®. The reaction mix was carried out in a total volume of 25 µL, which included: 4.2 µL of double distilled sterile water, 10 µL of dNTPs (500 µM), 2.5 µL of buffer 10X Tris-HCl 750 mM, pH 8.8; $(NH_4)_2SO_4$ 200 mM; Tween 20 at 1% (v/v); 1.0 µL of $MgCl_2$ (50 mM); 3.0 µL of primer at a concentration of 10 pM; 0.3 µL of Taq DNA polymerase enzyme (Fermentas, U.S.A)

Table 1. Geographic origin of genotypes used in the study.

Number	Genotype	Origin
1	CAMBRAY ROSA MORELOS	México
2	CRIOLLA EDO MEX	México
3	279 - 17	U.S.A
4	286 -31	U.S.A
5	263 -68	U.S.A
6	314 -47	U.S.A
7	ALFA GRANDE	Europe
8	ATLANTIC	U.S.A
9	ARMADA	Europe
10	FIANNA	Europe
11	LB5 -5	U.S.A
12	215 - 77	U.S.A
13	286 -60	U.S.A
14	306 -54	U.S.A
15	277 -35	U.S.A
16	263 -83	U.S.A
17	286 -35	U.S.A
18	286 -40	U.S.A
19	LB5 -92	U.S.A
20	286 -9	U.S.A
21	MONTESERRAT	Europe
22	254 -83	U.S.A
23	MUNDIAL	Europe
24	176 -62	U.S.A
25	GIGANT	Europe
26	ALFA CHICA	México
27	CAMBRAY BLANCA EDO MEX	México
28	TOLLOCAN	México
29	MOCHIS DF	México
30	CAMBRAY ROSA DF	México
31	CAMBRAY DF BLANCA	México
32	CRIOLLA BLANCA PUEBLA	México
33	CRIOLLA ROSA PUEBLA	México
34	PAPA CHICA	México
35	CAMBRAY ROSA EDO MEX	México

at a concentration of 5U µL^{-1}; and 4.0 µL of genomic DNA at a concentration of 10 ng µL^{-1}.

The reaction conditions were: one cycle at 94°C, 2 min; 38 cycles (94°C for 30 s, 40°C, for 30 s, 72°C for 90 s); with an extension of 72°C for 2 min. The amplified fragments were separated by electrophoresis using agarose gel at 1.2% (w/v) with TAE buffer (40 mM Tris-acetate, pH 7.6; 1 mM Na_2 EDTA), for 1 h at 120 V. The gels were stained with ethidium bromide (0.5 mg mL^{-1}) for 15 min; the excess stain was removed by rinsing in distilled water for 5 min and photographed under UV light. The amplification reactions were repeated at least twice.

Inter-simple sequence repeat (ISSR) reaction conditions

For the ISSR reactions, five primers were used (Table 3). The amplification reactions were performed in a similar reaction volume and with similar reagents to that used for RAPDs except for the reaction conditions, 94°C for 5 min, 35 cycles at 94°C for 30 s, specific annealing temperature for 45 s and 72°C for 2 min and a final extension at 72°C for 10 min. The annealing temperature ranged from 45 to 58°C and the cycles were reduced to 30. The amplified fragments were separated by electrophoresis using agarose gel at 1.5% (w/v) with TAE buffer (40 mM Tris-acetate, pH 7.6; 1 mM Na_2 EDTA), for 1 h at 120 V. The gels were stained with ethidium bromide (0.5 mg mL^{-1}) for 15 min; the excess stain was removed by rinsing in distilled water for 15 min and photographed under UV light. The amplification reactions were also repeated at least twice.

The materials were compared based on similarities and differences in band patterns. A value of 1 was assigned for the presence and 0 for the absence of bands. A data matrix was constructed in a page of calculations for obtaining a dendrogram of relations between the accessions with the coefficient of Jaccard (Jaccard, 1908) and with the Unweighted Pair Group Method using Arithmetic Averages) UPGMA. An analysis of resampling was done (Bootstrapping, 1000 repetitions) with the aim of obtaining consistent numeric data of the tree generated by the statistical programme Free Tree version 0.9.1.50 (Hampl et al., 2001). The Mantel test (Mantel, 1967) was conducted for correlation between similarity matrices, using the programme Mantel Nonparametric Test calculator for Windows Version 2.0 (1999 to 2007) (Liedloff, 1999). Nonmetric Multi-Dimensional scaling was carried out using NTSYS-pc version 2.1 (Rohlf, 2000).

RESULTS AND DISCUSSION

Random amplified polymorphic DNA (RAPD)

19 out of the 24 RAPD primers tested of the series RAPDs; A, B and D Operon were amplified (Table 2). The mean of the detected polymorphism by RAPD primers was 82.9%. Primers A19, D04 and D10 revealed 100% polymorphism. The mean number of bands revealed by a RAPD primer was 11.15, with oscillations between 4 and 17. A total of 222 *loci* were studied and 184 were polymorphic.

The analysis based on RAPD permitted the estimation of genomic similarities and differences. Figure 1A shows band patterns obtained by RAPD primer D10, which showed differences among the potato genotypes evaluated.

Figure 2A shows a dendrogram of relationships of similarities between different genotypes of potato using RAPD markers. The results show the formation of eight groups at a Jaccard coefficient of 0.60: Tollocan formed the first group, LB5-5 the second group, the third group included Monteserrat, the fourth group was 306-54 and the fifth group was made up of a homogeneous group with Cambray Rosa Morelos and Cambray Rosa Edo Mex, which probably represent the same genotype.

Gigant formed the sixth group and 176-62 the seventh group. The eight group included the rest of the accessions. This big group consisted of two clusters; the first cluster was divided into 5 sub- groups: the first sub group included Mexican white fleshed varieties (Cambray Blanca, Mochis, Criolla Blanca) and Atlantic which is from

Table 2. List of RAPDs primers and their sequences, number of amplified products, monomorphism and polymorphism.

RAPD Primer	Sequence (5´- 3´)	Amplification products	Monomorphism (%)	Polymorphism (%)
A02	TGC CGA GCT G	14	21.43	78.57
A3	AGT CAG CCA C	14	14.28	85.72
A05	AGG GGT CTT G	12	25	75
A13	CAG CAC CCA C	10	40	60
A19	CAA ACG TCG G	13	0	100
A09	GGG TAA CGC C	7	28.57	71.43
D05	TGA GCG GAC A	12	16.66	83.33
D07	TTG GCA CGG G	10	30	70
D04	TCT GGT GAG G	4	0	100
D10	GGT CTA CAC C	17	0	100
D11	AGC GCC ATT G	11	27.27	72.72
D18	GAG AGC CAA C	13	23.07	76.92
D20	ACC CGG TCA C	12	16.66	83.33
D01	ACC GCG AAG G	9	11.11	88.88
D03	GTC GCC GTC A	15	6.66	93.33
D06	ACC TGA ACG G	9	11.11	88.88
D09	CTC TGG AGA C	11	9.09	90.9
D02	GGA CCC AAC C	10	20	80
B05	TGC GCC CTT C	9	22.22	77.77
	Mean	11.15	17	82.98

Table 3. List of ISSR primers and their sequences, annealing temperature, number of amplified products, monomorphism and polymorphism.

ISSR Primer	Sequence (5´-3´)	TA	Amplification products	Mornomophism (%)	Polymorphism (%)
P1	GAGCAACAACAACAACAA	48	14	14.29	85.71
P2	CTGAGAGAGAGAGAGAGAG	58	18	22.23	77.77
P3	AGAGAGAGAGAGAGAGTG	45	21	14.29	85.71
P4	ATGATGATGATGATG	40	14	28.58	71.42
P5	AGAGAGAGAGAGAGAG	45	15	13.33	86.66
	Mean		16.4	18.54	81.45

U.S.A. The second consisted of European varieties, Alfa Grande, Fiana and Armada. The third, clones were from U.S.A (286-31, 215-77, 263-68). The fourth were, 314-47, 279-17, Criolla Edo Mex and Papa Chica. The fifth consisted of anthocianin coloured stem Mexican varieties (Criolla Rosa Puebla, Alfa Chica, Cambray Rosa DF and 254-83. The second cluster comprised of two sub groups: Mundial, the only variety from Europe, which was also relatively more divergent at 0.65, formed the first sub group. The second sub group included clones from U.S.A. (LB5-5, 286-35, 277-35, 263-83, 286-60, 286-40 and 286-9).

Average genetic similarity between pairs of plants was 0.61 ± 0.02, with a range of 0.46-0.87. The highest similarities were obtained among European varieties. Fiana and Armada recorded 0.87, Alfa Grande and Armada, 0.83, Alfa Grande and Fian,a 0.81 and Cambray Blanca DF and Mochis 0.81. The lowest similarities were recorded between LB5-5 and Tollocan (0.46) followed by Cambray Rosa Morelos (0.48), and Gigant and Tollocan (0.48). Tollocan was the most divergent at a similarity coefficient of (0.53) from the rest of the accessions (Figure 2A).

Multi-dimensional analysis (MDA) performed on 35 accessions of potato and 19 RAPD markers clearly divided them into two groups (Figure 3A): The first cluster was made up of clones from U.S.A (LB5-5, LB-92, 306-54, 263-83, 286-35, 286-60, 277-35, 286-31, 286-40 and 286-9). This cluster also included all of the 286 series clones. The second cluster included Mexican varieties, European varieties and the rest of the clones from U.S.A (Cambray Blanca Edo Mex, Criolla Blanca Puebla, Cambray DF Blanca, Mochis, Cambray Rosa DF, Cambray Rosa Morelos, Cambray Rosa Edo Mex, Criolla

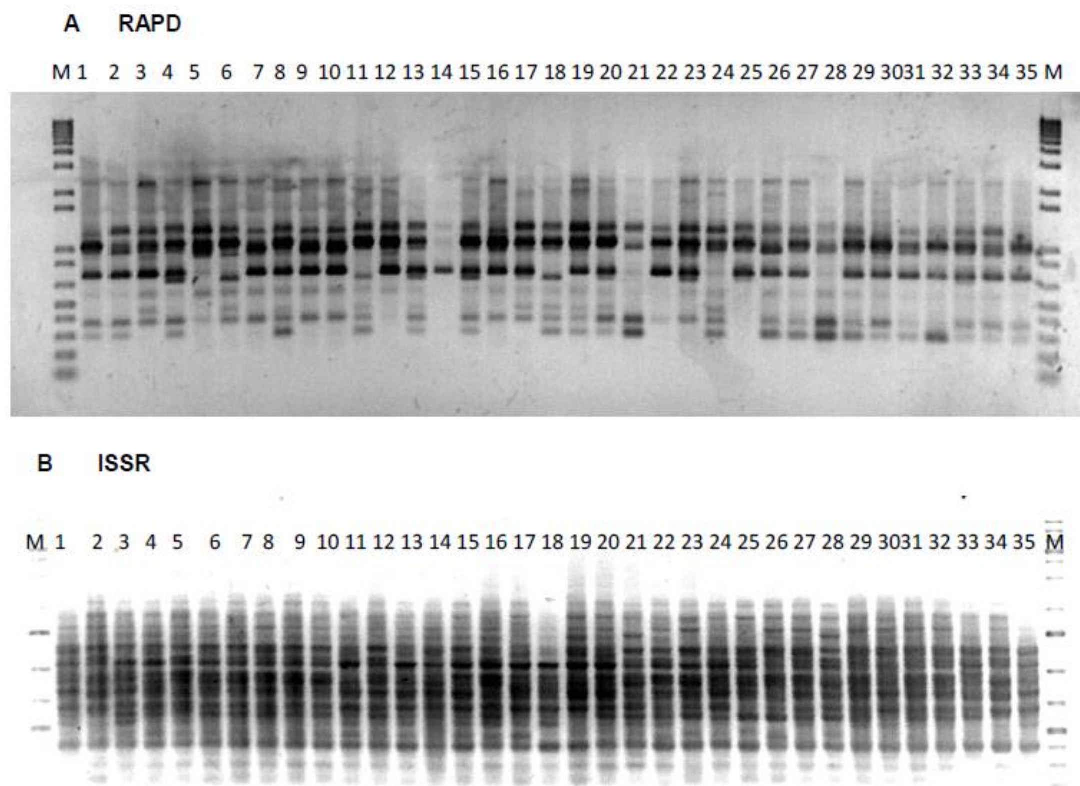


Figure 1. Gel electrophoresis of amplification products obtained with RAPD primers D10 (A) and ISSR primer P2 (B) in 35 (*Solanum tuberosum* L.) accessions as listed in Table 1.

Edo Mex, Criolla Rosa Puebla, Papa Chica and Alfa Chica, Tollocan, Fiana, Armada, Alfa Grande, Mundial, Gigant, Monteserrat, Atlantic, 279-17, 263-68, 215-77, 314-47, 254-83, and 176-62).

Inter-simple sequence repeat (ISSR)

Five ISSR primers were amplified (Table 3). The mean of the detected polymorphism by ISSR primers was 81.4%, which was comparable to the mean obtained by RAPD (82.9). The mean number of bands revealed by ISSR primers was 16.4, with oscillations between 14 and 21. A total of 82 *loci* were studied and 66.7 (81.4%) were polymorphic.

The analysis based on ISSR techniques permitted the estimation of genomic similarities and differences. Figure 1B shows band patterns obtained by ISSR primer P2, which showed differences among the potato genotypes evaluated.

Figure 2B shows a dendrogram of relationships of similarities between different genotypes of potato using ISSR markers. The results show the formation of seven groups at a Jaccard coefficient of 0.70: Cambray Rosa Edo Mex formed the first group and 286-35 the second. The third group consisted of two homogenous sub clusters: the first made of LB5-5 and LB5-92 and the second Criolla Edo mex and Papa Chica. Three homogeneous clusters of Montesserat and Tollocan, Cambray Rosa Morelos and Cambray Rosa DF, Gigant and 176-62, formed the 4th, 5th and 6th groups, respectively. Incidentally, Cambray Rosa Edo Mex formed the main out layer from the rest of the genotypes (0.47). The seventh group consisted of the rest of the accessions. This group was divided into two clusters; the first cluster consisted of potato clones from U.S.A (286-35, 286-31, 263-83, 286-60, 277-35, 286-9, 215-77, 254-83, 286-40, 306-54, 279-17, 263-68 and 314-47). The second cluster was made up of four sub groups; the first formed by Mundial, the second sub group consisted of accessions of European varieties (Alpha Grande, Armada, Fiana). Alfa Chica and Criolla Rosa Puebla which are Mexican anthocianin colored stem varieties formed sub group III. The fourth sub group consisted of Mexican white fleshed varieties Cambray Blanca Edo Mex, Criolla Blanca Puebla, Cambray DF Blanca, Mochis and Atlantic variety from U.S.A. The formation of the potato clone sub cluster from U.S.A was very distinct with the ISSR marker compared to RAPD.

The highest similarity was recorded between Cambray DF Blanca and Criolla Blanca Puebla (0.95), Cambray

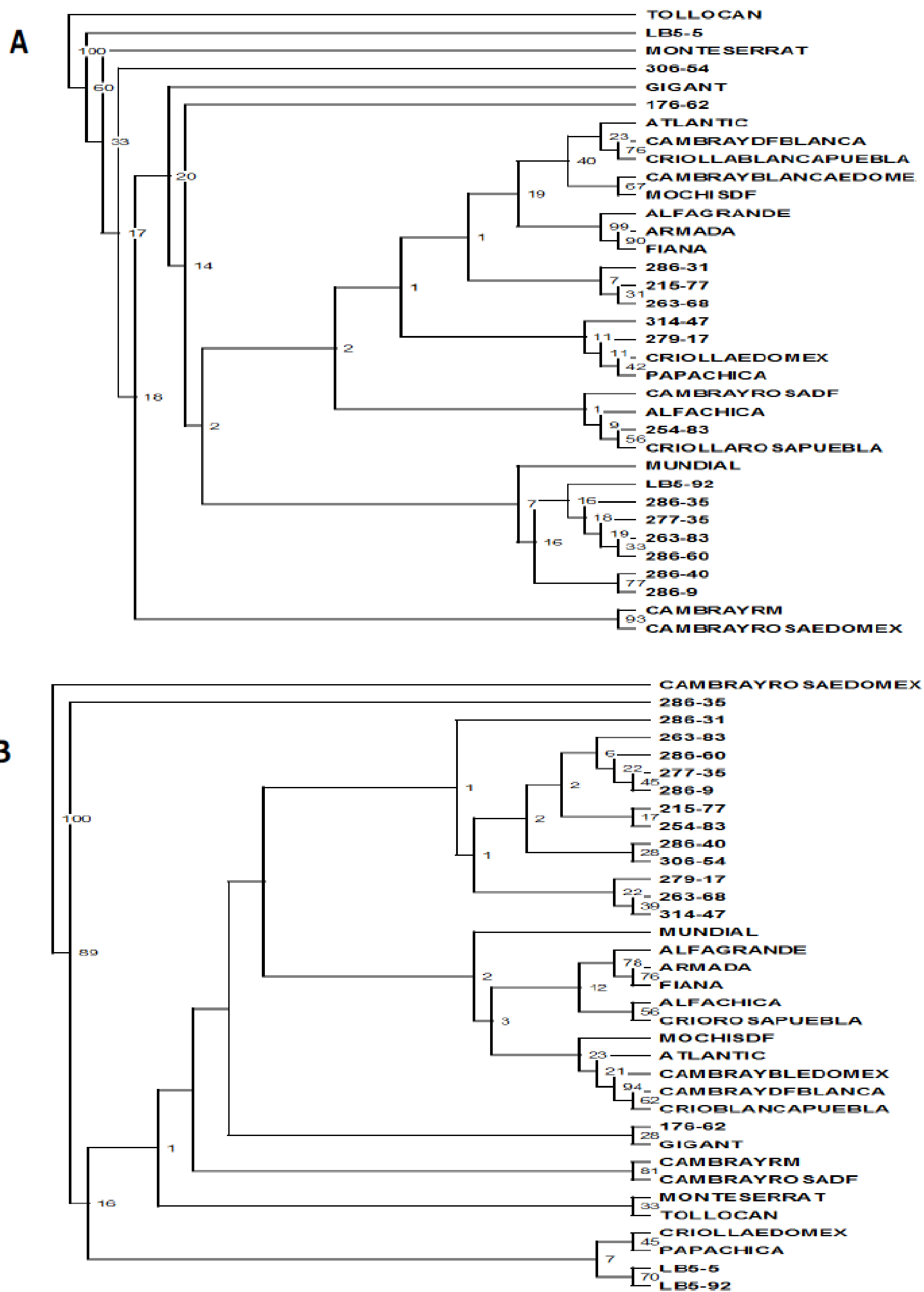


Figure 2. Dendrogram generated from RAPD (A), ISSR (B) and consensus data RAPD + ISSR (C) using Jaccard similarity coeficient.

Blanca Edo Mex and Criolla Blanca Puebla (0.94), Cambray Blanca Edo Mex and Cambray DF Blanca (0.92). The European varieties also recorded high similarity values: Fiana and Armada (0.92), Alfa Grande and Fiana (0.88) and Alfa Grande and Armada (0.86). The lowest similarity was obtained among Cambray Rosa

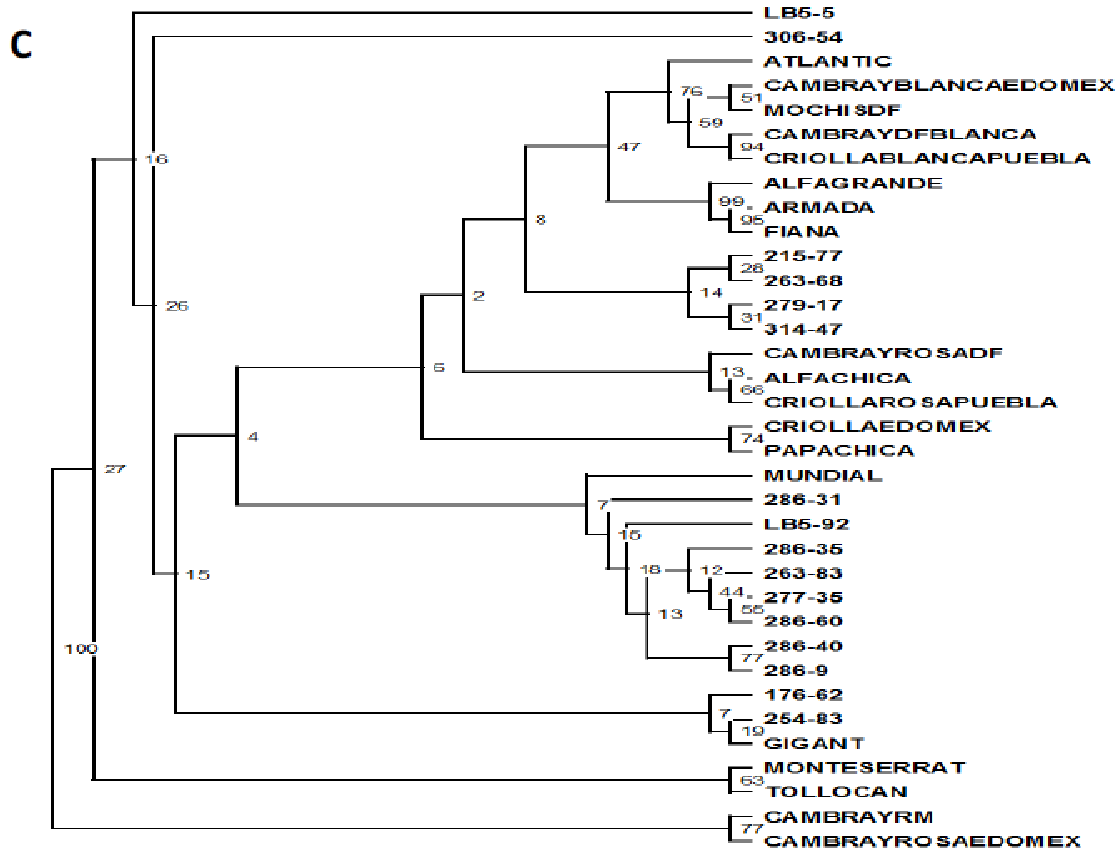


Figure 2. Contd.

Edo Mex and Monteserrat (0.40), Cambray Rosa Edo Mex and 263-68 (0.42) and Cambray Rosa Edo Mex and Armada (0.42).

Multi-dimensional analysis (MDA) performed on 35 accessions of potato and 5 ISSR markers clearly divided them into three distinct groupings (Figure 3B): the first cluster consisted of Cambray Rosa varieties from Mexico and clones from U.S.A (LB5-5, 279-17, 263-68, 254-83, 263-83, 277-35, 215-77, 314-17, LB5-92, 254-83, and 176-62). The second cluster was made up of all the European varieties and Mexican white skinned varieties (Fiana, Armada, Alfa Grande, Alfa Chica, Monteserrat, Mundial, Cambray DF Blanca, Criolla Blanca Puebla, Mochis, Cambray Blanca Edo Mex and Gigant) apart from Criolla Rosa Puebla which is red skinned and Atlantic from U.S.A. The third cluster was mostly made up of the 286 series from U.S.A (286-9, 286-60, 286-31, 286-40, 286-35 and 306-54).

Combined analysis

Figure 2C shows a dendrogram of relationships of similarities between different genotypes of potato using a combined analysis with RAPDs and ISSR markers. The results show the formation of six groups. The first group consisted of a homogenous group of Cambray Rosa Morelos and Cambray Rosa Edo Mex. Monteserrat and Tollocan formed the second group. The third group was made up of LB5-5, the fourth 306-54, the fifth 176-62, 254-83 and Gigant. The sixth group consisted of the rest of the accessions. This group was divided into two clusters. The first cluster consisted of a first sub group, made up of Atlantic from U.S.A and Mexican white fleshed varieties (Cambray Blanca Edo Mex, Mochis, Cambray DF Blanca and Criolla Blanca Puebla). The second consisted of European varieties (Alpha Grande, Armada and Fiana), and the third was made up of clones from U.S.A (215-77, 263-68, 279-17 and 314-47). The fourth sub group consisted of Mexican anthocianin pigmented stem varieties Cambray Rosa DF, Alfa Chica, and Criolla Rosa Puebla. The fifth sub group consisted of Mexican red skinned tuber varieties, Criolla Edo Mex and Papa Chica. The second cluster was made up of Mundial as the first sub group and the rest of the clones (LB5-92, 286-35, 286-83, 277-36, 286-60, 286-40 and 286-9), from U.S.A formed the second sub group. The combined analysis clearly grouped the 286 series clones from U.S.A into this second subgroup.

The highest similarity was recorded between

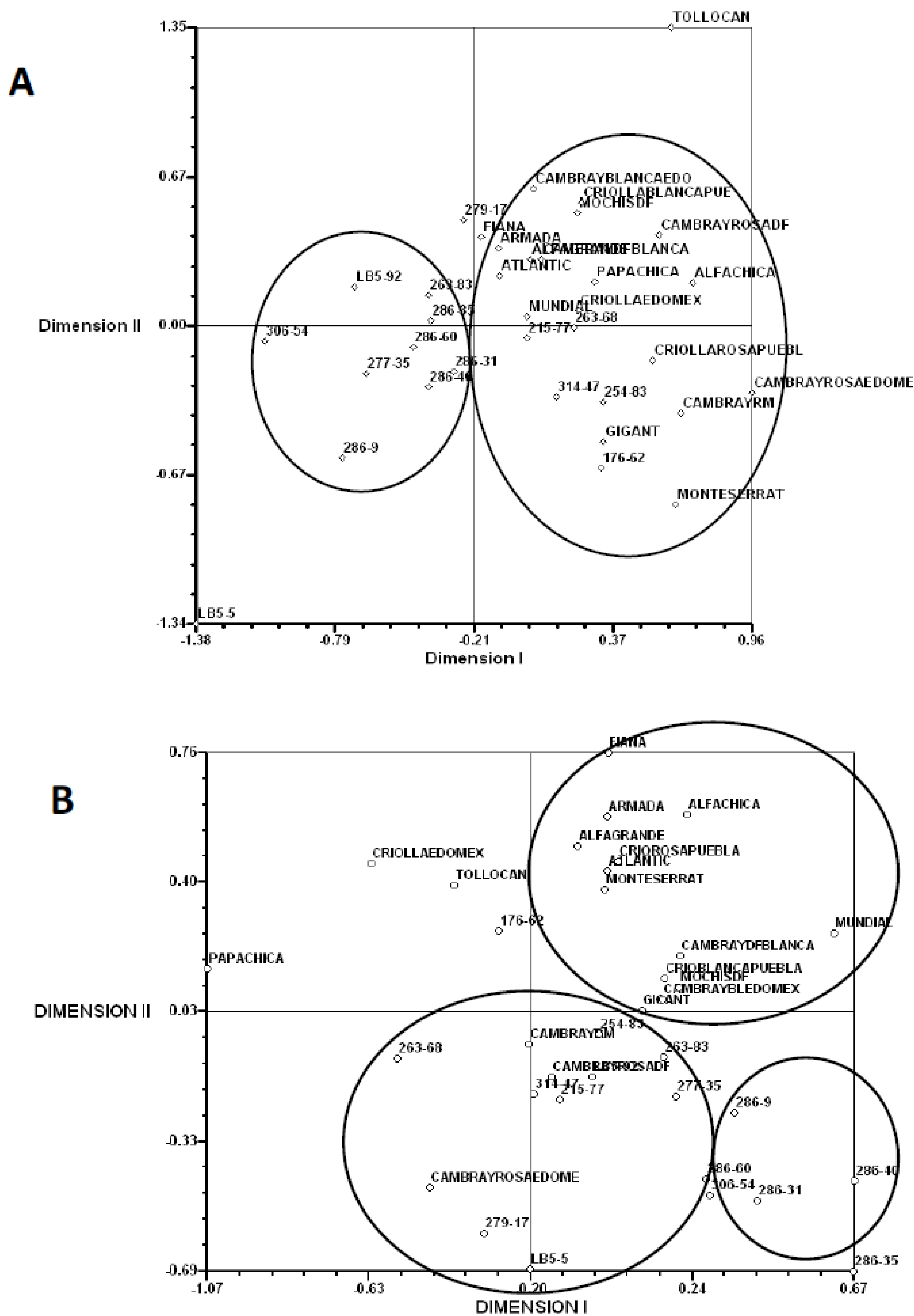
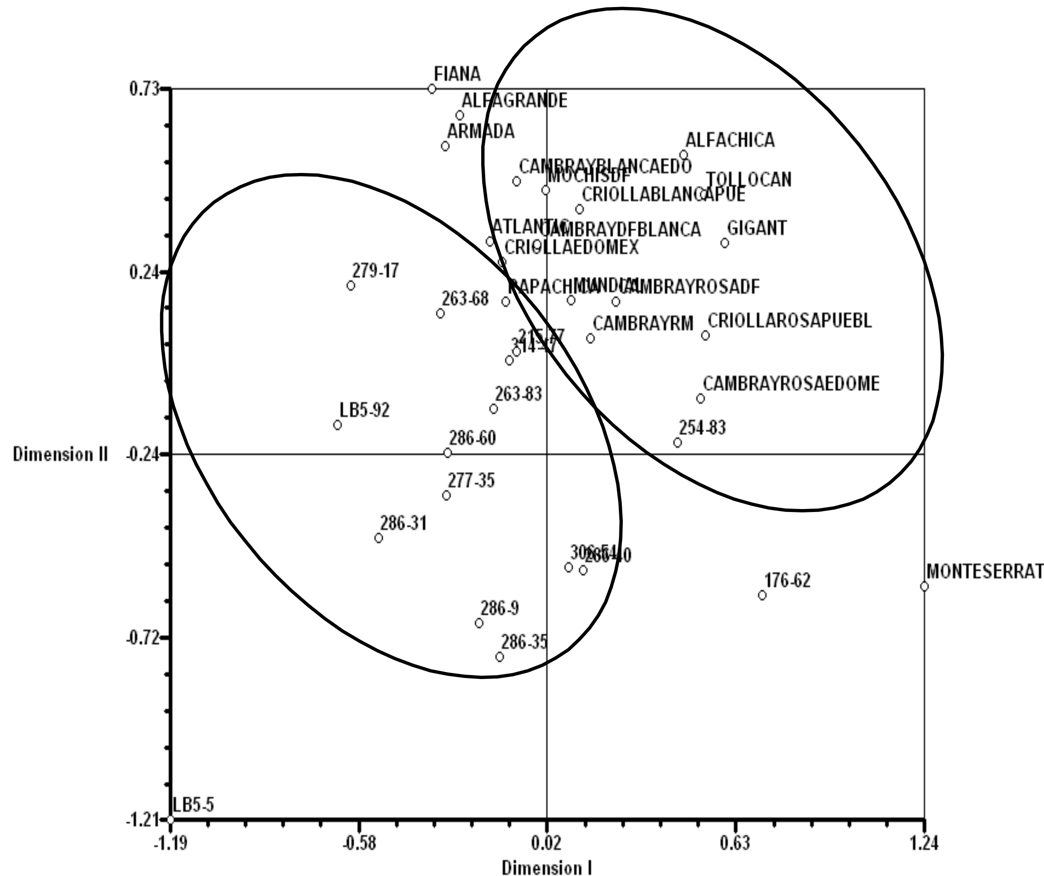


Figure 3. Scores plot of the Multi Dimension Analysis generated from RAPD (A), ISSR (B) and consensus data RAPD + ISSR (C) of all the 35 potato genotypes.



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European varieties: Fiana and Armada (0.88), Alfa Grande and Armada (0.84), Alfa Grande and Fiana (0.83) and Mexican white fleshed varieties: Cambray DF Blanca and Criolla Blanca Puebla (0.87), Cambray Blanca Edo Mex and Criolla Blanca Puebla (0.82) and Cambray Blanca Edo Mex and Cambray DF Blanca (0.80). The lowest similarity was obtained among Cambray Rosa Edo Mex and Tollocan (0.48), Cambray Rosa Edo Mex and LB5-5 (0.48) and Cambray Rosa Edo Mex and Mundial (0.49).

Multi-Dimensional Analysis (MDA) performed on 35 accessions of potato and 24 RAPD and ISSR markers clearly divided them into two distinct groups (Figure 3C). The first cluster was made up of potato clones from U.S.A. This cluster contained a defined subgroup of the 286 series potato clones. The second cluster consisted of all the potato varieties. Similar representation is shown in Figure 4 for three dimensional analyses.

DISCUSSION

The MD analysis provided additional information for genetic relationships among the potato accessions studied. Cultivar identification and genetic relationships using RAPD markers in potato is well documented (Mori et al., 1993; Charchravarty et al., 2003; Orona-Castro et al., 2006; Yasmin et al., 2006 and Rocha et al., 2010).

In the present study, high polymorphism levels were detected among potato genotypes using ISSR markers. In similar studies, the ISSR makers were quite promising in the identification of potato cultivars (Prevost and Wilkinson, 1999; Bornet et al., 2002).

The Mantel test (Mantel, 1967) carried out between RAPD and ISSR similarity matrices showed a positive relationship between the data but a low correlation (0.27). Previous studies of genetic diversity using PCR based molecular markers reported similar results. Rocha et al. (2010) and Ghislain et al. (2006), observed low correlation between RAPD and SSR. Similarly, Milbourne et al. (1997), obtained low correlation between RAPD, AFLP and SSR. The reason for these findings could be due to the kind of information obtained by different molecular markers since different molecular markers amplify different repetitive and non-repetitive regions of the genome (Ghislain et al., 2006). Further analysis between RAPD and RAPD + ISSR showed low correlation (0.47) and ISSR and RAPD + ISSR showed a

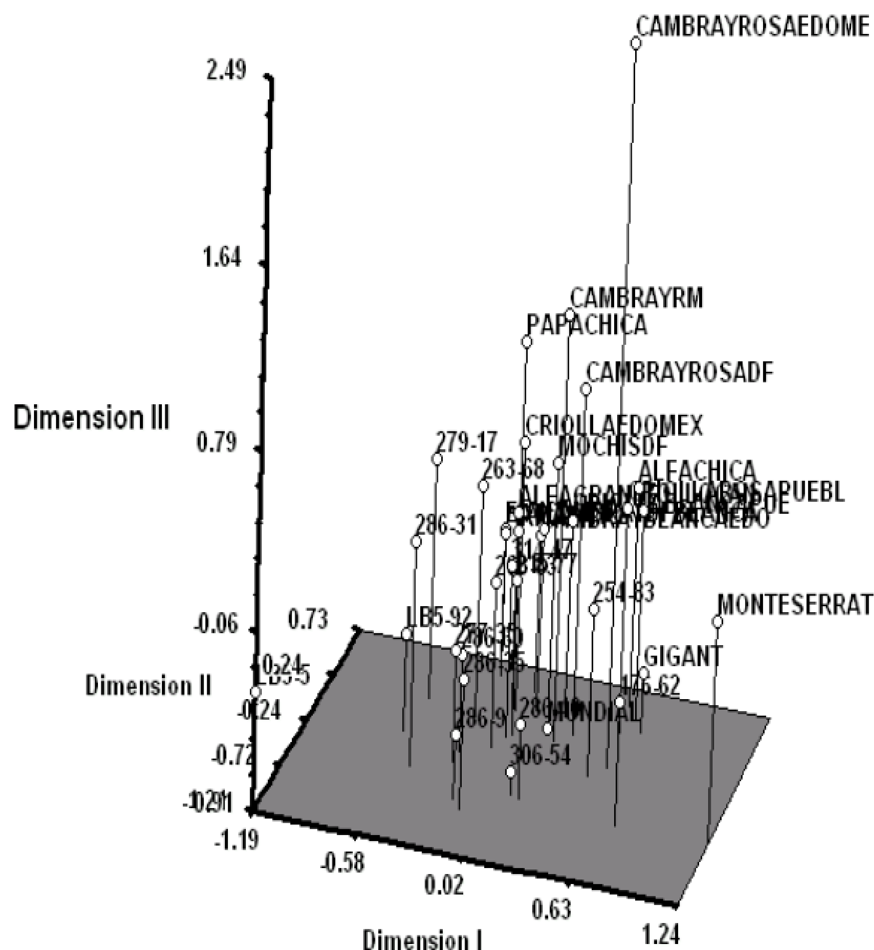

Figure 4. Tridimensional analysis generated from consensus data RAPD + ISSR of all the 35 potato genotypes.

high correlation (0.74). With only 5 primers, ISSR marker achieved comparable polymorphism (81.45%) to RAPD DNA marker (82.98%). The ISSR molecular marker has been reported to be powerful in analyzing genetic diversity in potato (Bornet et al., 2002), and in relation to RAPD marker in other plants (Rus-Kortekaas et al., 1994; Nagaoka and Ogihara, 1997; Raina et al., 2001). This may elucidate the high correlation between ISSR and RAPD + ISSR consensus data. The use of complimentary approaches, such as using both RAPD and ISSR data may provide more accurate information on genetic diversity.

Generally, the potato genotypes formed subgroups related to origin, probably due to shared parentage in breeding programs within a given region. Clustering of potato genotypes based on origin has been reported by several authors. Esfahani et al. (2009) reported clustering of potato genotypes based on origin from Europe and North America. Similarly, Bornet et al. (2002) discriminated potato genotypes based on origin from Europe and Argentina.

Conclusion

In conclusion, RAPD and ISSR markers successfully discriminated between the 35 different potato accessions used in the study. Consensus data provided more reliable information related to the genotypes. The results from this study will be useful for assisting in the selection of parental combinations for developing progenies with maximum genetic variability for genetic mapping or further selection.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The first author did this work as part of her doctoral studies at The Autonomus University of Chapingo,

Texcoco, Mexico. The authors are grateful to Dr. Hector Lozoya Saldaña, Department of Fitotecnia of the Autonomus University of Chapingo, Texcoco, Mexico, for providing potato tuber seeds. Appreciation to Foreign Affairs (Secretaria de Relaciones Exteriores (SRE), Mexico for funding the research study.

Abbreviations

ISSR, Inter-simple sequence repeat; **Ta,** annealing temperature; **RFLPs,** restriction fragment length polymorphism; **PCR,** polymerase chain reaction; **RAPD,** random amplified polymorphic DNA; **SSR,** simple sequence repeats; **AFLP,** amplified fragment length polymorphism.

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Vol. 15(22), pp. 1026-1034, 1 June, 2016
DOI: 10.5897/AJB2015.15175
Article Number: EAC802558673
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

In vitro regeneration of 'Feizixiao' litchi (*Litchi chinensis* Sonn.)

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Received 16 December, 2015; Accepted 28 April, 2016

A simple efficient *in vitro* plant regeneration system was developed by indirect somatic embryogenesis of 'Feizixiao' litchi (*Litchi chinensis* Sonn.). Pollen in the anther of monocytes was used to induce callus. Two auxins (naphthalene acetic acid [NAA] and 2,4-dichloriphenoxyacetic acid [2,4-D]), and two cytokines (kinetin [KT] and 6-benzyladenine [BA]) were tested to explore their influence on callus induction. MS medium supplemented with 2.22 µM BA, 2.69 µM NAA, 13.57 µM 2,4-D, and 0.4 g/L LH (lactalbumin hydrolysate) showed the highest callus induction frequency. The callus obtained from anther was subcultured in MS medium containing 4.52 µM 2,4-D to obtain synchronized friable embryogenic callus. Different developmental stages of SEs were obtained from the callus on MS medium containing 6% (w/v) sucrose and different PGRs (plant growth regulators). On MS medium containing 6% (w/v) sucrose and supplemented with 0.54 µM NAA, 23.23 µM KT, 0.4 g/L LH, 0.56 µM inositol, and 10% (w/v) CW (coconut water), a higher number of SEs (globular, heart, torpedo and cotyledonary embryos) was achieved than on other media. Plantlets were established onto half-strength MS medium containing 1.44 µM GA_3 (gibberellic acid) followed by successful acclimatization in the greenhouse. With flow cytometry and chromosome counting, ploidy analysis of regenerated plants revealed that the regenerated plantlets were all diploid. This study is the first report on somatic embryogenesis of 'Feizixiao litchi', providing an opportunity to improve the cultivar by biotechnology methods.

Key words: litchi (*Litchi chinensis* Sonn.), anther culture, callus, regeneration, somatic embryogenesis.

INTRODUCTION

Litchi (*Litchi chinensis Sonn.*), known as "the queen of fruit" (Menzel and Waite, 2005), is an important fruit tree in the tropical and subtropical regions of the world (Menzel, 1983). Given its long reproductive cycle and highly heterozygous genetic background (Litz, 1988; Raharjo and Litz, 2005), new litchi cultivars are difficult to create via conventional breeding methods. Modern breeding techniques such as gene manipulation have the advantages of high efficiency and directional improvement of specific traits, providing a new way for the improvement of litchi cultivars (Das and Rahman, 2012). The establishment of a regeneration system *in*

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vitro for litchi bioengineering breeding is vital. Many efforts have been made regarding the subject, but few successful results have been published. Fu and Tang (1983) reported that they obtained 24 plantlets via organogenesis from pollen callus. Subsequent reports were made on somatic embryogenesis and plantlet regeneration in litchi via the culture of anthers (Deng, 2005; Xie et al., 2006; Wang et al., 2013), immature embryos (Zhou et al., 1993, 1996; Kuang et al., 1997), or protoplasts (Yu et al., 1996). Das et al. (1999) reported a high proliferation rate from mature seeds using two kinds of methods. Yu et al. (2000) achieved somatic embryogenesis and plantlets from 'Xiafanzhi' litchi protoplasts isolated from embryogenic suspensions. Puchooa (2004) used young unvaccinated 'Tai So' leaves as explants to study various factors on the regeneration of the leaf blade and eventually obtained regenerated plants. By 'Heli' anther culture, Guo et al. (2014) obtained somatic embryos with root and no sprout. However, Huang and You (1990) and Yu (1991) and Guo et al. (2014) reported that the stem sprout buds, failed to produce (Huang and You, 1990; Yu, 1991). All the above results showed that the regeneration of different litchi varieties is inconsistent with their medium. The same culture medium plays different roles on the regeneration of different litchi varieties. Therefore, different genetic backgrounds of litchi varieties have a remarkable effect on *in vitro* regeneration ability. To date, only a few cultivars, such as 'Nuomici' (Kuang et al., 1997), 'Xiafanzhi' (Lai and Sang, 2003) and 'Hushanjiaohe' (Fu and Tang, 1983), have been successfully regenerated *in vitro*. 'Feizixiao' is an early maturity variety with a tender, juicy, sweet aril and high and stable yield. *In vitro* regeneration of 'Feizixiao' is potentially a valuable method for conservation, mass propagation, and genetic transformation of this species. Although 'Feizixiao' micropropagation has been described (Deng, 2005), no report exists regarding its *in vitro* plant regeneration by a somatic embryogenesis system.

This study aimed to develop a simple and efficient protocol for efficient plant regeneration rates via somatic embryogenesis from anther explants of 'Feizixiao', as well as to investigate the effects of plant growth regulators on the processes.

MATERIALS AND METHODS

Plant materials and callus induction

Branches with floral buds of 10-year-old 'Feizixiao' were collected from the base of Hainan Leihu Fruit Ltd. on March 12, 2011. Immature flowers were washed in running tap water to remove dust. They were then dipped into 70% (w/v) ethanol, and 0.1% (w/v) $HgCl_2$, followed by three rinses in sterile distilled water under aseptic conditions. The anthers were separated from the flowers for callus induction.

The anthers were cultured on MS (Murashige and Skoog, 1962) solid (containing 0.7% (w/v) agar) medium with 0.4 g/L LH and 30 g/L sucrose. The medium was supplemented with different concentrations and combinations of plant growth regulators [PGRs: BA (0, 0.89, 2.22, and 4.44 μM), KT (0, 2.32, 4.65, and 9.29 μM), NAA (0, 1.07, 2.69, and 5.37 μM), 2,4-D (0, 4.52, 9.05, and 13.57 μM)] for callus induction. An orthogonal experimental design was used for these experiments with three repeats for each treatment, seven bottles per repeat, and 9 to 11 anthers per bottle. The callus was record after 8 weeks of culture. The callus obtained from anthers was subcultured in MS medium containing 4.52 μM 2,4-D to obtain synchronized friable embryogenic callus.

Somatic embryogenesis

The 18-day-old friable embryogenic calli (light, yellow, vigorous, fine-grained) were transferred to MS medium supplemented with 6% (w/v) sucrose, 0.4 g/L LH, 0.56 μM inositol, 10% (w/v) coconut water, and a combination of auxin NAA (0–0.54 μM) and cytokines KT (13.94, 23.23, and 32.53 μM), ZT (13.68, 22.81, and 31.93 μM), and TDZ (13.62, 22.71, and 31.79 μM). A completely randomized design with 10 replications (0.1 g of fresh callus per replicate) for each treatment was used for these experiments. Different developmental stages of SEs (>0.3 cm) were recorded after 7 weeks of culture.

Plant regeneration

Different developed stages of SEs (>0.8 cm) were transferred to different MS basal media (MS, 1/2 MS) supplemented with various concentrations of GA_3 (0, 1.44, 2.89 and 5.77 μM) and 3% (w/v) sucrose for regeneration. A completely randomized design with 15 replications (4 to 6 explants per replicate) was used for these experiments. Regeneration was recorded after 9 weeks of culture. These explants were incubated under 16 h (lightness)/8 h (darkness) photoperiod (provided by cool-white fluorescent lamps at a photon flux of 27 $\mu mol/m^2s^1$) at 26±2°C.

All media were adjusted to pH 5.8 prior to autoclaving at 121°C for 20 min. All explants were incubated in the dark, unless stated otherwise.

Flow cytometry and chromosome counting

The ploidy level of regenerated plantlets was analyzed using flow cytometry. Root tips were chopped and measured using a Cell Lab Quanta SC (Beckman Coulter, CA, USA). Nuclei obtained from the root tip of air layering seedlings were used as a diploid control. Histograms of DNA content were evaluated using Cell Lab Quanta SC MPL Analysis software (Beckman Coulter, CA, USA).

Chromosome counts were carried out on root tip cells of the plantlet pretreated for 3 h with a saturated solution of 1,4-dichlorobenzene. Roots were fixed in 3:1 ethanol-glacial acetic acid, hydrolyzed in 1 M hydrochloric acid, and rinsed three times with distilled water. Each rinsed root tip was placed on a glass slide. The root cap was removed, and the remaining root was stained with a carbol fuchsin solution. Each root tip was moderately squashed as the cover slip was applied to help spread the chromosomes. Micrographs were taken with an FSX-100 microscope camera system (Olympus, Tokyo, Japan).

Specimen preparation for light microscopy

According to the methods described by He (1989) and Lee and Mu (1966), sections of friable embryogenic callus, white opaque SEs, non-friable embryogenic callus, different abnormal SEs, and vitrified somatic embryos were fixed in FAA for 20 h. The specimens were then rinsed three times in 70% (w/v) ethanol. For staining, the

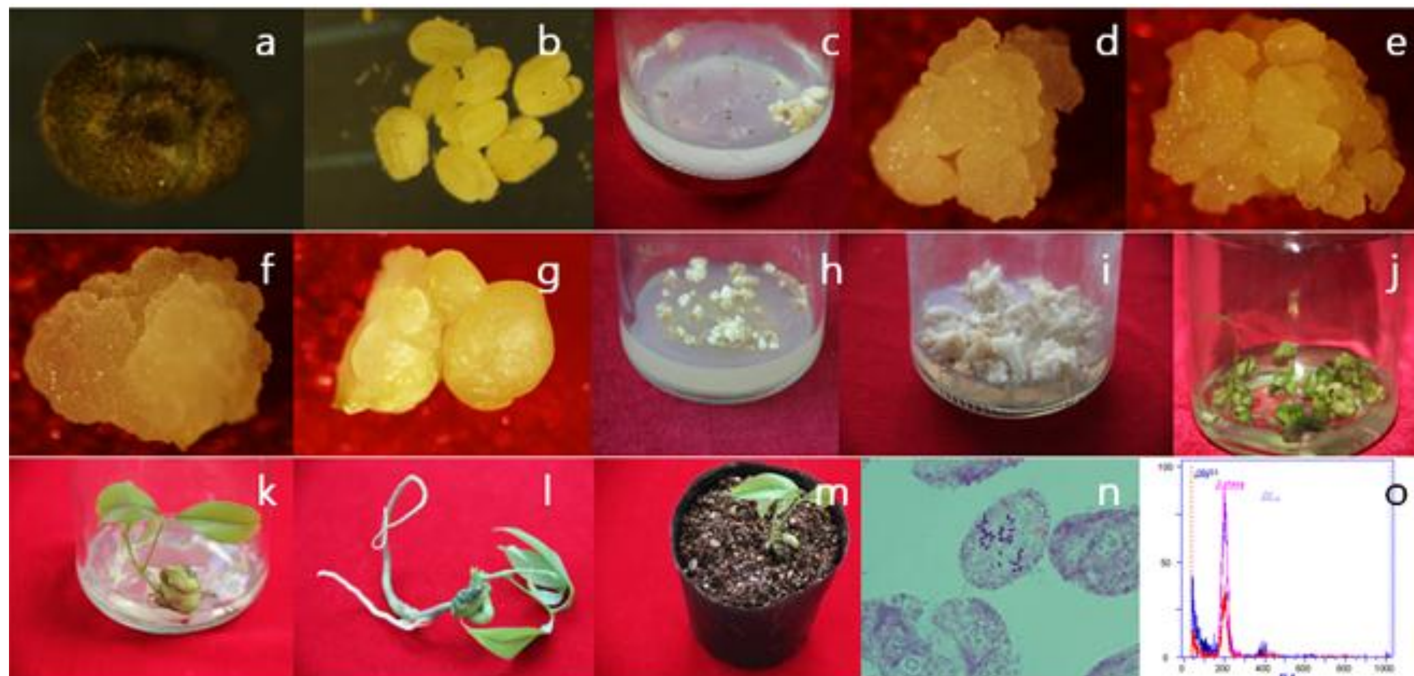

Figure 1. *In vitro* plant regeneration by indirect somatic embryogenesis from anthers of ‘Feizixiao’: (a) and (b) Immature flower buds and yellow anther (×5.6). (c) Different friable embryogenic callus developed. (d-g) Microscopic photograph of developmental stages of friable embryogenic callus (×2.5). (h and i) Induction of white somatic embryos on MS medium supplemented with 0.54 μM NAA, 23.23 μM KT, 4 g/L LH, 0.56 μM inositol, and 10% (w/v) CW. (j-l) Germination into plantlets in MS medium supplemented with 1.44 μM GA_3. m Acclimatized litchi plantlets in 1:1:1 (v/v/v) vermiculite: sand: coconut chaff mixture. n and o Determination of ploidy level of regenerated plants by flow cytometry and chromosome counting, displaying 2n = 2x = 30.

specimens were stained in Ehrilich’s dyeing liquid. The specimens were dehydrated in a range of different concentrations of dimethylbenzene and embedded in different concentrations of paraffin. The specimen block was roughly trimmed to exclude excess plastic matrix and expose the surface of the specimen. The roughly trimmed block was mounted into a Reichert Ultracut microtome, and 5 to 6 μM sections were cut using glass knives.

Statistical analysis

Data were statistically analyzed using ANOVA and are presented as the mean ± standard error of all independent experiments. Treatment means were separated using Duncan’s multiple range test at the 5% probability level and analyzed using DPS (version 2.0, Tang, 2010).

RESULTS AND DISCUSSION

Induction of embryogenic callus

Embryogenic calli were achieved from anther explants on MS medium, 3% (w/v) sucrose, and various PGRs in combination. One callus formed at the yellowish anthers after approximately 3 weeks of culture, other calli developed in all treatments, except the control, after 6 weeks (Figures 1a to c). The first callus was more light yellow in color, heavily watery, and non-granular (Figure 1d). The other was non-friable callus. Other calli emerged after 6 weeks. These calli were light yellow, compact, friable, tiny and/or nodular, and pre-embryogenic (Figures 1e to g). Amin and Razzaque (1995), Yu and Chen (1997), Puchooa (2004), Rahario and Litz (2007), Ma et al. (2009), Xu and Lai (2013), and Guo et al. (2014) also reported a similar type of callus development in other litchi cultivars. Similar phenomena were also found in longan (Lai et al., 1997), ‘Premier’ honey peach (Lai et al., 2008), *Heavea* (Tan et al., 2009), and *Pinus koraiensis* (Wang et al., 2015).

Different combinations of PGRs were used to test their effect on the induction of embryogenic callus. The experiment revealed significant differences in the number of callus produced among treatments, indicating that the combination and concentration of PGRs exerted a significant effect on callus induction (Table 1). LSD test revealed that a significantly higher number of callus was obtained when anthers were cultured in medium L9 (2.22 μM BA, 2.69 μM NAA, and 13.57 μM 2,4-D), followed by medium L8 (0.89 μM BA, 9.29 μM KT, 2.69 μM NAA, and 4.52 μM 2,4-D) and medium L3 (4.65 μM KT, 2.69 μM NAA, and 9.05 μM 2,4-D). No significant difference was observed among L4, L12, L14, and L15, which produced fewer FECs compared with the other media. Medium L1, which contained no added PGRs, produced no callus. Interestingly, medium L12, in which callus induction was low, produced callus similar to

Table 1. Effect of growth regulators combination on callus induction of litchi of 'Feizixiao'.

Medium	Growth regulators (µM)				Frequency of callus (%)	Types of callus
	BA	KT	NAA	2,4-D		
L1	0	0	0	0	0	Black, die
L2	0	2.32	1.07	4.52	79.52±0.16[abcd]	CII b
L3	0	4.65	2.69	9.05	82.31±0.17[abc]	CII b
L4	0	9.29	5.37	13.57	60.47±0.13[de]	CII a
L5	0.89	0	1.07	9.05	77.75±0.18[abcd]	CII b
L6	0.89	2.32	0	13.57	63.26±0.20[abcd]	CII b
L7	0.89	4.65	5.37	0	66.49±0.20[cde]	CII a
L8	0.89	9.29	2.69	4.52	87.07±0.13[abc]	CII b
L9	2.22	0	2.69	13.57	93.06±0.12[a]	CIII
L10	2.22	2.32	5.37	9.05	70.27±0.09[cde]	CII b
L11	2.22	4.65	0	4.52	80.13±0.18[abcd]	CII b
L12	2.22	9.29	1.07	0	44.28±0.09[e]	CII a
L13	4.44	0	5.37	4.52	77.46±0.12[ab]	CIII
L14	4.44	2.32	2.69	0	59.28±0.13[de]	CII a
L15	4.44	4.65	1.07	13.57	56.83±0.15[e]	CII a
L 16	4.44	9.29	0	9.05	79.82±0.16[abcd]	CII [b]
K1	1556.19	1737.98	1562.62	1190.48		
K2	2062.14	1906.43	1808.81	2269.40		
K3	2014.29	2000.48	2252.14	2171.19		
K4	1913.93	1901.67	1922.98	1915.48		
R	18.07	9.38	24.63	38.53		

Results are mean ± SD derived from 7 bottles / Repeated, 9 to 11 anthers / bottle. Means followed by the same letter do not differ significantly (p=0.05) as indicated by one-way ANOVA followed by Duncan's comparison test. CII a: Light yellow, compact, loose, coarser particles, many pre-embryos; CII b: Light yellow, compact, loose, Tiny and coarse particles, a little pre-embryo; CIII: Light yellow, hard, more tiny particles (Lai et al., 1997). K1, K2, K3 represents the sum of the indicators for each level of each factor repeated seven times, K1 represents the value sum of "1" level corresponding to the test index. BA: K1= (L1+L2+L3+L4)*7; KT: K1= (L1+L5+L11+L16)*7; R represents Range. Range = the maximum value of average yield- the minimum value of the average yield. BA: R= (K2-K1)/ (7*4); KT: R= (K3-K1)/ (7*4).

those in medium L9. These observations suggested that certain auxin-cytokinin ratios in medium may be necessary for callus induction from anther culture of litchi. In this study, R value revealed that 2,4-D was most effective in FEC induction, followed by NAA, BA, and KT. The effect of 2,4-D on FEC induction was also observed in other litchi species (Fu and Tang, 1983; Zhou et al., 1993). Yu and Chen (1997) reported that induction of embryogenic callus from immature embryos after NAA, instead of 2,4-D, was a failure. Su et al. (2004) showed that the callus in medium without 2,4-D did not survive the following experiment. Many species confirmed that FEC induction is influenced by 2,4-D, such as in pine (Yu et al., 2011), in longan (Lai et al., 1997), and *Hevea brasiliensis Mull. Arg* (Huang et al., 2014).

In the same medium, continuous EC subculture will lead to embryonic loss, thus, the addition of KT and $AgNO_3$ in the medium synchronized embryogenesis (Lai et al., 1997). The regulated EC differentiated into somatic embryogenesis after 18 days of culture. In the present study, three different combinations of PGRs were used in SE formation.

Somatic embryo formation

Somatic embryogenesis was achieved from EC on MS medium, 6% (w/v) sucrose, and various PGRs alone or in combination. The response of SEs to three different media was extremely variable, especially at five weeks after culture initiation (Figures 1 and i). Simon et al. (2007) also reported a similar type of embryo development in 'Brewster'. Duncan's multiple range test indicated that the medium supplemented with KT and NAA produced a significantly higher number of SEs compared with the other two media; the average SE induction numbers were 489, 344 and 101 FW/g in medium supplemented with KT and NAA, ZT and NAA, and TDZ and NAA, respectively. In this study, SE shapes varied after 7 weeks in treatments (Table 2). The embryo development stages observed were double cotyledon, no cotyledon embryo, single cotyledon, multi-cotyledon embryo, flake embryo,

Table 2. Effect of growth regulator combination on somatic embryogenesis of 'Feizixiao'.

Medium	Number	Growth regulators (μM)				Frequency of embryo formation (%)			
		NAA	KT	ZT	TDZ	Cotyledonary	Double-stages	Different stages of embryo	Means
T1	1	0	23.23	0	0	12.1±1.67[ab]	1.9±2.50[abc]	86±2.60[efg]	314
T2	2	0.54	13.94	0	0	12.7±1.92[ab]	2.5±2.48[abc]	84.8±1.73[g]	118
T3	3	0.54	23.23	0	0	13.3±2.50[a]	2±2.18[abc]	84.7±3.81[g]	489
T4	4	0.54	32.53	0	0	11.2±1.38[bc]	3.5±3.02[a]	85.3±3.75[f]	143
T5	5	0	0	22.81	0	11.1±0.79[bc]	1.1±1.78[c]	87.8±1.94[def]	181
T6	6	0.54	0	13.68	0	11.9±1.15[abc]	0.9±0.74[bc]	87.2±1.30[efg]	109
T7	7	0.54	0	22.81	0	12.8±1.81[ab]	2±0.99[ab]	85.2±2.06[g]	344
T8	8	0.54	0	31.93	0	10.5±1.73[cd]	1.5±1.61[abc]	88±2.63[de]	133
T9	9	0	0	0	22.71	9.2±1.10[de]	0±0.00[d]	90.8±1.10[bc]	87
T10	10	0.54	0	0	13.62	8.7±1.48[ef]	0±0.00[d]	91.3±1.48[bc]	23
T11	11	0.54	0	0	22.71	6.9±1.16[g]	0±0.00[d]	93.1±1.16[a]	101
T12	12	0.54	0	0	31.79	7.7±1.36[fg]	0±0.00[d]	92.3±1.36[ab]	117

Results are mean ± SD derived from 10 bottles (each 0.1 g FEC). Means followed by the same letter do not differ significantly (p=0.05) as indicated by one-way ANOVA followed by Duncan's comparison test. Different stages of embryo: globular embryos, heart-shaped stage, torpedo-shaped embryos and cotyledonary embryos.

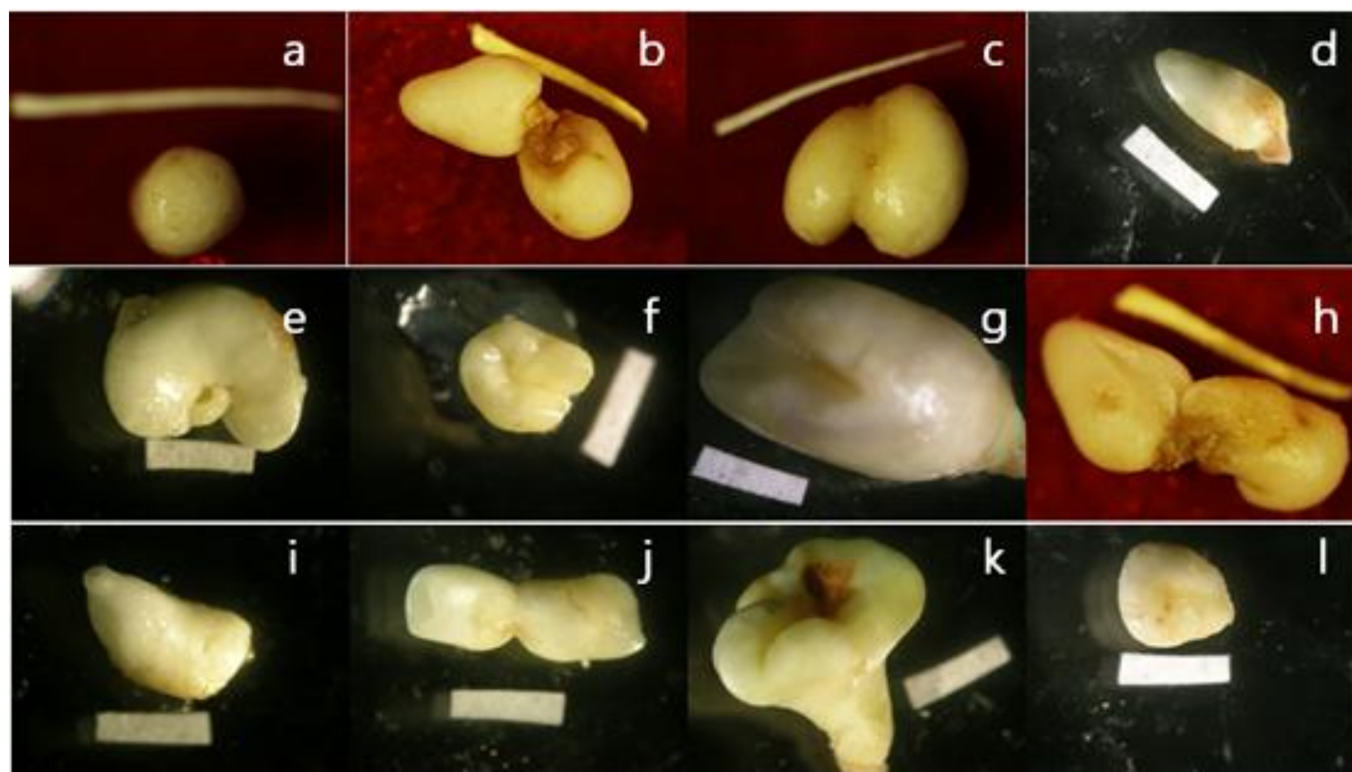

Figure 2. Microscopic photograph of developmental stage of somatic embryos. (a-d) Globular, heart, torpedo, and cotyledonary embryos. (a, d) (×2.5) and (b, c) (×2). Bar, 6 mm. No cotyledon, single cotyledon, multi-cotyledon, flake, goblet, conjoined, trumpet and mass embryo (×2.5). Bar, 3 mm.

conjoined embryo, goblet embryo, trumpet embryo, and mass embryo (Figure 2). Similar patterns of embryo development stages were reported for other plant species (Kuang et al., 1996; Tan et al., 2011).

These experiments resulted in significant differences in the number of SEs produced among treatments,

Table 3. Effect of combination of different basic medium with different regulator on regeneration.

Medium	Number	Basal medium	Growth regulators (μM)	Number of culture	Number of root	Number of regeneration	Frequency of root (%)	Frequency of regeneration (%)
		MS	GA3					
R1	1	1	0	88	12	1	13.78±9.58[b]	1.11±4.30[b]
R2	2	1	1.44	91	9	2	10±15.17[b]	2.22±5.86[ab]
R3	3	1	2.89	71	14	5	19.67±22.87[b]	6.67±12.34[a]
R4	4	1	5.77	76	8	0	10.44±14.74[b]	0±0[b]
R5	5	1/2	0	71	16	0	23.67±25.15[b]	0±0[b]
R6	6	1/2	1.44	73	50	1	68.33±10.97[a]	1.33±5.16[b]
R7	7	1/2	2.89	81	12	1	15.33±22.46[b]	1.33±5.16[b]
R8	8	1/2	5.77	85	20	0	22.89±21.41[b]	0±0[b]

Results are mean ± SD derived from 15 bottles / Repeated, 4 to 6 anthers / bottle. Means followed by the same letter do not differ significantly (p=0.05) as indicated by one-way ANOVA followed by Duncan's comparison test.

indicating that the combination and concentration of PGRs had a significant effect on SE yield (Table 2). For double cotyledonary embryos, Duncan's multiple range test revealed a significantly higher number of SEs when FECs were cultured in medium T3 (0.54 μM NAA and 23.23 μM KT), followed by medium T7 (0.54 μM NAA and 22.81 μM ZT) and medium T2 (0.54 μM NAA and 13.94 μM KT). No significant differences were observed among T8, T9, T10, T11 and T12 media which produced fewer SEs compared with the other media. Interestingly T1 and T4, with only cytokinin (KT or ZT) and no auxin (NAA) added, produced smaller and fewer SEs but medium T9 displayed contrasting results. These observations suggested that certain auxin-cytokinin ratios in medium may be necessary for SE formation.

Plant regeneration

Successful plant regeneration on the plant induction medium was achieved from post-somatic embryogenetic developmental stages: Globular, heart, torpedo, cotyledonary, and plumule embryos. Most double cotyledonary and conjoined mature embryos germinated to produce complete plantlets after 4 weeks in culture. Most abnormal embryos failed to germinate on the entire medium.

Half-strength solid MS medium produced a higher frequency of rooting (23.67%) but a lower frequency of germinated plantlets, whereas MS medium produced a lower frequency of rooting (13.78%) and well-germinated plantlets (1.11%) (Table 3). The frequency of rooting in half-strength solid MS medium was higher than that in full-strength solid MS medium in *Pyrus communis L.* (Predieri et al., 1999). Different salt concentrations would change the osmotic pressure, thereby affecting nutrient absorption and release substances into the medium. Perhaps the decrease in the concentration of nitrogen in half-strength solid MS medium stimulates rooting.

Meanwhile 2.89 μM GA_3 produced higher frequency of rooting (68.33%) but lower germination (1.33%) in half-strength solid MS medium, whereas full-strength solid MS medium produced lower frequency of rooting (19.67%) and well germinated plantlets (6.67%). The beneficial effect of gibberellins on somatic embryo germination has been reported in other plant species. For example, in *Clematis*, enhanced frequency of shoot development and greater internode elongation resulted from the influence of GA_3 (Wang et al., 2014). Compared with other studies on shoot bud differentiation such as *Quercus* (Vengadesan and Pijut, 2009) and *Ammopiltanthus mongolicus* (Yang et al., 2014), in this paper, the tap root of litchi produced black spots (1.5 cm), which ultimately led to plant death. Thus, GA_3 promoted rooting, inhibited root growth, and suppressed lateral root growth.

Successful germination and plant regeneration via SEs have been reported with combinations of KT and NAA in ginger and buffel grass (Lincy et al., 2009; Carloni et al., 2014). After germination, GA_3 is required for plant development in buffel grass. Well-developed plantlets were separated (Figure 1l) and then transferred to plastic pots containing a 1:1:1 (v/v/v) vermiculite: sand: coconut chaff mixture. The plantlets were successfully acclimatized in a greenhouse (Figure 1m) with a 100% survival rate.

The present work is the first report on plant regeneration via indirect somatic embryogenesis from anther explants of 'Feizixiao' litchi. Promising plant regeneration from SEs via indirect somatic embryogenesis was remarkably influenced by PGRs. The system will be helpful for conservation, mass clone propagation, production of bioactive compounds, and genetic transformation studies.

Ploidy level of regenerated plant

All the plantlets were diploid. Ploidy levels of regenerated plants were determined by flow cytometry, and chromosome numbers were determined by chromosome counting (Figures 1n and o). 'Feizixiao' litchi has a

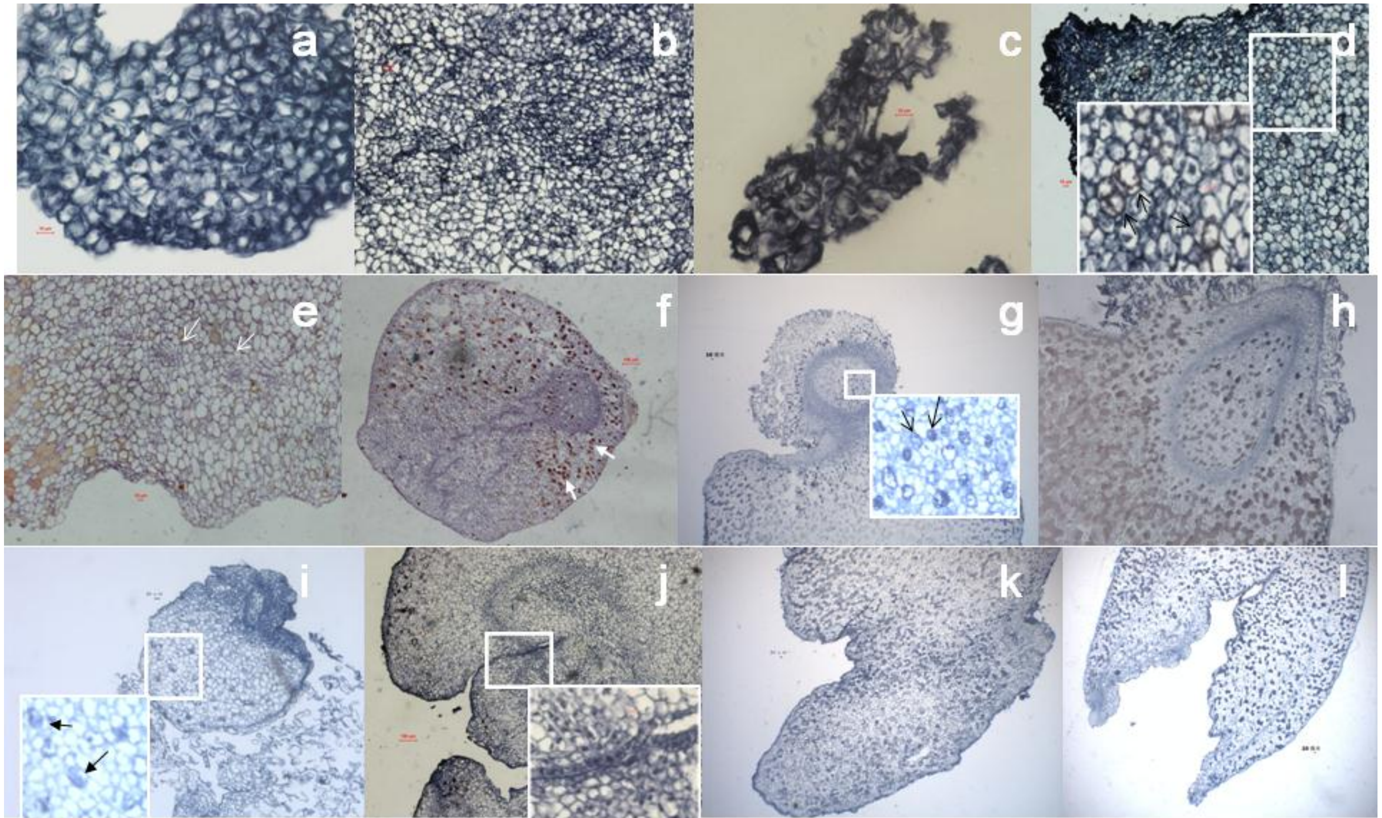

Figure 3. Histological observation of different calli and developmental stage of somatic embryos. (a) The NFEC cytoplasm was sparse or almost absent, and the cell nucleus was relatively small. (b) Cell walls are relatively thick and deeply dyed. (c) FECs that split vigorously contained more embryonic cells, which had small, high nuclear/cytoplasm ratios. (d-j) One embryonic cell divided several time to form multicellular proembryo. The development of the multicellular proembryo was followed by proembryo formation of globular embryo, heart embryo, torpedo embryo, and cotyledon embryo. g Vitrified somatic embryo, arrow indicates tannins. k and l Different developmetal stages of malformation embryo.

chromosome numbers of 2n = 2x = 30. The same chromosome numbers were confirmed in the diploid plants.

Somatic embryogenesis

Histological observations via microscopy showed that different FECs exhibited various morphologies. In general, these FEC cell walls were thick and deeply dyed (Figure 3a). The FECs that were split vigorously contained more embryonic cells, which were small with high nuclear/cytoplasm ratios (Figure 3b). The NFEC cytoplasm was sparse and even disappeared, whereas the cell nucleus was relatively smaller (Figure 3c) compared with the control.

One embryonic cell divided several times to forming a multicellular proembryo. The development of multicellular proembryos was followed by proembryo formation of globular embryo, heart embryo, torpedo embryo, and cotyledon embryo (Figures 3d to j). The somatic embryogenesis of several species was similar to that of litchi; these species included *Eucalyptus globules* and *E. saligna* × *E. maidenii* (Corredoira et al., 2015), *Hevea brasiliensis* (Wang, 2004), and *Liriodendron* hybrids (Chen et al., 2012). Embryo development was similar to zygote embryo development. In this paper, the litchi embryonic cells initially appeared in the interior and then continue to develop somatic embryos (Figure 3d). Therefore, somatic embryogenesis was of similar origin and occurred for single cells. A similar method was also observed with other woody species, such as ‘Honghezi’ longan (Chen, 2001). However, this phenomenon was different from that in other species, such as *Quercus variabilis* (Zhang et al., 2007), oil palm (Kanchanapoom and Domyoas, 1999), and *Quercus robur* (Corredoira et al., 2006). Zeng et al. (2002) reported that embryogenic cell ‘Yuanhong’ litchi is of a different origin. Lai and Sang (2003) reported that litchi in low 2,4-D is of a different origin.

Normal somatic embryos are ivory, whereas treated somatic embryos are vitrified. Histological observations showed that the early normal somatic embryonic cells were of uniform size, compact, possessed thick and cell

walls, and deep dyes; starch grains were evenly dispersed within the cell (Figures 3 and i). Moreover, the cambium was obvious. Starch grains accumulated near the earth in mature white cotyledon embryo cells, and bud primordial were obvious (Figure 3j). Vitrified somatic embryos exhibited different cell stages, and the cells were loosely arranged with thin cell walls. A small number of cell walls underwent autophagy and contained numerous brown tannins (Figure 3f).

Different developmental stages, either bud primordium or asymmetric leaf primordium, were lacking in deformed embryos. Moreover, abnormalities in the procambium prevented further development (Figures 3k and l).

Conflict of Interests

The authors hereby declare that no conflict of interest exists among them.

ACKNOWLEDGEMENTS

The financial support was from China Agriculture Research System (CARS-33-02) and Hainan natural science foundation of China (311066). The authors are grateful to Dr. Cheng Bai for his critical reading and revision of the manuscript.

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Vol. 15(22), pp. 1035-1040, 1 June, 2016
DOI: 10.5897/AJB2016.15232
Article Number: 295D03958676
ISSN 1684-5315
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http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Standardization of DNA extraction from invasive alien weed *Parthenium hysterophorus*

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Received 20 January, 2016; Accepted 23 March, 2016

DNA isolation from the weed, *Parthenium hysterophorus* is complicated due to the presence of high amount of allelochemicals in the form of secondary metabolites that causes hindrance in extraction and enzymatic reactions. A modified and efficient DNA extraction from *P. hysterophorus* leaf has been developed. The present protocol is a modified version of cetyltrimethylammonium bromide (CTAB) method constituting high salt concentration to remove polysaccharides. The increased concentration of β-mercaptoethanol, polyvinylpolypyrrolidone (PVPP), and phenol/chloroform/isoamyl alcohol extractions eliminated protein and phenolic compounds well. Good amount and quality DNA was obtained by this method. The resulted genomic DNA showed fine random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR) banding pattern, polymerase chain reaction (PCR) amplification of actin gene and restriction digestion confirm the efficiency of modified procedure.

Key words: Allelochemicals, DNA extraction, *Parthenium hysterophorus*, stress tolerance.

INTRODUCTION

Parthenium hysterophorus is an annual invasive weed of asteraceae. It spreads rapidly worldwide in a short period due to its great structural and physio-chemical flexibility, vegetative multiplication, effective dispersal of seeds, longer seed dormancy, stress tolerance and presence of secondary metabolites (Patel, 2011). *P. hysterophorus* exhibited wide adaptability towards various environmental conditions which enabled this plant to grow successfully in drought and heat prone areas (Hedge and Patil, 1982; Shubneet et al., 2014; Kumar, 2014). Apart from these properties, *P. hysterophorus* also displayed antibacterial, antioxidant, and cytotoxic activities (Kumar et al., 2013). The occurrence of diverse alkaloids, terpenoids, flavonoids, hydrocarbons and fatty acids in its tissues may execute these properties (Panwar et al., 2015). However, no molecular information is available on *P. hysterophorus*. Keeping the above facts in mind, *P. hysterophorus* can be used as a good source for identification of valuable genes, proteins and metabolites that is responsible for diverse properties of this weed. Therefore, molecular characterization of this weed is necessary for further use in transgenic approach. The quality as well as quantity of DNA is a matter of great concern in functional genomics. Fine quality DNA is an

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essential requirement in all the manipulations related to recombinant DNA technology. A fast, simple and reliable DNA isolation method is immensely required for this purpose since similar DNA isolation protocols may not be suitable for all plants (Akhtar et al., 2013), because size, content and organization of genome and metabolic setup of different plant systems differ from each other (Sangwan et al., 1998). Many problems were faced in the isolation and purification of DNA which include degradation of DNA, contamination with highly viscous polysaccharides, polyphenols, tannins, terpenoids, flavonoids, quinines and other allelochemicals which affects the downstream application (Kotchoni et al., 2011). Therefore, a genomic DNA isolation protocol was modified from the earlier method (Doyle and Doyle, 1990) to simplify and optimize the procedure for *P. hysterophorus.*

MATERIALS AND METHODS

Collection of plant sample

Seeds from authenticated *P. hysterophorus* were collected from Jamia Millia Islamia University campus, New Delhi, India (Latitude 28.6° 4'N and Longitude 77.2°) growing in the alluvial soil. The plant was further identified with authentic specimen and deposited with accession no. 115597 at Botanical Survey of India, Dehradun, India. Seed sterilization was done by soaking of seeds in 0.3% $KMnO_4$ for 10 min, and then rinsed ten times with sterilized distilled water. Germinated seed (five days old seedlings) were transferred to Soilrite™ (Keltech Energies Ltd., India) containing pots (6"× 6", 300 g/pot) and cultured in 14 h/10 h light/dark at 25°C with 250 µmol photons m-2 s -1 for 55 days. Thus, two months old plants were used in the present study.

Solution used

Extraction buffer was 2.5% cetyl trimethyl ammonium bromide (CTAB), 100 mM Tris HCl (pH 8.0), 1.5 M NaCl, 25 mM ethylenediamine tetraaceticacid (EDTA), 3% polyvinylpolypyrrolidone (PVPP), 0.4% β-mercaptoethanol (added just before use). Isopropanol 70% ethanol (v/v); Phenol: Chloroform: Isoamyl alcohol (25: 24: 1, v/v); Chloroform: Isoamyl alcohol (24:1, v/v); 0.3 M sodium acetate (pH 5.4); TE (Tris/EDTA) buffer: 10 mM Tris-HCl (pH 8.0); and 1 mM EDTA (pH 7.4).

DNA extraction procedure

(1) 1.0 g of plant material (*P. hysterophorus* leaves) was immediately placed in pre-chilled (-80°C) mortar and pestle and ground thoroughly. The ground sample was transferred to 30 ml sterile centrifuge tube containing 5 ml preheated extraction buffer and mixed by inversion. The slurry was formed and tube incubated at 65°C for 60 min in water bath with occasional gentle swirling.
(2) Next, incubated sample was extracted with equal volume of chloroform: isoamyl alcohol (24:1) and mixed gently by inversion thoroughly for about 30 min at room temperature. The mixture was centrifuged at 12,000 rpm (10 min, 25°C) for phase separation.
(3) After the two phases were separated visibly of which top clear aqueous phase was carefully transferred to a new sterile centrifuge tube and equal volume of chilled isopropanol was added to precipitated nucleic acid. The whole preparation was mixed gently and kept at -20°C for 1 h.
(4) The mixture was centrifuged at 10,000 rpm for 15 min. The supernatant was removed carefully and pellet was washed with 70% ethanol, air dried at room temperature for 1 h and dissolved in 400 µl TE buffer. Next, 5 µl RNase A (1 µg/µl) was added and incubated at 37°C for 1 h.
(5) For further purification, mixture was extracted with an equal volume of phenol: chloroform: isoamyl alcohol.
(6) The upper aqueous layer was transferred to another sterile 2.0 ml micro-centrifuge tube and further extracted with equal volume of chloroform: isoamyl alcohol. It was then centrifuged at 10,000 rpm for 10 min
(7) In the next step, the top aqueous layer was picked carefully and transferred to fresh sterile 2.0 ml micro-centrifuge tube and precipitated the DNA by addition of 1/10th volume of sodium acetate (3 M pH 5.2) with equal volume of chilled isopropanol. The tube was inverted thoroughly and kept at -20°C for at least 1 h. Later, the mixture was centrifuged at 10,000 rpm for 15 min.
(8) The supernatant was decanted and pellet was washed with 70% (v/v) ethanol. Resulted air dried pellet was dissolved in 100 µl TE buffer.

Quantification of DNA

The DNA (5 µl) was added to 495 µl TE, and mixed by vortexing. The absorbance was read in spectrophotometer (SICAN 2301, India) by setting the blank against TE. The observations were taken at 260 and 280 nm. The ratio of $OD_{260\ nm}/OD_{280\ nm}$ provides an estimate of purity of nucleic acid. Pure preparation of DNA have OD_{260}/OD_{280} ratio of 1.8 to 2.0 (Sambrook et al., 1989). The DNA concentration (µg/µl) was calculated by using the following formula:

$$\text{DNA concentration } (\mu g/) = \frac{OD_{260} \text{ x Dilution factor x } 50}{1000}$$

Qualitative analysis of DNA

Qualitative analysis of DNA isolated from fresh leaves was done by 0.8% agarose gel electrophoresis. Gels were stained with ethidium bromide and documented using gel documentation system (Bio-Rad, USA).

Restriction digestion

The composition of reaction mixture and procedure used for the restriction digestion is shown in Table 1. Genomic DNA (4 µg) was used in restriction digestion mediated by *Eco* R I (Figure 2).

RAPD amplification

Random decamer primers synthesized from Life Technologies (India) were used in random amplified polymorphic DNA (RAPD) amplification. The amplification of template DNA was carried out by using a total volume of 25 µl containing 2.5 µl reaction buffer (10X), 2.0 µl dNTPs mix (200 µM each), 30 ng of decamer primer, 50 ng of genomic DNA and 0.5 µl (3U μl^{-1}) *Taq* DNA polymerase. Thermal cycler (VeritiR, Applied Biosystem, USA) was used to perform PCR reactions having an initial denaturation at 95°C for 5 min, denaturing at 94°C for 1 min, annealing at 40°C for 30 s and polymerization at 72°C for 2 min and final extension 72°C for 5 min. After amplification, the PCR product was resolved on 1.5% agarose gel in 1X TAE buffer. DNA was stained with ethidium bromide and visualized under UV light using gel documentation system (Bio-Rad, USA) (Figure 3).

Table 1. Composition of reaction mixture and procedure used in restriction digestion.

Reaction mixture	Quantity
DNA	2 µl
Buffer	1 µl (10 X)
Enzyme	1 µl (20 units/µl)
Sterilized doubled distilled water	6 µl
Total volume	10 µl
Procedure	
The reaction mixture was prepared as above in an Eppendorf tube	
The mixture was mixed gently	
Then it was incubated at 37°C for 2 h	
The mixture was taken out after 2 h and the digestion was monitored on 1.5% agarose gel running at 50 V for 45 min	

ISSR amplification

Six inter simple sequence repeat (ISSR) primers were employed with genomic DNA of *P. hysterophorus* for the amplification. The PCR reaction was performed by using mixture that consisted of 50 ng genomic DNA, 2.0 mM $MgCl_2$, 1X PCR buffer, 100 mM of dNTP, 25 ng primer and 1.5 U *Taq* polymerase in 25 µl volumes. PCR programme had an initial denaturation for 5 min at 95°C, followed by 40 cycles for 1 min at 94°C denaturation, annealing at 50°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 7 min. The amplification was conducted in a thermal PCR (VeritiR, Applied Biosystems, USA). After amplification, 10 µl of PCR products were loaded on the 1.5% agarose gel and electrophoresed in 1X TAE buffer. The image was taken under UV-light using gel documentation system (Bio-Rad, USA).

PCR amplification of actin gene

The gene specific primer for actin gene was used in PCR amplification. A reaction tube mixture contained 40 ng of DNA, dNTP mixture (100 mM each), Taq DNA Polymerase (1.0 U), 10X reaction buffer and gene specific primers (30 ng). The thermal cycling of DNA was performed with program as follows 95°C for 5 min (Initial denaturation), 35 cycles at 95°C for 1 min (denaturation), 52°C for 1 min (annealing), 72°C for 1 min (extension) and final extension at 72°C for 5 min. The amplified PCR product was resolved on 1.2% agarose gel in 1X TAE buffer. The gels were examined under UV light and photographed by documentation system (Bio-Rad, USA).

RESULTS AND DISCUSSION

In the present study, optimized CTAB method was used for isolation of DNA from parthenium leaf. The main complications of plant DNA isolation were associated with the presence of polyphenolic compounds, terpenoids and polysaccharides (Kotchoni and Gachomo, 2009). Weed plant such as *P. hysterophorus* showed strong allelopathic character which is associated with secondary metabolites (Bhadoria, 2011; Mawal et al., 2015). Thus, quality and amount of DNA were greatly affected by these interfering compounds which co-precipitate during the DNA isolation from *P. hysterophorus* leaf. Extraction using high salt CTAB buffer was done to remove polysaccharides. CTAB also acts as a good detergent that solubilizes cell wall, lipid membrane and denatures protein (Clarke, 2009). Polyphenols associated with DNA cause hindrance in polymerase chain reaction and restriction digestion. DNA molecules are damaged by reactive oxygen species produced by oxidized product of phenolics (Li and Trush, 1994). Plant cells were effectively lysed on increasing the temperature (65°C) of heat treatment. Increased amount of PVPP (3%) was used to support elimination of phenolic compounds mediated by hydrogen bond formation with them. Oxidation of polyphenol as well as browning of the DNA is effectively prevented by PVPP (Guillemaut and Maréchal-Drouard, 1992; Borse et al., 2011). On the other hand, increased concentration of β-mercaptoethanol (0.4%) in modified protocol also improves the quality of DNA because β- mercaptoethanol works as a strong reducing agent which contributes in removal of polyphenols from the crude extract of plant and facilitates the linearization of proteins by disruption of disulphide bonds between cysteine residues (Nalini et al., 2004). Elimination of protein was carried out by phenol: chloroform mixture followed by centrifugation steps. Long term treatment using chloroform: isoamyl alcohol was done to get rid of pigments. Isolation of RNA free DNA was achieved by 1 h RNase treatment at 37°C. The use of liquid nitrogen was substituted by prechilled mortar and pestle stored overnight at −80°C. The shearing of DNA was not visible in the present method (Figure 1), which proved the good quality of extracted DNA.

Finally, white pellet of DNA was obtained. The value of A260/280 ratio was 1.79±0.005 (Table 2) showing the absence of contaminants. Suitability of *Eco*RI mediated restriction digestion (Figure 2) of DNA isolated by modified method indicating the efficiency of this method. Clear, reproducible and consistent banding patterns yield demonstrated its compatibility for polymerase chain reaction using RAPD (Figure 3) and ISSR primers (Figure 4). A distinct PCR amplification of actin gene (Figure 5) was also obtained, which further proved that a good

Table 2. Quantification of DNA extracted by Doyle and Doyle's (1990) and modified methods from leaf of *Parthenium hysterophorus*.

Protocol used	Concentration of DNA (μg/μl)	Concentration of DNA (μg/g FW)	A_{260}/A_{280}	A_{260}/A_{230}
Doyle and Doyle's (1990)	1.23±0.005	122±0.578	1.64±0.005	1.86±0.008
Modified	2.1±0.057	199±0.577	1.79±0.005	2.01±0.006

n=3, P ≤ 0.05; FW, fresh weight.

Table 3. Comparison of components of two DNA extraction methods.

Doyle and Doyle (1990)	Modified protocol
2% CTAB	2.5% CTAB
100 mM, Tris HCl (pH 8.0)	100 mM, Tris HCl (pH 8.0)
1.4 M NaCl	1.5 M NaCl
0.2%, β-mercaptoethanol	0.4%, β-mercaptoethanol
20 mM, EDTA	25 mM, EDTA
PVP (1%)	PVPP (3%)
60°C/60 min (Heat treatment)	65°C/60 min (Heat treatment)
2.5 M, Ammonium acetate (pH 7.7)	0.3 M, Sodium acetate (pH 5.4)
Tissue/ Buffer (w/v), 1:10	Tissue/ Buffer (w/v), 1:5

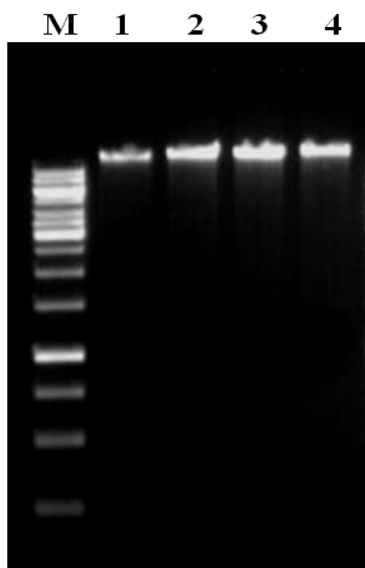

Figure 1. Total DNA (Lanes 1, 2, 3 and 4) extracted from *P. hysterophorus* leaf using present modified method visible on 1% agarose gel stained with ethidium bromide. Lane M is 1Kb ladder.

Figure 2. Restriction enzyme digestion of *P. hysterophorus* genomic DNA with *Eco* RI (Lanes 4, 5, 6).

quality of DNA was obtained. Several independent extractions and replicates proved the reliability of the modified method over Doyle and Doyle's (1990) (Table 3).

Conclusion

Conclusively, a modified CTAB method for DNA isolation from weed plant *P. hysterophorus* was described. It is reliable and cost-effective and does not require liquid

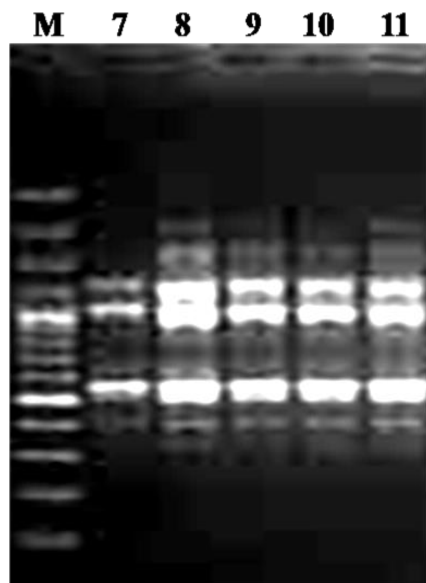

Figure 3. RAPD profile (lanes 7, 8, 9, 10 and 11) of *P. hysterophorus* obtained with primers LC-71, 72, 73, 78 and 93. Lane M is 100 bp ladder.

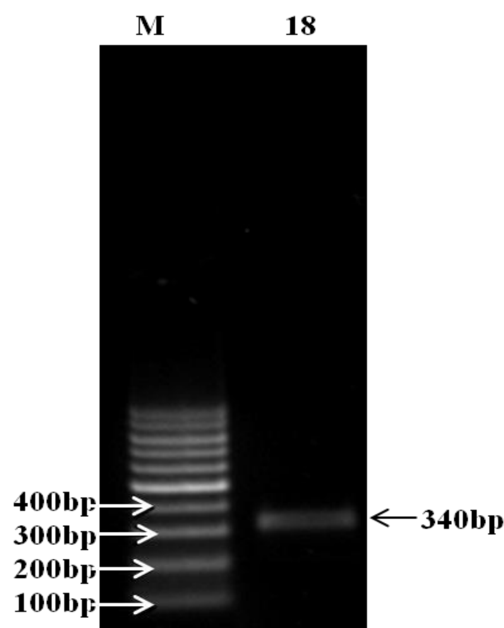

Figure 5. PCR amplification of actin gene on 1% agarose gel stained with ethidium bromide with arrow indicates the actin gene product (lane,18); DNA ladder (M: 100 bp ladder).

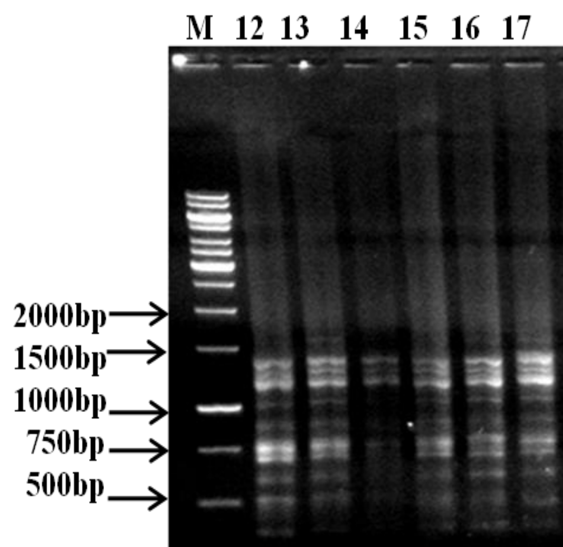

Figure 4. ISSR profile (lanes 12, 13, 14, 15, 16, and 17) of *P. hysterophorus* obtained with primers IR-1, 2, 3, 4, 8, 9. Lane M is 1Kb ladder.

nitrogen. The quality as well as yield of extracted DNA is also not compromised. Absorbance ratio in purity range and sharp band intensity on agarose gel electrophoresis indicated the effectiveness of the optimized method for DNA isolation. The extracted DNA product is suitable for molecular studies such as RAPD, ISSR, PCR amplification and restriction digestion analysis.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for supporting this work through research group no. RGP-297.

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Vol. 15(22), pp. 1041-1049, 1 June, 2016
DOI: 10.5897/AJB2016.15316
Article Number: 575CEB158680
ISSN 1684-5315
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http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Subcritical water extraction of bioactive compounds from dry loquat (*Eriobotrya japonica*) leaves and characterization of triterpenes in the extracts

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Received 2 March, 2016; Accepted 6 May, 2016

Medicinal properties of loquat leaf extracts (LLEs) are associated with their constituents of phenolic compounds and triterpenes. In this study, the efficacy of subcritical water extraction (SWE) technique was assessed by comparing with conventional solid-liquid extraction (CE) and Soxhlet extraction (SE). Results showed that the highest yields of total polyphenols were 82.7 ± 1.5 mgGAE/g leaf weight (LW), total flavonoids (54.1 ± 4.1 mgQE/g LW) and total triterpenoids (37.5 ± 3.2 mgUAE/g LW) were obtained by SWE compared to total polyphenols (61.8 ± 3.3 mgGAE/g LW), total flavonoids (43.2 ± 0.6 mgQE/g LW) and total triterpenoids (28.7 ± 2.3 mgUAE/g LW) extracted by SE and total polyphenols (50.3 ± 1.8 mgGAE/g LW), total flavonoids (40.4 ± 2.1 mgQE/g LW) and total triterpenoids (22.9 ± 3.2 mgUAE/g LW) obtained by CE. The extraction efficiency of triterpenes using SWE was about 1.7 times higher than those obtained using traditional extraction methods, and their main structural pattern of the cured extracts was comparable to the extracts obtained using traditional extraction methods.The infrared spectra obtained from the three extraction techniques appeared identical, but the variation in the intensity of the peak of absorption was visible among the three extraction techniques. The similarity of the infrared spectral pattern (peak coincided peak by peak) implies that the triterpenes in the extract obtained by the three techniques were identical by LC/MS. The findings of this study have demonstrated that SWE can be employed as an alternative green extraction technology to get important phytochemicals from plant sources.

Key words: Chinese loquat leaf, *Eriobotrya japonica*, subcritical water extraction, triterpene.

INTRODUCTION

Chinese loquat (*Eriobotrya japonica*) leaves have a high potential of bioactive compounds required in the growing pharmaceutical industries. They have been used as nutrition supplements for chronic bronchitis, coughs,

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asthma, phlegm, high fever and gastroenteric disorders (Hong et al., 2008a; Hong et al., 2008b; Chang et al., 2011). They also contain some phenolics and triterpenes which have promising potential for anticancer, anti-inflammation and hypoglycemia (Cha et al., 2011; Zong and Zhao, 2007).

Bioactive compounds from plant sources are extracted by various classical extraction techniques such as Soxhlet extraction, maceration and hydro distillation (Azmir et al., 2013). For the extraction of bioactive compounds from loquat leaves, conventional methods have been used for many years based on solid–liquid extraction using organic solvents (Thien et al., 2012). However, these techniques are time consuming, laborious, lack automation, and ultimately have low reproducibility. In addition, they are also less selective which leads to low extraction yields (Singh and Saldaña 2011). To address the shortcomings of classic extraction techniques, considerable research work by various researchers has been done on alternative extraction techniques, especially, non-conventional such ultrasonic-assisted extraction and microwave-assisted extraction (Vetal et al., 2012, Vetal et al., 2013, Bera et al., 2015), and combined ultrasonic/ microwave assisted extraction (Cheng et al., 2011). These techniques have been known to improve the extraction yields and extraction time. However, the use of conventional solvents like methanol poses environmental safety concern. In view of concerns and limitations associated with the use of solvents, the use of subcritical water extraction (SWE) is an attractive alternative to obtain bioactive compounds from loquat leaves.

To overcome the solvent limitations, the extraction of active ingredients from loquat leaves have necessitated the development of green and novel techniques which neither pollutes the environment nor damages the target bioactive compounds. In view of such development, SWE can be an ideal candidate. SWE is a technique which can extract both polar and non-polar compounds (Kwon and Chung, 2015; Luong et al., 2015), therefore it is an attractive alternative to obtaining bioactive compounds from loquat leaves. The SWE technique uses subcritical water (a green solvent) during the extraction process. The subcritical water refers to water at high temperature, mostly from its boiling point (100°C) to below the critical temperature (374°C), but with moderate pressure (below critical juncture at 22.4 MPa) that maintains it in the liquid phase.

The critical point of water is at 22.4 MPa and 374°C and water below these critical points has demonstrated its ability to extract different classes of compounds depending on pressure and temperature programming (Duba et al., 2015; Liu et al., 2015). At subcritical condition, more polar compounds are extracted at low temperature while the less polar compounds are extracted at higher temperatures into subcritical water (Luong et al., 2015). The dielectric constants of liquid water change with temperature which in turn facilitates the release of different bioactive compounds in the plant material based on solubility. It is generally well recognized that loquat leaf extract consists of different kinds of bioactive constituents such as polyphenols, flavonoids, and triterpenes.

The aim of this current study was to compare the extraction yields of polyphenols, flavonoids, and triterpenes obtained by CE, SE and SWE, and the characterization of major triterpenes in the loquat leaf extracts obtained by SWE, which was then compared to other extraction techniques.

MATERIALS AND METHODS

Loquat leaves used in this study were bought from a local pharmacy store (Wuxi, Jiangsu, China).

Sample preparation

The dried loquat leaves were taken to the laboratory where they were ground using a grinder DYF-200 (Linda Machinery Company, Zhejiang, China). Grinding was done to attain appropriate particle size (80 mesh) for traditional extraction methods. The dried loquat leaves portioned for SWE, were crushed into the proper size of SWE filter (12 mesh). The samples were either used immediately or kept under low-temperature storage (-18°C) for further uses.

Standards and solvents

Standard CA, OA and UA with 99.9% purity and gallic acid (90%), quercetin (85%), and Folin-Ciocalteu reagent were purchased from Sigma (Shanghai, China). HPLC grade organic solvents such as methanol and ethanol were purchased from Amethyst Chemicals J & K Scientific Ltd (Beijing, China). Other chemicals used were of analytical grade and were purchase from a local chemical store (Wuxi, China). Double distilled water used in SWE technique was prepared within the laboratory.

Conventional solid-liquid extraction

Conventional solid-liquid extraction method reported by Singh and Saldaña (2011) was used with a slight modification as described in our previous work (Mlyuka et al., 2015). The dry loquat leaves powder was extracted twice with 90% ethanol (solid to solvent ratio of 1:20) at 80°C for 2 h. The supernatant from each extraction was mixed before centrifugations (6000 rpm for 15 min). After centrifugation, the supernatant was concentrated in a rotary evaporator at 60°C until dry and then the total extraction yield was obtained by the mean value of the total extracts divided by the mass of dry loquat leaves used.

Soxhlet extraction

Extraction was carried out using the method of Zhao and Zhang (2014) with a slight modification. 7.5 g of ground loquat leaves were placed in a cellulose thimble and transferred to a Soxhlet extractor. The extractor was filled with 150 mL of 90% ethanol heated for 24 h at 90°C. The extraction under the set conditions was performed in triplicate, and the combined extract was

concentrated in a rotary evaporator at 60°C until dry. The total extraction yield was obtained by the mean value of the total extracts divided by the mass of dry loquat leaves used.

Subcritical water extraction

Subcritical water extraction was carried using an extractor (Hangzhou Huali Co. Ltd, Hangzhou, China). The extractor sample cell had a capacity of 2000 mL. Samples were placed at the bottom of the extraction unit. The cell was then placed in the extractor followed by pressurizing the system at 10 MPa while heating, and the inlet valve remained opened until the temperature rose to the set temperature and for an additional of 5 min after the set temperature was attained (Luong et al., 2015; Mlyuka et al., 2016). The mode of extraction in this study was static, and water remained in the extraction unit for set durations. To investigate the effects of temperature, static extraction duration was fixed at 20 min for 100, 150, 180 and 200°C. To examine the effects of time, extraction at 200°C was also performed for 30, 45 and 60 min. At the end of the extraction time, both the outlet valve and point collection valve were opened simultaneously. The extract was collected in a one-liter glass bottle. Each subcritical water extract obtained were carried out in triplicate. The extract were then concentrated in a rotary evaporator at 60°C (at reduced pressure 72 mbar, with circulating cool water at 20°C) until dry and then the total extraction yield was obtained by the mean value of the total extracts divided by the mass of dry loquat leaves used.

Determination of total polyphenol content

Total phenolic contents (TPC) in loquat extract were evaluated using the Folin-Ciocalteu assay, which was adopted from Khanizadeh et al. (2008) with some modifications as described by Khanam et al. (2012). The sample mixture was allowed to stand at room temperature for 30 min. Absorption was measured at 765 nm in an UV/Visible an Alpha-1102 spectrophotometer Laxco^{TM} (Shanghai, China). Quantification was based on the standard curve generated with 15 to 200 µg/ml of gallic acid and the TPC was expressed as mg gallic acid equivalents (GAE) /g leaf weight (LW).

Determination of total flavonoid content

Total flavonoid contents (TFC) were determined using the aluminum ion colorimetric method (Kim et al., 2009a). 0.5 mL of leaf extract diluted with 90% ethanol was transferred to a test tube followed by 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. After 30 min of incubation at room temperature (25°C), the absorbance of the reaction mixture was measured at 415 nm using a UV/Visible an Alpha-1102 spectrophotometer LaxcoTM (Shanghai, China). Quantification was based on the standard curve generated with 50 to 1000 µg/ml of quercetin and the TFC was expressed as mg quercetin equivalent (QE)/g LW.

Determination of the total triterpenes

The total triterpenes in crude loquat extract were determined by colorimetry (Fan and He, 2006; Grishkovets and Gorbacheva, 1997). The crude loquat extract was diluted to 5 mL with glacial acetic acid, and its absorbance was measured at 548 nm. Quantification was based on the standard curve generated with 100 to 1000 µg/ml of UA and the total triterpenes were expressed as mg ursolic acid equivalent (UAE)/g LW.

Characterization and separation of triterpene acids

Characterization and separation of triterpene acids were carried out by LC/MS analysis according to He et al. (2014) with slight modification. An individual triterpenoid was determined by an HPLC equipped with an MS/MS detector (Quattro micro API, United Kingdom) using Venusil XPC C18 column (100 mm × 2.1 mm) at 35°C. Chromatographic separation was carried out by using mass spectrometry (MS/MS) detection. It was obtained by electrospray ionization (ESI) source operated in negative ionization mode, and by using selected ion recording (SIR) function.Two channels were used: channel one and two were used to detect OA/UA, and CA, respectively. Data acquisition and analysis were performed using MassLynx 4.1 software with QuanLynx program.

Fourier transform infrared spectrometer analysis of loquat leaf extracts

Loquat leaf extracts were further examined by the Fourier transform infrared spectrometer (FTIR-650) technique to compare IR chromatograms of the extracts obtained by different methods (Soxhlet extraction, conventional solid-liquid extraction, and subcritical water extraction). Loquat leaf extracts from each of the methods were finely ground with powdered potassium bromide, followed by pressing the mixture under high pressure to produce KBr pellet which was inserted into a holder in the spectrometer. IR chromatograms were obtained using the FT-IR, the spectra corresponded to the sum of 32 scans at a 1.5 cm^{-1} spectral resolution.

Statistical analysis

Yields data were analyzed by one-way analysis of variance (ANOVA) with the statistical package for the social sciences (SPSS) version 19 (SPSS Inc., USA). Tukey method was used to determine significant differences of bioactive yields at 95% confidence interval. Sigma plot software version 10 was used in graphical data presentation.

RESULTS AND DISCUSSION

Total bioactive yields

Results for the extraction yields of different bioactive compounds obtained by various extraction techniques are presented in Figure 1. The total polyphenol yields in the extracts obtained by SWE at 200°C were comparatively higher than the yields achieved by the other techniques utilized in this study (CE & SE). The SWE increased the extraction rate of total polyphenol from loquat leaves when the extraction was performed at high pressure (10 MPa) and temperature. Additionally, a higher temperature at subcritical condition has been reported to affect the polarity of water (Carr et al., 2010). It also lowers the polarity of the solvent by weakening the hydrogen bonds. Furthermore, it has been reported to produce a series of effects including improved mass transfer and solubility (Carr et al., 2010) of the compounds in the extract. Moreover, it lowers the surface tension of the water leading to improved penetration into the sample matrix. Therefore, by careful and strategic temperature

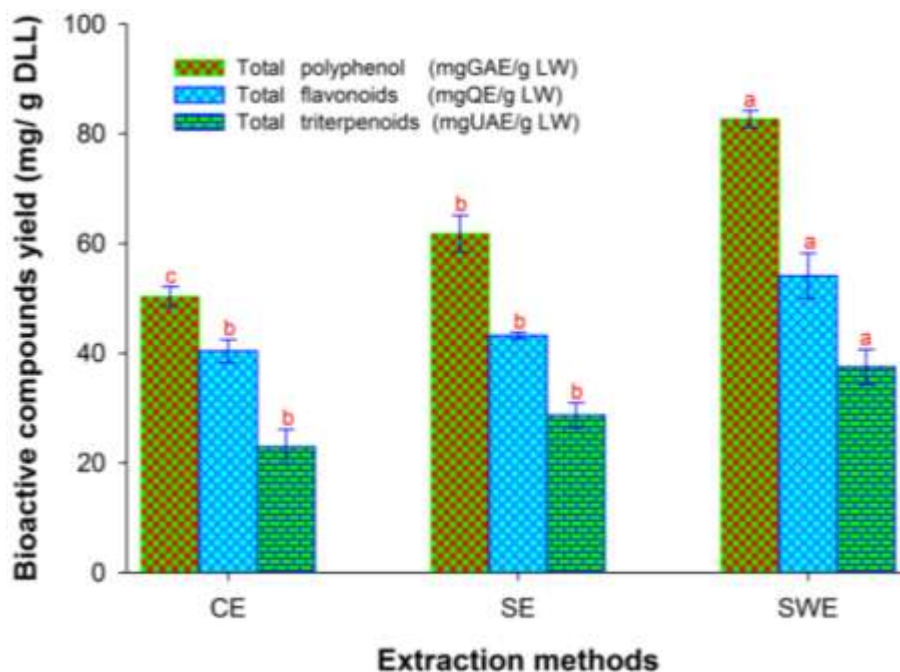

Figure 1. Extraction yield of bioactive compounds obtained from dry loquat leaves by different extraction methods. CE, Conventional extraction technique at 4 h; SE, Soxhlet extraction at 24 h; SWE, Subcritical extraction at 200°C for 20 min. GAE, Gallic acid equivalent; QE, Quercetin equivalent; UAE, Ursolic acid equivalent; LW, leaf weight. Extraction methods for similar phytochemical with the same letters are not significantly different according to the Tukey test ($p<0.05$).

programming, SWE can offer advantages when compared to the other extraction techniques tested in this study (Figure 1).

The time required to obtain total polyphenol yield from loquat leaves varied depending on the extraction technique used. The longest extraction time was spent when using SE (24 h) followed by CE (4 h) and SWE registered the shortest time (60 min), but within SWE duration variation did not result in a significant ($p>0.05$) yield of total polyphenol from loquat leaves (Figure 2). It was further found out that extending the extraction to 60 min resulted in a slight decline of total polyphenol yield which could be due to degradation of bioactive compounds at subcritical condition (Yang et al., 2007).

Results for the total flavonoids contents in the extracts obtained by CE, SE, and SWE at 200°C are presented in Figure 1. Total flavonoids obtained by CE and CE were not significantly different ($p>0.05$). In addition, these conventional methods used the same extraction solvent (90% ethanol) which might have influenced the performance of both CE and SE. In SWE, water was used as an extraction solvent. Water is considered safe for human use and has been employed previously for extraction of bioactive compounds from plant samples (Kim et al., 2009a; Mlyuka et al., 2016; Singh and Saldaña, 2011). The total flavonoids obtained by SWE were significantly ($p<0.05$) higher than the yields obtained by conventional extraction methods (Figure 1). This is because SWE is more selective and efficient technique. Tunable properties of subcritical water account for the selectivity of SWE. Furthermore, properties of water such as dielectric constant, surface tension, viscosity, and dissociation constant can be varied by adjusting extraction temperature at moderate pressure to keep water in the liquid state. Consequently, the maximum yield of total flavonoids was achieved when the SWE was performed at 200°C and 10 MPa for 45 min, but statistically ($p>0.05$) was not different from other tested times (Figure 2). This observation is consistent with what was reported by Kim and others on the extraction nutraceutical compounds from citrus pomaces (Kim et al., 2009a). It has been shown that bioactive compounds in plants could be extracted by subcritical water better than when using organic extraction techniques as demonstrated by the results of this study (Figures 1 and 2).

From the total triterpenoids yield comparison between extraction techniques (CE, SE & SWE), SWE obtained the highest yield (37.5 ± 3.2 mgUAE/g LW), which was significantly different ($p< 0.05$) from the other two techniques (Figure 1). These results indicated that SWE yields were strongly influenced by the temperature which is known to exert an effect on the dielectric constant of water (Kim et al., 2009a; Singh and Saldaña, 2011). The highest yields of total triterpenoids (41 ± 1 mgUAE/g LW)

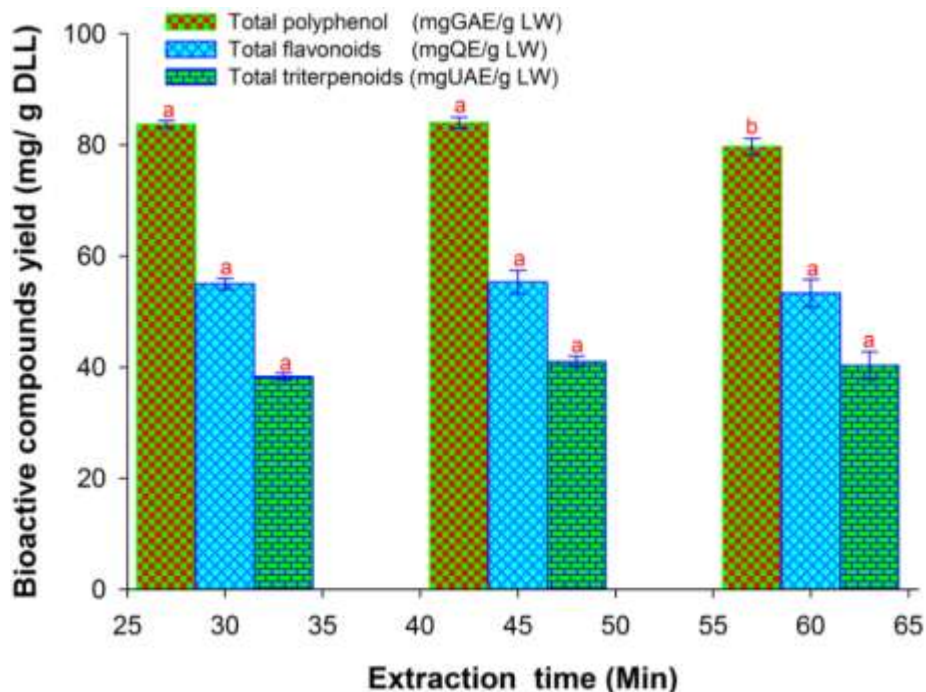

Figure 2. Extraction yield of bioactive compounds obtained from dry loquat leaves with subcritical extraction technique as a function of time. Extraction temperature and pressure were kept constant at 200°C and 10 MPa respectively. GAE, Gallic acid equivalent; QE, quercetin equivalent; UAE, ursolic acid equivalent; LW, leaf weight. Extraction times for similar phytochemical with the same letters are not significantly different according to the Tukey test ($p<0.05$).

were obtained at 200°C and 10 MPa for 45 min, but were not significantly different ($p>0.05$) from the other yields (40.3 ± 2.5 mgUAE/g LW) determined at the same temperature and pressure, but at different extraction time (60 min) as shown in Figure 2. This result is in agreement with the findings reported by other authors on the efficiency of SWE which was demonstrated in the process to obtain bioactive components yield from *Centellaasiatica* (Kim et al., 2009b). In our study, total triterpenoids increased with an increase in temperature (Figure 3) and time but slightly decreased at 60 min likely due to degradation of triterpenoids at high temperature and extended extraction time (Yang et al., 2007).

Characterization and separation of triterpene acids

Results on identification and quantification of triterpene acids are shown in Figure 4. HPLC conditions were optimized to provide reproducible separation triterpenes particularly CA, OA, and UA within a reasonable separation time. The separation of triterpenes was achieved after 16 min under optimal chromatographic conditions, compared to 22.5 and 25.03 min retention time previously reported by other authors (Olszewska, 2008; Xu et al., 2012). This separation time is consistent with the suitability property of fast separation of triterpene acids, which has recently been reported by Lesellier et al. (2012). Under current chromatographic conditions, OA and UA were not completely separated with CE and SE, but were separated at base level with SWE (Figure 4). It was not possible to completely separate OA from UA in loquat leaf extracts obtained by CE and SE. Therefore, only CA was successfully separated from OA/UA for the extract obtained by conventional extraction methods (Figure 4). The separation of OA from UA was not achieved for the extract obtained by CE and SE because these techniques likely were not able to initiate separation of isomeric triterpenes (UA and OA).

Additionally, OA and UA have been previously reported to always exist in the same plant (Lesellier et al., 2012; Olszewska, 2008), and are difficult to separate them completely by LC/MS (Lesellier et al., 2012), but in this study separation of the three triterpene acids was achieved by LC/MS only for the extract obtained by SWE (Figure 4). This separation was possible for the extract obtained by SWE which can be attributed to the fact that this technique probably initiated separation of the isomers at extraction stage as influenced by unique properties of water at higher temperatures. The selective extraction of triterpenes from dry loquat leaves was significantly influenced by SWE extraction mode and parameters (Liu

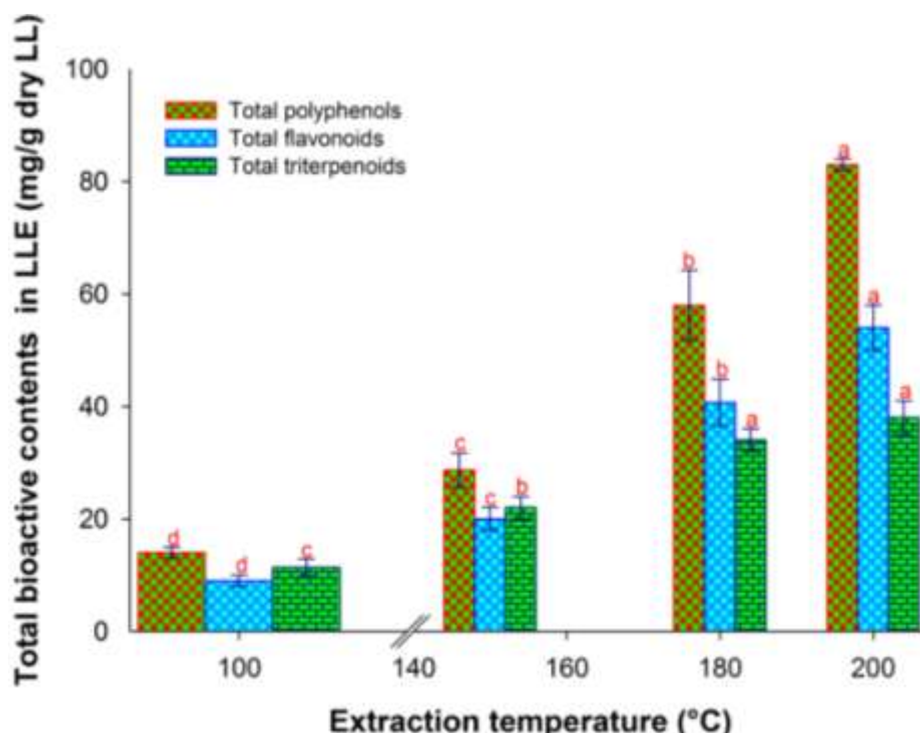

Figure 3. Total bioactive compounds of dry loquat extract by the subcritical water extraction as a function of temperature at 10 MPa and 20 min. LLE, Loquat leaf extract; LL, Loquat leaves. Extraction temperatures for similar phytochemical with the same letters are not significantly different according to the Tukey test ($p<0.05$).

et al., 2015).

The comparison of triterpenes contents of loquat leave extract with other plant materials

There has been a growing interest in triterpenes due to their beneficial health effects, such as anti-inflammatory, anti-diabetic and anti-tumoral (Lesellier et al., 2012, Giménez et al., 2015). They are widely found in more than 1620 plant species and resinous natural materials (Fujiwara et al., 2011; Pollier and Goossens, 2012, Rhourri-Frihet al., 2012). They have also been reported to be found mainly in the bark of trees such as plane, cork, and birch, liquorice roots, but also in the leaves (Rhourri-Frih et al., 2012). Triterpenes contents in extracts can vary due to plant species, geographical origin and extraction techniques used (Hong et al., 2008a; Huang et al., 2013). In our previous study, CA yield was found to be 9.63 ± 0.33 mg/g while UA was found to be 15.04 ± 0.81 mg/g as main PTTs extracted from dry loquat leaves at 180 and 200°C, respectively using static-dynamic mode (Mlyuka et al., 2016). The quantity of UA was higher than the quantity of CA at the same experimental condition and this might be attributed to the fact that dry loquat leaves have higher quantity of UA than CA. Similar results were reported by Olszewska (2008) on corosolic, oleanolic, and ursolicacids in *Prunus serotina* Ehrh. Other authors working on the process for the preparation of high purity corosolic acid and ursolic acid using traditional extraction method also reported higher UA proportional than CA in loquat leaves (Yoshida et al., 2012). In addition, Wei and Yang (2014) reported a 12% higher yield of OA and UA from *Hedyotisdiffusa* obtained by using the hyphenated ultrasound-assisted extraction. Vetalet al. (2012) reported the maximum yield of ursolic acid from *Ocimum sanctum* leaves (16.47 mg UA/g) produced at optimum extraction conditions (extraction time 12 min, solid to solvent ratio 1:30, temperature 45°C and frequency of 25 kHz). The observed differences of triterpenes from different plants source could be attributed to differences in plant species as well as the extraction technique employed. Therefore, extraction techniques have shown to have a significant impact on the yields of triterpenes from different plant sources.

Fourier transform infrared spectrometer analysis of loquat leaf extracts

Loquat leaf extracts obtained by different extraction techniques were further analyzed by FT-IR over a KBr window to compare the IR of the loquat extracted by various extraction techniques (SWE, SE and CE) as

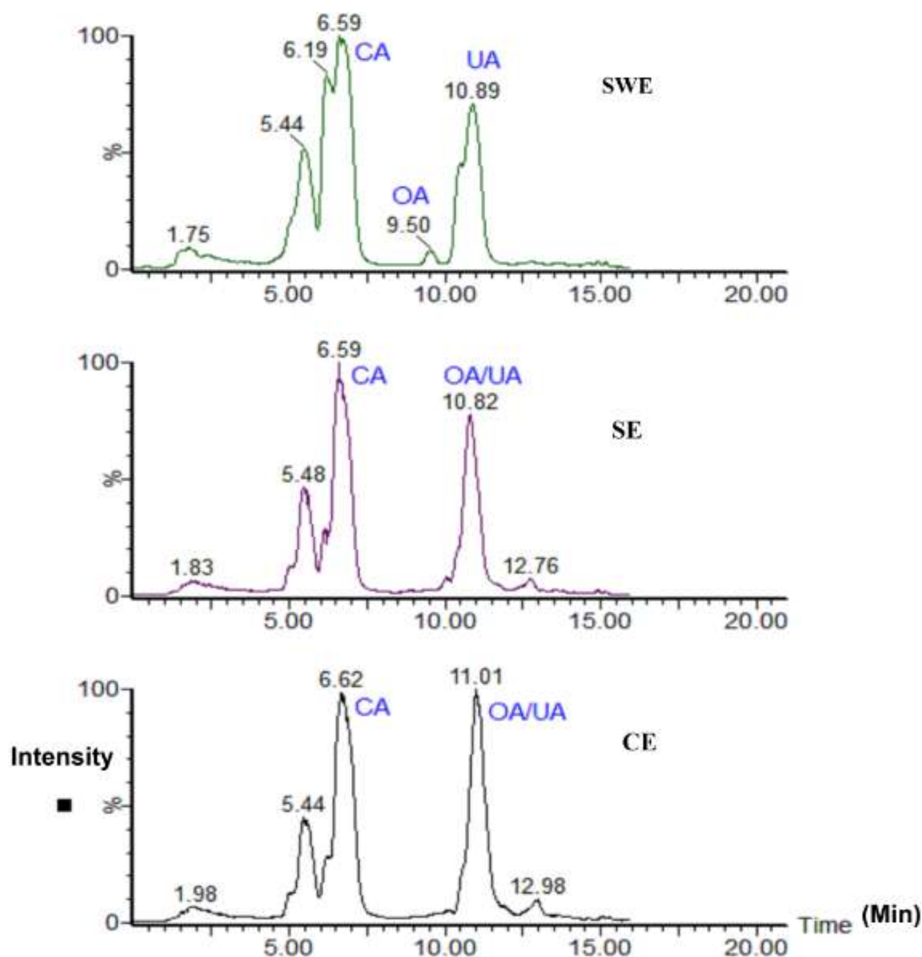

Figure 4. HPLC chromatograms of the triterpene acids from loquat leaf extract. SWE, subcritical water extraction: SE, Soxhlet extraction: CE, conventional extraction technique; CA, corosolic acid; OA, Oleanolic acid; UA, ursolic acid.

shown in Figure 5. The FT-IR employed in this study has emerged as an efficient tool for the characterization of extraction technique - matrix interaction (Lachos-Perez et al., 2015; Liu et al., 2014). The infrared spectra obtained from the three extraction techniques looks identical, but the variation in the intensity of the peak of absorption is visible among the three extraction techniques. The similarity of the peak pattern implies that the compounds in the extract obtained by the three techniques seem identical because their infrared spectra coincided peak for peak (absorption for absorption). Based on these observations, triterpenes could have contributed significantly to the signal recorded by FT-IR as supported by LC/MS results (Figure 4). These FT-IR results helped us to determine structural information about the molecules in the loquat leaf extracts. The absorptions of the double bond in triterpene acids have medium to weak absorption in the range of 1680 to 1600 cm^{-1}, as shown in Figure 5. All the three methods have shown characteristics absorption in this region, but SWE showed relatively stronger absorption when compared to the other extraction techniques employed.

Conclusions

In this study, the efficiency of SWE technique to obtain bioactive compounds yield from dry loquat (*Eriobotrya japonica*) leaves was compared with traditional extraction methods (CE and SE). The crude extracts obtained by both SWE technique and traditional extraction methods (CE and SE) mainly consisted of polyphenols, flavonoids as well as triterpenes. The extraction efficiency of triterpenes using SWE was found to be about 1.7 times higher than those obtained using the traditional extraction methods. In addition, the main structural patterns of the cured extracts obtained by SWE technique were comparable to extracts obtained using the conventional methods and the only extract obtained by SWE, UA and OA were able to separate completely using LC/MS.

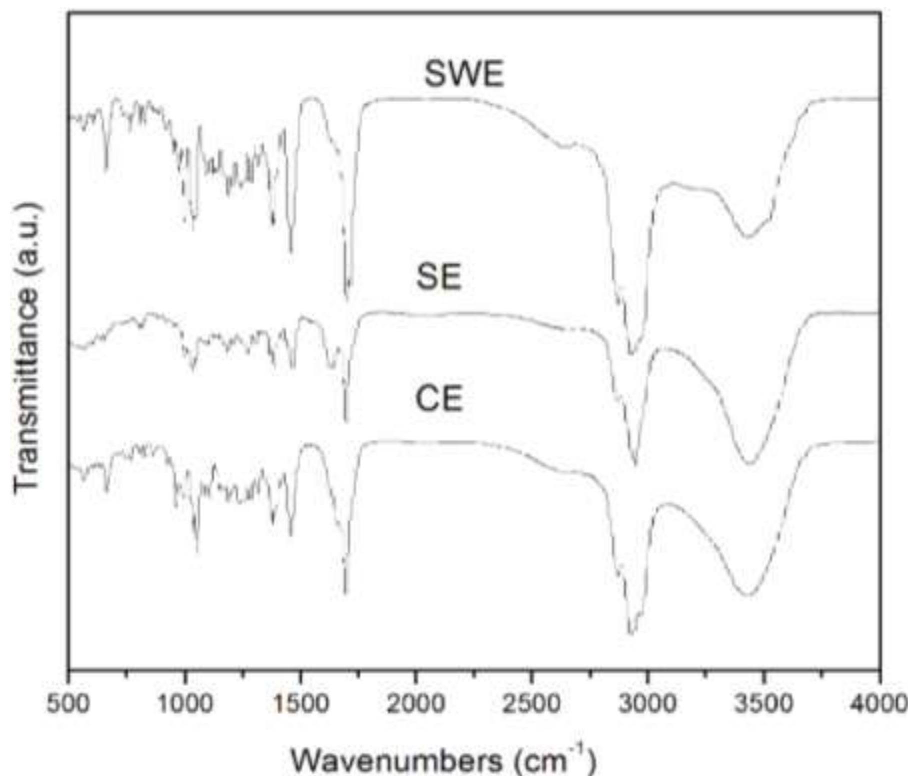

Figure 5. Fourier transform infrared spectra of dry loquat leaf extracts obtained by different extraction techniques. SWE, Subcritical water extraction; SE, soxhlet extraction; CE, conventional extraction.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The study was supported by National Natural Science Foundation of China (Grant No. 31000751), 973 projects from the Ministry of Science and Technology (No. 2012CB720801), Doctoral Funds from the Ministry of Education (No. 20100093120005) and the Fundamental Research Funds for the Central Universities (No. JUSRP21108).

Abbreviations

LLEs, Loquat leaf extracts; **CA,** corosolic acid; **DLL**, dry loquat leaves; **PTTs**, pentacyclic triterpenoids; **SWE**, subcritical water extraction; **CE**, conventional solid-liquid extraction; **UA**, ursolic acid; **LC/MS**, liquid chromatography-mass spectrometry; **SE**, soxhlet extraction; **TPC**, total phenolic contents; **OA**, oleanolic acid; **TFC**, total flavonoid contents; **FT-IR**, fourier transform infrared spectrometer.

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Vol. 15(22), pp. 1050-1057, 1 June, 2016
DOI: 10.5897/AJB2015.14976
Article Number: 945E19158683
ISSN 1684-5315
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http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Nutritional profiles of tiger Nut (*Cyperus esculentus*) plant organs during its growth cycle

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Received 17 October 2015, Accepted 27 November, 2015

This study was carried out to determine major nutrient profiles changes of tiger nut plant during its growth period. The plant leaves, roots, tuber moisture, starch, fat and protein were analyzed by oven drying, enzymatic hydrolysis, glucose assay, soxhlet extraction and kjeldahl methods. The results show the moisture content was decreased during its growth cycle but varied with different plant organ. For leaves, the starch content was increased with reducing oil content. For roots, oil content was highest (8%) at the 100th day, and it was gradually decreased (3%) till harvest time with non-significant changes of starch content. For tuber, reducing sugar and protein content was insignificant where the starch and oil content increased significantly but the changes were irregular in the middle growing. For optimum macronutrient yields, it is recommended to harvest the plant at 142nd day for starch. The delayed harvesting may lead to increase in oil content while reducing its total starch contents. For the starch purpose, the harvest time could be around 142 days. However, harvest time could require staying longer in soil.

Key words: Tiger nut, oil, starch, growth cycle, nutrients enrichment.

INTRODUCTION

Tiger nut (*Cyperus esculentus* L.) is an edible perennial grass-like C_4 plant of the sedge family (Turesson et al., 2010). Tiger nut is tuber usable grass and also called chufa, nut grass, yellow nut sedge, earth almond, edible galingale and ground almond (Defelice, 2002; Sanchez-Zapata et al., 2012). It is widely used for human and animal consumption as a nutritious food and feed in Africa, Europe and America (Sanchez-Zapata et al., 2012).

Tiger nut is rich in starch, oil, minerals, and vitamins E and C. The starch and oil are major macronutrients in the tiger nut tuber. High starch content of this plant provide unique functional properties (Manek et al., 2012), cold storage stabilities, and preserves organoleptic properties of foods (Jing et al., 2012). The tiger nut oil also has high monounsaturated fatty acids, similar to olive, avocado and hazelnut oil (Ezeh et al., 2014). These monounstaturated oil has high unsaponifiable matter, phospholipids and other bioactive compounds such as tocopherols, phytosterols and polyphenols (Sanchez-

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Zapata et al., 2012; Ezeh et al., 2014). Although tiger nut oil fatty acid profile is similar to olive oil, nut oil has unique gold-yellow color, neutral taste properties, high in phytosterols (Sanchez-Zapata et al., 2012), and better deep frying stability (Sanchez-Zapata et al., 2012; Lasekan and Abdulkarim, 2012) .The nutritional profiles and unique functional properties have made tiger nut as unique food (Ekeanyanwu and Ononogbu, 2010) like beverage, flour (Oladele and Aina, 2007; Chinma et al., 2010), edible oil (Muhammad et al., 2011; Lasekan and Abdulkarim, 2012), and a feed source (Sanchez-Zapata et al., 2012).Although there are numerous example of plants that accumulate high amount of starch or sugars in roots and tubers, tiger nut accumulates a substantial amount of oil in such tissues. As an high oil yield and more adaptable crop, tiger nut have more potential usage as food and industrial materials. Current research focused on functional properties, organoleptic properties, biochemical actives, oil extraction and nutritional value of tiger nut but the information on changing nutrient patterns during its growing cycle is meager. Therefore, the current study was carried out to determine macronutrient profile changes of the field grown tiger nut plant parts during its full growth cycle.

MATERIALS AND METHODS

Tiger nut tubers were provided by the Karamay Huili CO., LTD (Karamay, Xinjiang, China). Before planting, the tubers were rinsed with tap water for 2 h, and then soaked at 45°C for 24 h changing the water in the middle. Then it was drained out for backup. After that the tubers were planted with spacing 20×40cm under the condition of sandy soil. The germination appeared after planting of 10days. It was watered at 10 days interval according to their real growth status. After the growth of 60 days, the tiger nut was harvested at 3 days interval. After harvest, the tubers, root and leaves were separated and wrapped in aluminium foil and stored at -80°C.

Instrument and equipment

Soil shovel, magnetic stirrer, resistivity meter, PH Meter (HANAHI98183), paraffin pan, electric oven blast, analytical balance, dryer, weighing bottle, Soxhlet extractor, constant temperature water bath, formwork units, Kjeldahl flask, adjustable electric, water bath, grinder, oven, electronic balance (0.0001 g), conical flask (250ml), volumetric flask, reflux device, acid burette, filtration device and microwave were used in this study.

Preparation of sample

Parts of tiger nuts was slowly thawed and then oven dried, and splintered with grinder.

Analyses of tiger nut

Measurement of soil physical and chemical properties

The soil physical and chemical properties were measured according to the method of Bao (2000). The quantitative analysis of water, fat, total protein, starch and sugar were determined according to the method of Jing (2012) and Nielsen (2010) without modification.

Statistical analysis

The data was replicated three times, and the mean data analyzed by statistical package for the social sciences (SPSS) 17.0.

RESULTS

Soil physical and chemical properties

Soil salt content decreased from top to bottom with soil depth, and its surface clustering. Their effect was relatively strong because the soil water soluble salt increased with the water evaporates and stays around 0 to 20 cm in the strong solar radiation. So, there was no risk of soil salinization. Considering the vertical distribution of salt ions content, the composition of the soil soluble salt ions took SO_4^{2-} relatively large proportion than K^+ and Na^+. The soil organic matter content declined from top to bottom with depth, and therefore enriched the soil. The vertical distribution of soil moisture belonged to bottom poly-type soil moisture profile. The type of soil moisture profile was characterized by its various levels of soil water content which was relatively small, and surface soil moisture was low than others. So, the soil moisture from 20 to 40 cm was a linear upward trend than 0 to 20 cm, but the changing trend decreased with increasing soil depth. Considering the surface soil water content, the soil moisture content of 20 to 40 cm increased to 95.67%, whereas the 40 to 60 cm layer increased to 10.86% (Table 1).

The moisture content (%)

The moisture content of each part of tiger nut was reduced within its growth cycle, but there were differences among the different parts of the tiger nut. The lower trend of moisture content was observed between the leaves and tuber with the increasing of harvesting time. On the other hand, the moisture content of the roots was increased before the 89th day, and was decreased with the advancement of harvesting periods after the time (Figure 1).

Oil content (%)

In growth cycle, Figure 2 shows that there was changing trend of oil content among the leaves, root and tubers of the tiger nut. The oil content increased until 98th days for both leaves and roots and after that, it gradually reduced. In roots, the oil content changed dramatically. In the

Table 1. Physical chemical properties of the tested soil.

Parameter	Salt content	pH	K^+	Na^+	Ca^{2+}	Mg^{2+}
0-20 cm	1.26	7.57	2.78E-05	3.16E-04	6.60E-05	1.55E-05
20-40 cm	0.54	7.47	2.32E-05	3.04E-04	6.60E-05	1.61E-05
40-60 cm	0.45	7.16	4.23E-05	4.18E-04	2.14E-04	3.92E-05
Parameter	**Cl^-**	**CO_3^{2-}**	**HCO_3^-**	**SO_4^{2-}**	**Soil organic matter**	**Soil moisture content（%）**
0-20 cm	6.38E-06	0.00E+00	4.88E-07	5.21E-03	52.81	21.99
20-40 cm	3.59E-06	0.00E+00	2.75E-07	3.48E-03	43.11	43.04
40-60 cm	9.38E-05	0.00E+00	1.22E-07	1.98E-02	29.46	47.71

Unit: mg/kg for ions; g/kg for soil organic matter

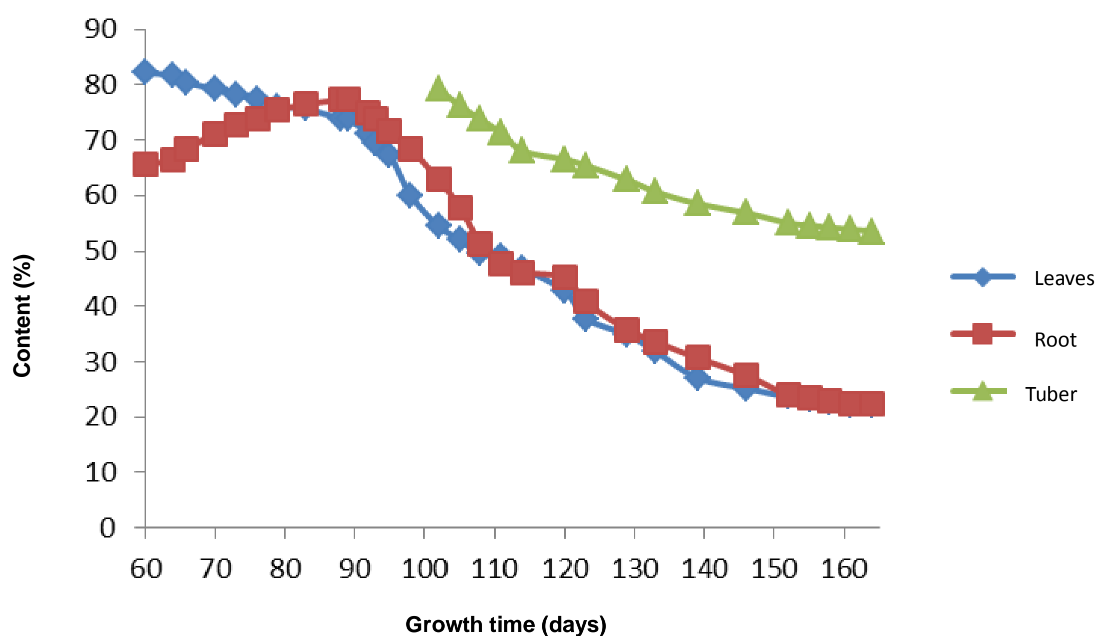

Figure 1. Moisture content of each parts of tiger nut.

tuber, the oil content increased with the growth of tiger nut. The highest oil content was also recorded in tuber (30%) followed by leaves (8.9%) and root (8.3%) respectively in the whole growth cycle of the tiger nut.

Protein content (%)

The protein content was significantly different between the leaves and tubers of the tiger nut (Figure 3). Initially, the protein content of the leaves was lower until 111 days (0.11±0.00) but after that, it increased until 120 days (2.19±0.02). In case of tuber, there was an increasing trend until 152 days (1.11±0.00) and after that, it decreased gradually with the advancement of growing periods.

Starch content (%)

There was an increasing trend of starch content at different parts of the tiger nut among the leaves, roots and tubers, but the increasing values were irregular among the leaves, roots and tubers (Figure 4). The highest content of starch was recorded before harvest, and the values were lower among the leaves, roots and tubers after harvest.

Sugar content (%)

The increasing trend of reducing sugar content of tiger nut was not very clear between leaves and tubers fluctuation was observed. This phenomenon might be

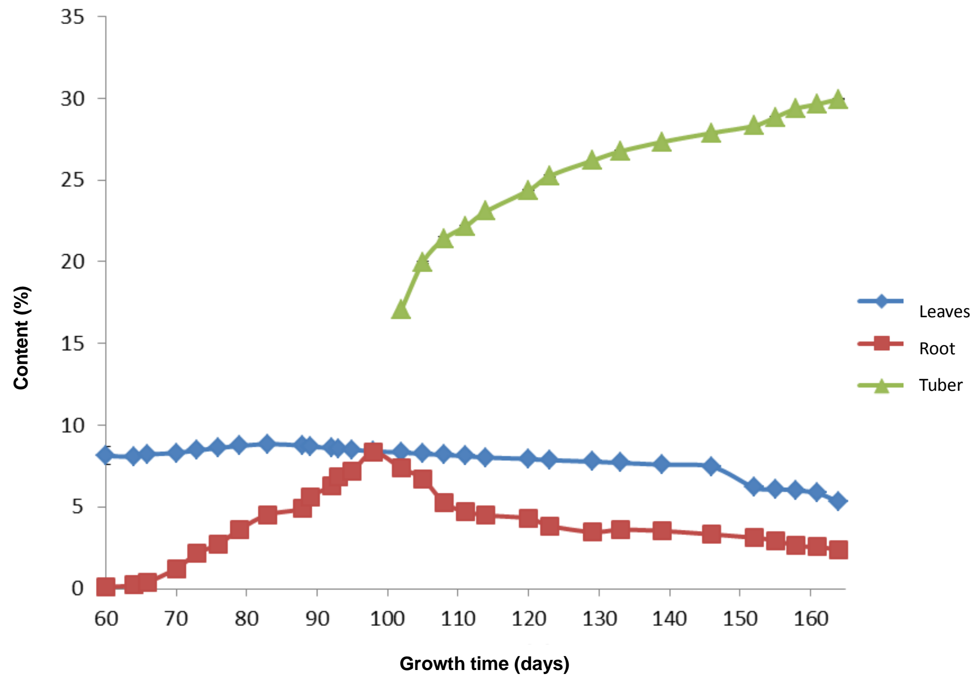

Figure 2. Oil content changes of the each part of tiger nut (dry mass).

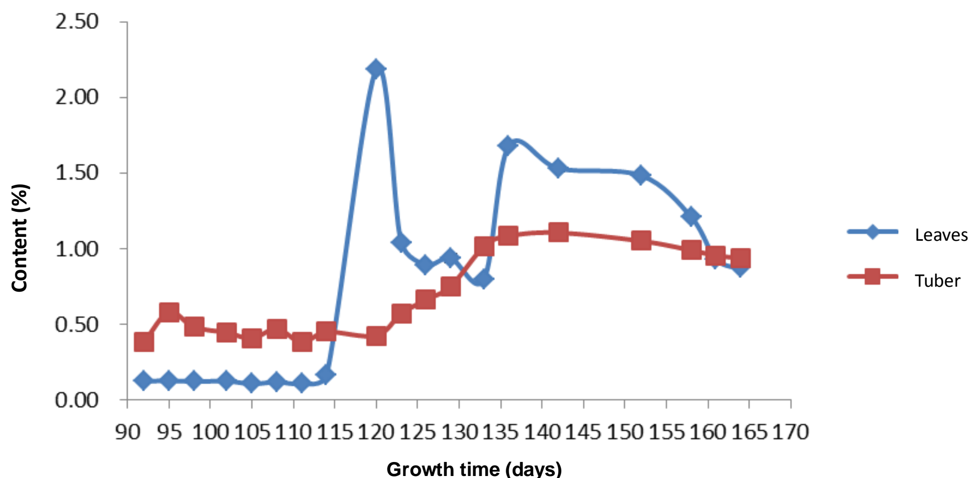

Figure 3. Protein content of the each part of the tiger nut (dry mass).

due to their irregular changes during the growing of middle time. The highest reducing sugar content (6.99±0.57) was recorded in tubers at 155 days whereas the lower one was recorded (5.73±0.27) for leaves (Figure 5).

DISCUSSION

The primary aim of this investigation was to study the biochemical changes in developing tiger nuts in field, to assess tiger nut carbon allocation into different organ and to get best harvest time.

Very interestingly, tuber is the main storage organ in tiger nut, but other organ including leaves and roots store high amount of starch. The highest starch content of the tiger nut leaves was increased gradually until 150 day, and after that the changes were irregular and reached the lowest level of 19% (Figure 6). For the oil content of leaves, it increased from the beginning of 85th day with

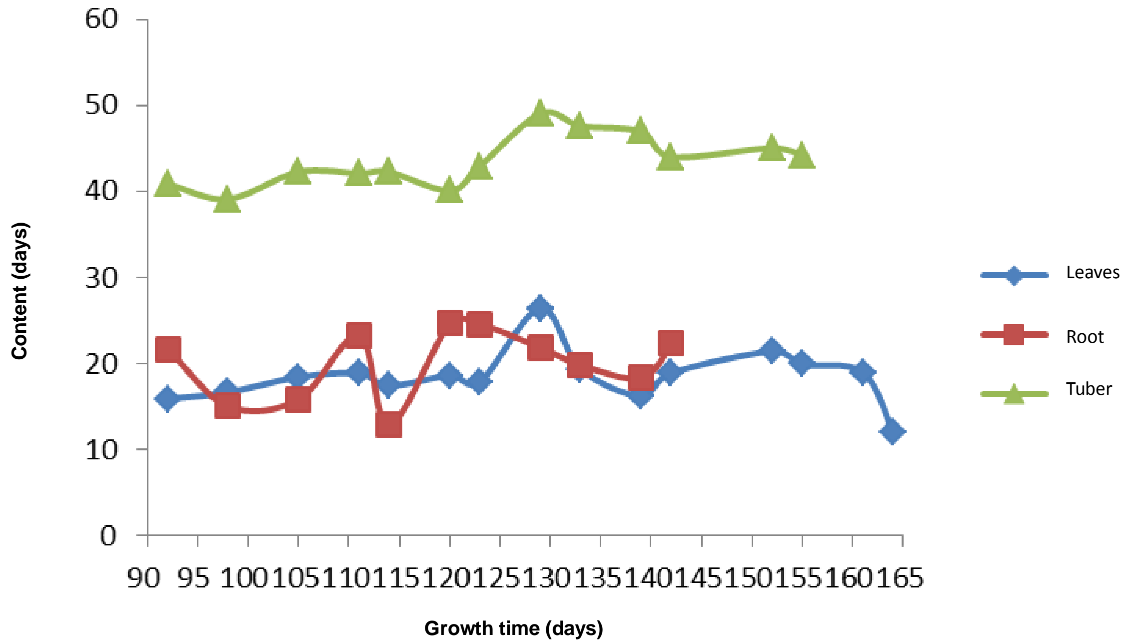

Figure 4. Starch content of the tiger nut (dry mass).

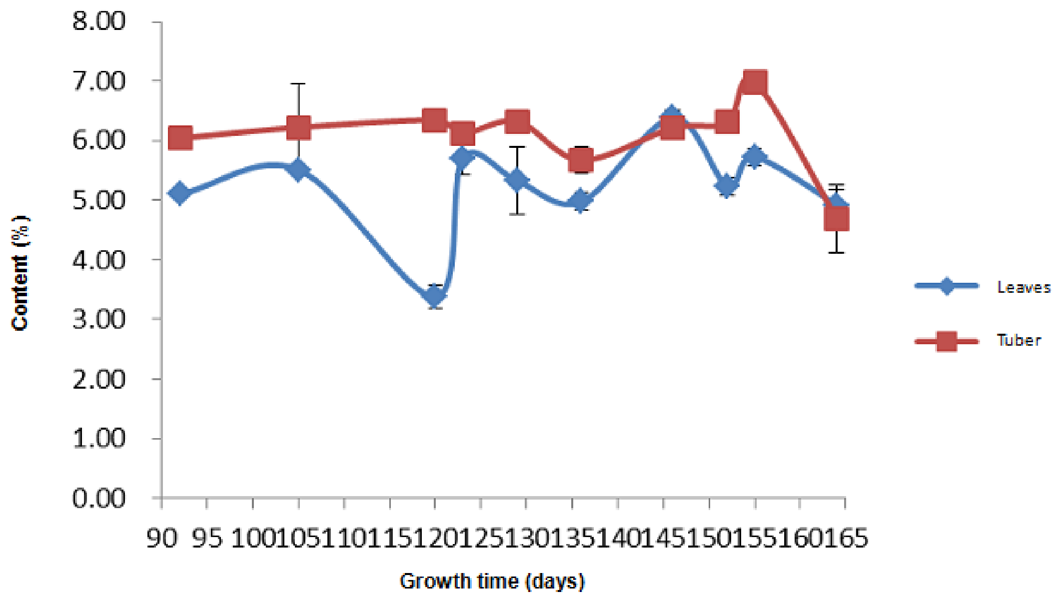

Figure 5. Reducing sugar content of the tiger nut (dry mass).

the highest level of 9%. From that time to harvest, it began to decrease and got the lowest level of 5% at the end (Figure 6) of the harvest. For sugar content of leaves, there was an increasing trend with irregular changes while the highest level was recorded at 6.4% and the lowest level was 3.4% (Figure 6). The leaves' protein content was very low until the 110th day, and reached the highest level of 2.19% at the 120th day. After that, it decreased with irregularities (Figure 6). Generally, starch, sugar and protein content of the tiger nut increased whereas the oil content decreased before the 120 to 130th day. From 130 day, the oil, sugar and protein content decreased while the changing trend of starch content was not clear. However, the starch content

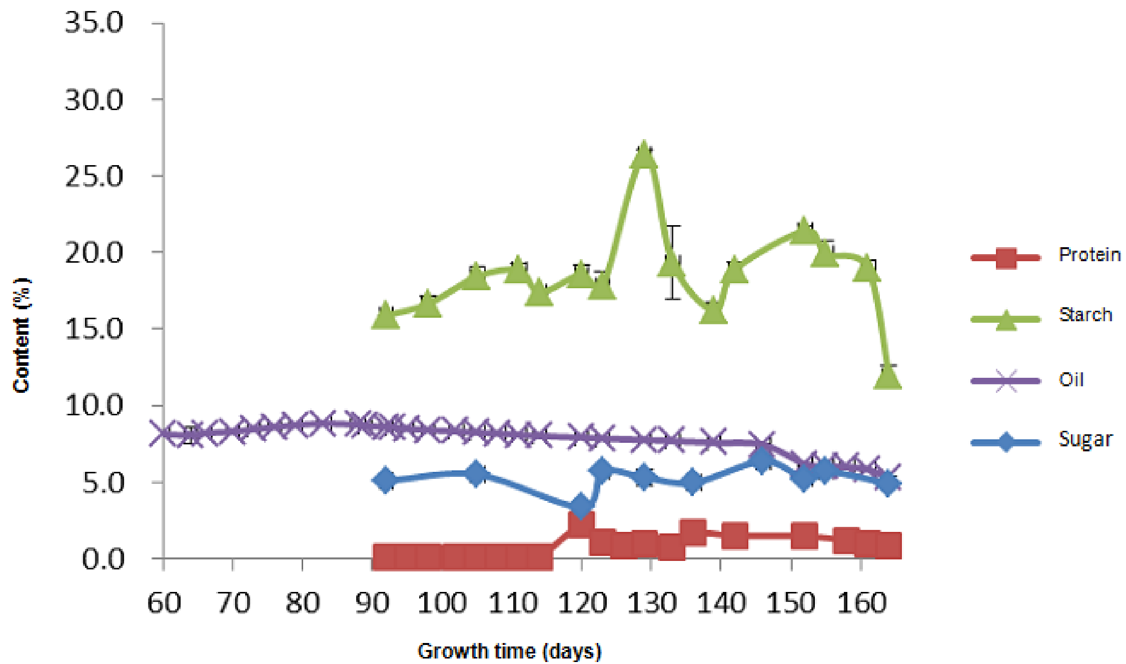

Figure 6. Nutrient contents of the tiger nut leave (dry mass).

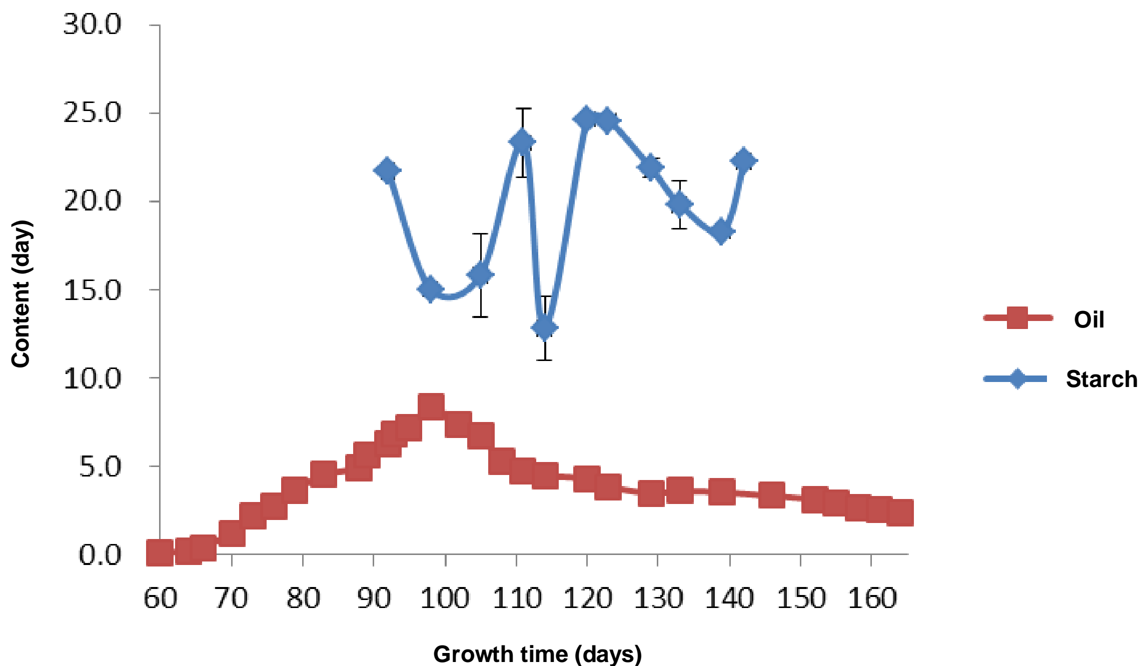

Figure 7. Oil and starch content changes of the tiger nut roots (dry mass).

increased whereas the oil content of the tiger nut decreased.

The changing trend of the root oil content was clear in whole growth cycle from the beginning of 100 day with the highest level of 8% and decreased from 100 day to the harvest time (Figure 7). During harvest, the oil content recorded 3% while the starch content has higher value of 25% with the irregular changing trend (Figure 7).

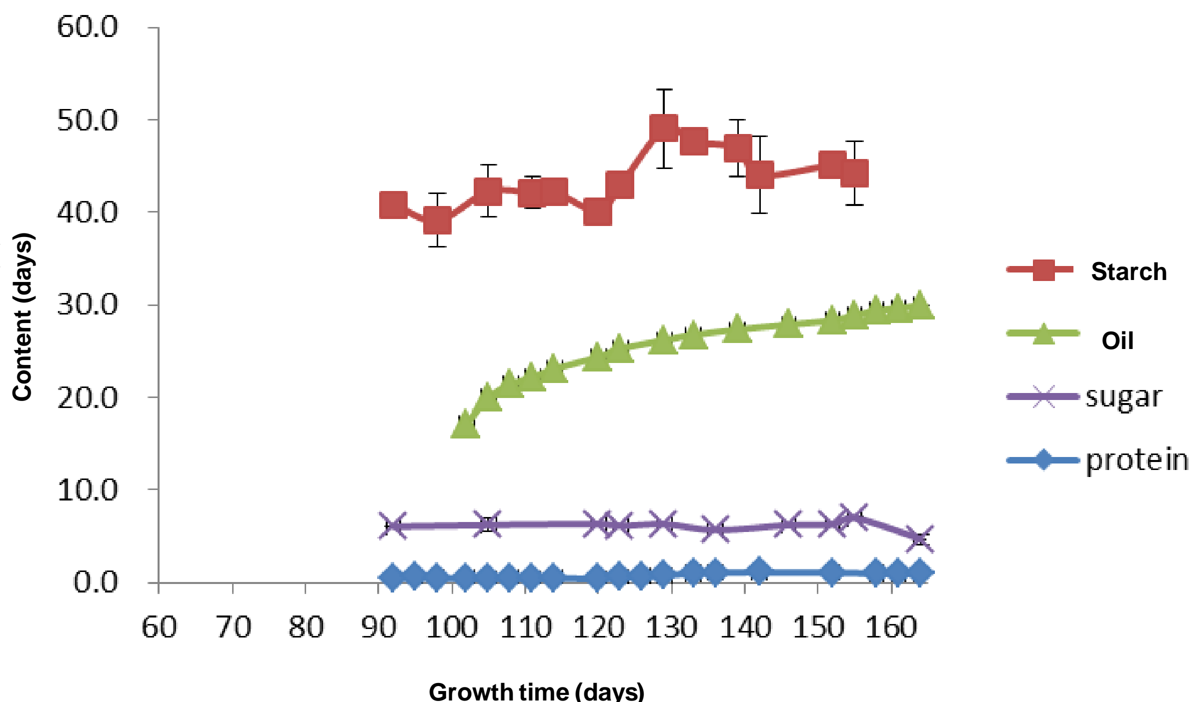

Figure 8. Nutrient content of the tiger nut tuber (dry mass).

However, the starch content of the root was higher as compared to oil content.

Tiger nut tuber development consists of at least two phases. In the first phase, root elongation ceased, and its apex started to swell and eventually developed into a tuber (Turesson et al., 2010). This development is similar to potato tuber development (Viola et al., 2001). For tuber nutrition content changes, starch and oil content accumulation pattern were same with aeroponic system (Turesson et al., 2010). The starch content increased before the 142 day with the highest level of 49% and after that day it decreased with the increasing of growing period (Figure 8). However, tuber oil content gradually increased in entire growth cycle and attained the highest level of 30% during harvesting (Figure 8).

The sugar content pattern was different from aeroponic system due to different sampling way. In the field growing system, although at the beginning sampling was same with aeroponic system, different development phase tuber occurred at the same time. So, the sugar content of the tuber was increased until the 152th days and after that, the decreasing trend was observed (with high sugar content at the early phase and low sugar content at the later phase). The protein content of tuber was very low, and remained at the same level in whole life cycle than other nutrient content (Figure 8) (Turesson et al., 2010).

For moisture content, tiger nut's leaf and tuber moisture content reduced from the beginning. However, the moisture content of roots increased before the 85th day and then began to reduce (Figure 1). Although the highest moisture content recorded were 82.25, 77.49 and 79.31% respectively for leaves, roots and tubers, the moisture content in tuber was higher than in leaves and root (Figure 1).

Conclusion

The moisture content of the tiger nut decreased throughout its growth cycle with time with different pattern in different organs. For leaves, moisture content was decreased until harvest time. And it was gradually decreasing before 90th day, and sharply decreasing after that day. The starch content was increased with reducing of fat content. There was no changing trend in sugar content, but protein content was increased around 115th day, and remaining in that level until harvest. The maximum starch, fat, total sugar, and protein percentages were 26.4, 9, 6.4, and 2%, respectively.

For root, moisture content increased till 90th day, and decreased until harvest time. The oil content showed upheaval features with the rapid increase before 100th day with the maximum value of 8% and gradually was decreased till harvest time. The starch content changed with irregularities, but always kept higher than oil content. Tuber, starch and oil content increased with the increasing of growing time but in the middle of their growth cycle, the changes were irregular. Reducing sugars and protein content of the tuber also showed no significant change. The maximum starch, fat, total sugar, and protein

percentages were 49, 30, 7 and 1.1%, respectively. For tuber optimum yields, it is recommended to harvest the plant at 142nd day for starch. The delayed harvesting may lead to increase in oil content while reducing its total starch contents.

Tiger nut has the potential to provide novel information that can significantly widen understanding about the synthesis of storage reserves, regulating and directing into specific tissues and organs. This kind of information is valuable for work aimed at either increasing the oil content in presently used oil crops or searching for best ways for oil accumulation in organs or tissues that normally do not store oil. Because of this unique nutritional composition of different plant parts, it has the potential to become a model plant to study oil accumulation in non-seed tissues.

ACKNOWLEDGEMENTS

The authors are grateful to the financial support of the National Science Foundation of China- A Mutual Fund Project in Xinjiang (No. U1303103), Program from the Chinese Ministry of Education to Promote Research Collaboration with the United States and Canada and High-Level Personnel Training Project. We thanks to the Karamay Huili CO., LTD for supplying tiger nut tubers. Their sincere thanks go to Molla Mohammad Mainuddin, Ph.D. student, College of Food Science and Nutritional Engineering, China Agricultural University (CAU), Beijing, China and other Ph.D. students for their valuable suggestions and technical support.

Conflict of Interests

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

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Vol. 15(22), pp. 1058-1068, 1 June, 2016
DOI: 10.5897/AJB2015.15092
Article Number: 6F0156E58687
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Bioremediating silty soil contaminated by phenanthrene, pyrene, benz(a)anthracene, benzo(a)pyrene using *Bacillus* sp. and *Pseudomonas* sp.: Biosurfactant/*Beta vulgaris* agrowaste effects

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Received 9 November, 2015; Accepted 21 March, 2016

Polycyclic aromatic hydrocarbons (PAHs) are recalcitrant contaminants which are routinely found in numerous environmental matrices, contributing to ecological degradation. In this study, the removal of LMW and HMW PAHs with 4- and 5-benzene rings, by *Bacillus licheniformis* STK 01, *Bacillus subtilis* STK 02 and *Pseudomonas aeruginosa* STK 03, was evaluated in silty soil for a period of 60 days. Subsequently, a biosurfactant produced from *Beta vulgaris* agrowaste was used to augment the removal of the aforementioned PAHs in mono- and co-cultures. The isolates proved to be proficient in removing the contaminants, with *B. licheniformis* STK01 cultures achieving the highest removal rates. Biosurfactant supplementation significantly enhanced the removal of benzo(a)pyrene- a 5-ring benzene HMW PAH. The highest removal rates achieved in biosurfactant-supplemented cultures were: 100% for phenanthrene, 95.32% for pyrene, 82.71% for benz(a)anthracene and 86.17% for benzo(a)pyrene. The kinetic data used to simulate removal rates were suitably described by first-order kinetics, with the rate constants showing that phenanthrene removal was rapid in cultures without biosurfactant (k = 0.0620 day^{-1}) as well as with biosurfactant (k = 0.0664 day^{-1}), while the removal rates for others followed in the order of their increasing molecular weight. The synergy of the bacterial isolates and the biosurfactant produced from *B. vulgaris* agrowaste could be used in environmental bioremediation of PAHs even in silty soil.

Key words: Benz(a)anthracene, benzo(a)pyrene, bioremediation, biosurfactant, *Beta vulgaris*, polycyclic aromatic hydrocarbons.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous and recalcitrant contaminants, released into the environment through natural and anthropogenic sources (Sánchez et al., 2015). These sources are mainly biogenic, petrogenic and pyrolitic (Harvey, 1998). Owing to their hydrophobicity, they tend to fuse to non- and

porous particulate matter, making soil and sediment a suitable repository. Several PAHs have been identified as potential human mutagens and carcinogens (Grimmer, 1983). Chemical and biological methods have been used to remediate PAH-contaminated matrices, with the bioremediation approach being deemed suitable because it is environmentally benign and less invasive. Of the sixteen PAHs classified as priority pollutants by the United States Environmental Protection Agency (IRIS: EPA's Integrated Risk Information System, 1997), eight were identified as potential human carcinogens. These eight PAHs belong to the high molecular weight (HMW) class, a group associated with a higher tendency to bioaccumulate in environmental matrices.

Recent research studies have focused on the sequestration of these HMW PAHs by biologically-evolved microbial species and the exploration to enhance their bioavailability for subsequent removal from environmental matrices (Mishra and Singh, 2014; Wang et al., 2014; Moscoso et al., 2012; Lu et al., 2014; Chaudhary et al., 2011). Although several bacterial strains have shown an ability to remediate matrices contaminated by low molecular weight (LMW) PAHs, a few have demonstrated a similar ability for HMW PAHs with more than three benzene rings. Among these bacterial strains are many Gram-negative and a few Gram-positive species. These species include *Bacillus* sp., *Pseudomonas* sp., *Rhodococcus* sp. and *Acinetobacter* sp. (Mishra and Singh, 2014; Dandie et al., 2004; Boonchan et al., 2000), with degradation rates achieved ranging from 28 to 85% predominantly for 4-benzene ring PAHs such as pyrene and benzo(a)anthracene.

Moreover, a number of these studies often focused on the removal of PAHs in the aqueous phase as a secondary remediation process after extraction from solid matrices such as soil, that is, using washing as an extraction method prior to degradation due to the sequestration of these contaminants in soil. As a result of the small intraparticle pores in soil grains, Gram-negative bacteria appear to be better degraders of PAHs in such soil due to their thinner cellular membrane which may assist in higher PAH mass transfer across the cellular membrane, thereby facilitating sorption subsequent to intracellular degradation (Ma et al., 2013). Moreover, earlier studies had reported that most indigenous bacteria may be physically precluded from some intraparticle soil grain pores because of the mean diameter of the pores that the immobilized bacteria require to penetrate soil grains in order to access bound pollutants (Lawrence et al., 1979; Alexander, 1961).

This perhaps elucidates the limitations associated with the bioremediation of contaminated soil with a high fraction of clay and silt. Putatively, low bioavailability and mass transfer limitations are challenges to PAH bioremediation processes, particularly in soil. These limitations are often influenced by the molecular structure, weight and weathering of the contaminants including the soils' physicochemical characteristics. Several methods have been adopted to circumvent these challenges such as extraction (Lau et al., 2014; Silva et al., 2005; Song et al., 2011; Yap et al., 2012), adsorption/biosorption (Chang et al., 2004; Vidal et al., 2011; Kaya et al., 2013), co-metabolism (Reda, 2009) and biosurfactant application (Whang et al., 2008; Kang et al., 2009; Franzetti et al., 2010). Rather than utilizing these methods, an approach that is less intrusive and harmful to the environment is often preferred, such as the utilization of biosurfactants.

The application of biosurfactants to increase the bioavailability of pollutants in the environment seems to be a suitable method, considering that these surface chemistry modifying agents are benign. In addition, the availability of an array of suitable agrowaste for biosurfactant synthesis provides an alternative to mitigate the cost associated with their production. Several studies have reported the enhancement of PAH availability and subsequent biodegradation in the presence of biosurfactants (Jorfi et al., 2013; Husain, 2008). Naturally, PAH-degrading bacteria are able to access hydrophobic substrates in the environment through the synthesis of biosurfactants and their ability to directly attach to the hydrophobic substrate by modifying their cell membrane hydrophobicity (Das and Mukherjee, 2007). Biosurfactant-enhanced bioavailability often occurs via two mechanisms: 1) pre-micellar lowering of the surface tension, thereby enhancing the mobilization of the contaminants from particulate matrices resulting in increased sorption mass transfer and 2) micellar solubilization (Amodu et al., 2013).

Another less invasive approach for enhanced biodegradation of recalcitrant PAHs is by microbial co-cultivation using liquefied substrate with a lower surface tension. The biodegradation of phenanthrene, pyrene and benzo(a)anthracene by a bacterial consortium of *Staphylococcus warneri* and *Bacillus pumilus* increased from a maximum of 85% for mono-septic cultures obtained for each PAH, to biodegradation rates greater than 90% when co-cultivated cultures were used in a bioreactor system containing a mixture of PAHs, with a lowered surface tension aqueous phase (Moscoso et al., 2012). Although significant removal rates were achieved in these studies for some HMW PAHs, particularly those with less than 4-benzene rings, the degradation of 5- or more membered benzene ring PAHs, such as benzo(a)pyrene, is scarcely reported.

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In the authors' previous studies, novel bacterial strains were isolated that showed abilities for biosurfactant synthesis, with significant hydrocarbon emulsification indices and surface tension reduction under various environmental conditions (Amodu et al., 2014). In the present study, the effectiveness of these strains to remediate silty soil in which a mixture of PAHs is present, was investigated. The synergy of biosurfactant amendment with microbial co-culture cultivation on PAH-removal and the effect of contaminant co-metabolism were also studied. Finally, the dynamics of the bioremediation of the PAH contaminated soil was studied by monitoring the reduction kinetic rates for each of the PAH being studied.

MATERIALS AND METHODS

Microorganism and inoculum

Bacillus licheniformis STK 01 and *Bacillus subtilis* STK 02 were originally isolated from decaying wood chips and roadside coal tar respectively, both within the proximity of the Cape Peninsula University of Technology, Cape Town, South Africa. No sampling permission was required in these locations. However, *Pseudomonas aeruginosa* STK 03, which was isolated from a restricted location (an oil spill site at the Pipeline and Petroleum Product Marketing Company (PPMC) depot, Shagamu, Ogun State, Nigeria), and permission to sample was given by the Head of the depot (Area Manager). The bacterial strains were identified by morphological as well as 16S Ribosomal deoxyribonucleic acid (rDNA) sequence analysis. In addition, the *Bacilli* sp. were identified as Gram positive, while the *Pseudomonas* sp. was identified as a Gram negative strain. The sequences were registered in the NCBI with GenBank, with accession numbers KR011152, KR011153 and KR011154 for *B. licheniformis* STK 01, *B. subtilis* STK 02 and *P. aeruginosa* STK 03, respectively. They were maintained on nutrient agar slants at 4°C and subsequently subcultured every three weeks.

Chemical reagents

Phenanthrene (Phe), pyrene (Py), benz(a)anthracene (BaA) and benzo(a)pyrene (BaP) were all certified reference materials purchased from Sigma Aldrich Chemical Co. (Germany). Hexane (> 97%), dichloromethane (≥ 99.8%) and anhydrous sodium thiosulfate (> 98%) were also obtained from Sigma Aldrich, while a C-18 Solid Phase Extraction (SPE) 6 ml glass cartridge (0.5 g solid phase) was purchased from SUPELCO (Bellefonte, PA, USA) and utilized for the clean-up and concentration of the PAHs under evaluation.

Soil characterization

Uncontaminated soil sample was obtained within the vicinity of the university (CPUT). The soil was characterized using an American Society for Testing and Material method (ASTM method DIN-4188) coupled with a United Soil Classification system. The soil contained 30% clay, 20% silt (half passing through no. 200 sieve), 20% fine and 30% coarse sand (half being retained on no. 4 to 200 sieve). The soil was classified as a silty soil. It was collected, sterilized and stored at 4°C until use.

Bioremediation of PAHs in soil experiment-sample preparation

The bioremediation of PAHs containing soil was determined in the absence of indigenous microorganisms. Two hundred grams of soil was autoclaved twice at 121°C for 30 min, within a 12 h interval. The soil was spiked with a mixture of 40 mg PAH per kg of soil, using each of the following PAHs: Phe, Py, BaA, and BaP. The spiking process was carried out as described by Brinch et al. (2002), with a minor modification, that is, 20% of the soil was treated with the PAH mixture in acetonitrile. After the solvent has volatilized, the PAH-contaminated soil was mixed with 50% of the uncontaminated soil sample. After adequate mixing, the contaminated portion was then mixed with the remaining soil to ensure a uniform distribution of the contaminants in the soil. Ten grams of the contaminated soil was weighed into 100 mL Erlenmeyer flasks (covered with foil on the exterior), subsequent to incubation at 37°C in a dark, static incubator. The soil samples were inoculated with 8% (v/w) of overnight microbial cultures grown in nutrient broth. The concentration of the culture grown overnight was determined to be 10^8 CFU mL^{-1} by cell count using a Quebec Darkfield Colony Counter.

Different cultures were studied, viz the monocultures of each of the isolate (without supplementation with either biosurfactant or *B. vulgaris* agrowaste extract) and a co-culture of the isolates (consortium, without supplementation). The best performing culture was then supplemented with; 1) *B. vulgaris* waste (5%, w/w) agrowaste extract, and 2) the crude biosurfactant produced from *B. vulgaris* (5%, v/w) agrowaste extract.

Moreover, the concentration of PAHs in each flask was determined prior to incubation in order to assess the recovery efficiency of the extraction method used as well as the removal rate at the end of each experiment. The soil moisture content was maintained at 60% holding capacity as reported by Acevedo et al. (2011), by adding 5 mL of sterile water to each flask at 10 day intervals. Control experiments were prepared in a similar manner without an inoculum to account for the disappearance of PAHs due to abiotic factors. Each experiment was carried out in triplicate. Samples were incubated for 60 days; initially without periodic assessment of the degradation efficiency, but subsequently, the experiment was repeated with periodic sampling.

PAH extraction, clean-up and quantification

PAHs were extracted using an ultrasonication method. At the end of the experiment of 60 days, and during intermittent sampling, samples were transferred from Erlenmeyer flasks into 100 mL amber bottles and extracted with 20 mL of hexane for 20 min at 25°C in an ultrasonic bath. During the sonication, sample bottles were swirled intermittently to avoid the soil settling at the bottom. This step was repeated twice for each sample while the supernatants were pooled into another bottle and centrifuged at 5,000 rpm for 10 min, in preparation for the clean-up stage.

Clean-up procedure: Solid phase extraction

Solid phase extraction (SPE) column LC 18 cartridge was preconditioned with 30 mL of hexane (HEX) and dichloromethane (DCM). The order followed was: DCM – DCM/HEX – DCM, with a volume of 10 mL being loaded at a time for each preconditioning step. The DCM and HEX were mixed in a ratio 2:3 by volume. Sodium thiosulfate (1.0 g) was added to the top of the solid phase in the cartridge prior to conditioning. The supernatant collected from centrifugation was passed through the conditioned SPE cartridge followed by the elution of the PAH analytes with DCM and HEX, using a 7.5 mL of the eluent each time. The eluant collected was dialysed in a rotary evaporator; thereafter, the residue was

reconstituted in DCM to 1 mL in an amber vial, followed by analysis with a gas chromatography-flame ionisation detector (GC-FID).

GC-FID analysis – instrument operation

The GC-FID-analysis was performed using a 7890A Series GC-system (Agilent Technologies, CA, USA) equipped with a flame ionisation detector, an Agilent capillary column USB499114H (20 m x 180 µm x 0.14 µm) and an auto sampler. The oven programme was 170°C followed by ramping at 5°C min^{-1} up to 300°C with each ramping step being maintained for 3 min. Once a temperature of 300°C was reached, the temperature was increased to 310°C and held for 5 min. The total run time was 36 min. The post run time was used to wash the column with a DCM/HEX mixture, to remove any residual analytes before subsequent analyses. The carrier gas used was nitrogen, while a split mode injection was used with the injector temperature set to 250°C. A calibration curve was plotted using calibration standards, with a concentration in the range of 0.5 to 100 mg L^{-1} (R^2 = 0.9996), which was used to quantify the concentration of each analyte in order to evaluate the extraction method and to determine the level of abiotic disappearance of PAHs.

Bioremediation studies: Removal rate kinetics of PAHs from soil

Subsequent to the observation that biosurfactant supplementation resulted in increased removal efficiency, the kinetics of the reduction of PAHs and thus the effect of biosurfactant addition to the cultures on the bioremediation profile, was investigated. Hence, only the mono-cultures of *B. licheniformis* and with biosurfactant supplementation were used in this second stage of the study. The contaminated soil used contained an initial concentration of 50 mg per kg of soil for each of the PAHs, that is, Phe, Py and BaA and 25 mg of BaP. Culture preparation was done as described earlier. After soil spiking with PAHs, 50 g of the contaminated soil was transferred into a 250 mL Erlenmeyer flask covered with aluminium foil to prevent the disappearance of PAHs through UV irradiation. The flask was then incubated at 43 ± 2°C in a dark, shaking incubator at 180 rpm for 60 days. The temperature used here was the predetermined optimum temperature for *B. licheniformis* STK 01 growth and biosurfactant production in our previous report (Amodu et al., 2014). Samples were analyzed periodically and prior to sampling, the flasks were swirled thoroughly to ensure homogeneity. Two control experiments were used, one for each culture. All experiments were carried out in triplicates for this set of experiments. Samples were taken periodically to determine the concentration of PAHs in each flask, including uninoculated cultures (controls) and, by mass balance analysis, removal rates were evaluated. Extraction of analytes, clean-up and analysis using GC-FID were performed as described in earlier. The rate constant (*k*) was determined using a first-order decay rate expression (Equation 1):

$$-\frac{dC}{dt} = kC^n \quad (1)$$

where *C* is the concentration of PAH (mg L^{-1}), *t* is the time (day), *k* is the PAH removal rate constant (day^{-1}) and, *n* the reaction order, which is unity for first order kinetics (Kwon et al., 2009).

The above equation was integrated, while the logarithm of the ratio of PAH-concentration at the various sampling times to the initial concentration was plotted as a function of time. Hence, the disappearance rate, being the gradient of the plot, was determined.

RESULTS AND DISCUSSION

Bioremediation of PAHs from the soil

The biodegradation of phenanthrene, pyrene, benz(a)anthracene and benzo(a)pyrene by *B. licheniformis* STK 01, *B. subtilis* STK 02 and *P. aeruginosa* STK 03, is shown in Table 1. The cultures containing *B. licheniformis* were supplemented with biosurfactant and with an agrowaste (*B. vulgaris*) extract in order to investigate the effect of co-metabolic substrate utilization on the bioremediation of PAH contaminated soil. The biosurfactant used was produced by the *B. licheniformis* strain mentioned above from *B. vulgaris* waste extract, without supplementation with refined carbon sources or trace elements.

Table 1 shows the concentration of each PAH compound in the different cultures determined by the GC-FID prior to incubation as compared to the initial 40 mg kg^{-1} spiked into the soil. Hence, the recovery efficiency of the extraction method was evaluated using the experimental samples prior to incubation and it was found to be between 86 and 90% for Phe, Py, B(a)A and B(a)P. The PAH removal rates decreased with an increase in the molecular weight of the contaminants, for all the cultures studied with an exception being observed for the culture containing *P. aeruginosa*, whereby the degradation of the 5-benzene ring PAH (BaP) was higher than PAHs with 4-benzene rings (BaA and Py).

For all the experiments, the removal rate ranged from 73.97 to 96.88% for phenanthrene, 69.15 to 88.58% for pyrene, 62.21 to 83.30% for B(a)A, and 54.90 to 75.40% for B(a)P. The recalcitrance of PAHs to microbial sequestration often decreases with increasing molecular weight, as observed in this study and as reported elsewhere (Lors et al., 2012; Chaudhary et al., 2011). This phenomenon was expected to occur in the bioremediation of PAHs contaminated soil because, as the molecular weight increases, the tendency for the compound to sequester in a soil matrix and become non-bioavailable increases. Similarly, other researchers have also reported certain cases whereby HMW PAHs were degraded more than the LMW PAHs. Zhang et al. (2009), for instance, reported 97.7% degradation for pyrene and 82.1% for anthracene. Additionally, Acevedo et al. (2011), in a study of the biodegradation of some HMW PAHs, reported a degradation level of 60% for pyrene and 75% for B(a)P. Further research work may be required in this area to explicate the effects of structural symmetry of the pollutants on their biodegradation.

Comparing the removal rates in monoseptic cultures, it was observed that *B. licheniformis* achieved higher bioremediation levels than the other two isolates for all the PAHs. Generally, the microbial isolates used were all found to demonstrate a higher ability for the removal of the HMW PAHs. Few bacterial species have demonstrated a similar proficiency in soil environment. In

Table 1. Bioremediation of PAHs by: a) mono-septic cultures of *Bacillus licheniformis, Bacillus subtilis* and *Pseudomonas aeruginosa*; b) in co-cultures (consortium) using isolates, *Bacillus licheniformis* and *Bacillus subtilis,* including cultures augmented with *Beta vulgaris* waste extract and biosurfactant produced from the *B. vulgaris* waste extract. These samples were incubated for 60 days without periodic sampling.

PAH	*B. licheniformis*			*B. subtilis*			*P. aeruginosa*		
	C_i	C_f	R_{bd} (%)	C_i	C_f	R_{bd} (%)	C_i	C_f	R_{bd} (%)
Mono-septic cultures									
Phe	38.20	3.28	91.43	34.03	5.16	84.83	34.21	5.79	83.97
Py	38.71	8.38	78.35	28.56	7.44	73.96	35.61	10.99	69.15
B(a)A	35.55	8.86	75.07	35.11	13.27	62.21	34.11	12.59	63.09
B(a)P	36.96	11.59	68.63	33.60	15.16	54.90	36.34	8.94	75.40*

Co- and augmented cultures

PAHs	Co-culture			*B. licheniformis* and *B. vulgaris*			*B. licheniformis* and biosurfactant		
	C_i	C_f	R_{bd} (%)	C_i	C_f	R_{bd} (%)	C_i	C_f	R_{bd} (%)
Phe	34.56	3.34	90.34	37.18	3.69	90.07	38.84	1.21	96.88
Py	36.74	8.99	75.54	35.50	5.39	84.82	35.11	4.01	88.58
B(a)A	35.20	9.74	72.34	38.51	6.47	83.03	34.46	8.16	76.31
B(a)P	36.01	10.01	72.20	32.74	10.72	67.27	35.71	8.82	75.29

Phe– Phenanthrene, Py– pyrene, BaA– benz(a)anthracene, BaP– benzo(a)pyrene, %R*bd*- percentage biodremediation; Ci/f– initial and final concentration (mg/L). *Outlier- B(a)P samples had a higher margin of variability in terms of the quantified concentration.

a bioremediation study of BaP, it was found that *Pseudomonas aeruginosa* PSA5 and *Rhodococcus* sp. NJ2 reduced about 88 and 47% of the contaminant respectively, during a 25-day incubation period in a mineral salt medium (Mishra and Singh, 2014).

Considering the biosurfactant supplemented *B. licheniformis* cultures and the mono-culture without biosurfactant supplementation, it was observed that the reduction of pyrene and B(a)P increased from 78.35 to 88.58%, and 68.63 to 75.29%, respectively. Several other studies have reported biosurfactant-enhanced bioremediation of soil contaminated with PAHs. For example, Husain (2008) observed that a rhamnolipid emulsan produced by *Pseudomonas fluorescens* increased the removal of pyrene from 91 to 98% after 10 days of bioremediation studies. Similarly, the addition of biosurfactant synthesized by *P. aeruginosa* SP4 to a soil artificially contaminated with pyrene, was found to enhance removal rates by 25% (Jorfi et al., 2013).

The positive synergistic effects of *B. licheniformis* and *B. subtilis* in a co-culture, as well as the supplementation with the *B. vulgaris* agrowaste extract on the soil bioremediation process, was promising. It was observed that by supplementing the soil with the *B. vulgaris* agrowaste extract, could have served as a cometabolic substrate, thus enhancing the remediation process– an approach that has been reported for enhanced culture performance during the bioremediation of soil contaminated with organic compounds such as PAHs (Moscoso et al., 2012; Reda 2009). The presence of other easily metabolizable PAHs, that is, phenanthrene or other LMW PAHs, can reduce bioremediation rates. However, in such a situation, optimization of culture parameters may be required to control the microbial metabolic activity in order to avoid cell proliferation at the expense of bioremediation (Brinch et al., 2002). In a study of BaP degradation by *Lasiodiplodia theobromae* that lasted for 35 days, the presence of Phe in the culture was found to inhibit BaP degradation since phenanthrene, being a LMW PAH, is easier to metabolize (Wang et al., 2014). If the experiment had lasted longer, the microorganisms may adjust to the nutrient-limiting conditions and thus metabolize B(a)P. Usually, in a culture medium, the tendency is for the microorganisms to first metabolize a readily-accessible substrate and, under the deficiency of certain nutrient elements, such as nitrogen, the organisms can therefore manipulate their metabolic pathways by producing surface active agents extracellularly to solubilize the non-readily available substrate.

Bioremediation kinetic rates for PAHs in soil

As observed in the results shown in Table 1, *B. licheniformis* STK 01, as well as its supplementation with biosurfactant, demonstrated higher bioremediation levels for most of the PAHs studied than the other cultures. Hence, biosurfactant-supplemented cultures were used to study the reduction levels of PAHs with time, as shown in Table 2.

From the bioremediation profiles of the contaminants

Table 2. Reduction levels of PAHs with time by *B. licheniformis* STK 01 with and without biosurfactant supplementation, with periodic sampling.

Organism/days		Bioremediation (%)								
		3	8	15	21	28	35	42	50	60
Bacillus licheniformis STK 01	Phe	7.10	17.17	54.68	66.48	81.86	84.60	93.36	96.81	97.44
	Py	6.88	4.36	34.47	53.08	55.14	63.36	70.93	78.12	89.12
	BaA	7.45	8.25	51.84	56.05	66.40	67.54	73.23	74.02	76.03
	BaP	5.82	6.85	26.05	32.47	52.54	57.33	70.34	82.36	83.05
Bacillus licheniformis STK 01 with Biosurfactant	Phe	1.55	14.23	30.89	46.30	67.46	83.69	96.78	98.31	100
	Py	4.65	7.90	18.38	25.91	41.43	76.18	82.99	91.82	95.32
	BaA	9.85	12.24	29.86	56.58	65.81	70.76	78.80	81.90	82.71
	BaP	6.79	7.53	21.32	38.49	40.04	43.65	66.47	85.39	86.17

Phe– Phenanthrene, Py– pyrene, BaA– benz(a)anthracene, BaP– benzo(a)pyrene.

(Figures 1 and 2), a brief lag phase was noticeable up to day 7, prior to a decrease in concentration of the PAHs in the soil. Moreover, the profiles showed that most of the PAHs were sequestrated between days 7 and 40. For example, about 70% of Phe was reduced within the first 21 days (Figure 1a) while the BaA and BaP reached an equilibrium only after 50 days (Figures 1 and 2). As the concentration of the contaminants decreased, sorption into soil intrapores increased, causing the remaining fractions to become less bioavailable. This phenomenon is common in PAH-bioremediation studies; an observation particularly associated with HMW PAHs, as equally observed for B(a)A and B(a)P in this report. Furthermore, for B(a)P (Figure 2d), the bioremediation process seemed to undergo different phases– a seemingly stationary phase followed by a first-order decrease in PAH concentration between day 10 to 20 and between day 35 to 50, respectively. This may be attributed to the biosurfactant supplementation which seemed to increase the bioavailability of the contaminant from the soil matrix in a discrete form.

By mass balance analysis and also considering the periodic determination of the concentration of PAHs in the control experiment, the percentage removal rate for each of the contaminants in soil after the experiment after 60 days was 97.44, 89.12, 76.03 and 83.06% for Phe, Py, BaA and BaP respectively, for cultures without biosurfactant supplementation. Furthermore, the addition of biosurfactant slightly enhanced the degradation levels to 100, 95.32, 82.71 and 86.17% for Phe, Py, BaA and BaP, respectively. In all the studies, abiotic loss of PAHs, as determined in the uninoculated control flasks, was in the range of 4 to 10%. Hence, the overall concentration of PAHs obtained over the study period in the inoculated flasks was basically due to bioremediation by *B. licheniformis* strain STK 01. The effect of adsorption of PAHs to bacterial biomass on the overall loss may be considered to be negligible since the extraction solvent was added directly to the soil/culture samples. Therefore, the solvent was expected to solubilize both residual PAHs in solution and those adsorbed on to the biomass, as sonication is well known to effectively lyse biomass.

Previous studies on the kinetics of the bioremediation of PAHs in soil have reported results comparable to some of the results presented here. A study by Acevedo et al. (2011) on the rate kinetics of PAH reduction in soil for 60 days, reported that most PAH compounds studied were degraded within 14 to 35 days, while 75 and 60% degradation was achieved for Py and BaP respectively. Lors et al. (2012) also investigated the degradation kinetics of 16 PAHs in soil for 200 days and observed that the highest rates occurred in the first two months for most of the PAHs, with the LMWs being degraded within 7 to 34 days. In the same study, an average of 90% reduction was reported for most PAHs studied, with 85 and 35% being recorded for the 4- and 5- ring PAHs, respectively. For this study, the range was high (85%) for Phe and low (44%) for BaP, after 35 days. Table 3 shows the summary of some of the studies that have reported significant degradation of PAH compounds in soil environment.

It was also observed from the analyses that the rate kinetics showed that the soil containing phenanthrene was rapidly remediated both for cultures without biosurfactant (k = 0.0620 day^{-1}) and with biosurfactant (k = 0.0664 day^{-1}) as compared to the rate constants determined for Py, BaA and BaP (Figures 3 and Table 4). The rate constant values obtained in the culture with biosurfactant supplementation were in the order: $k_{phe} > k_{py} > k_{BaA} > k_{BaP}$, while for the culture without biosurfactant addition, the order observed was $k_{phe} > k_{py} > k_{BaA} = k_{BaP}$.

Conclusion

The bacterial isolates, *B. licheniformis* STK 01, *B. subtilis* STK 02 and *P. aeruginosa* STK 03 used in this study,

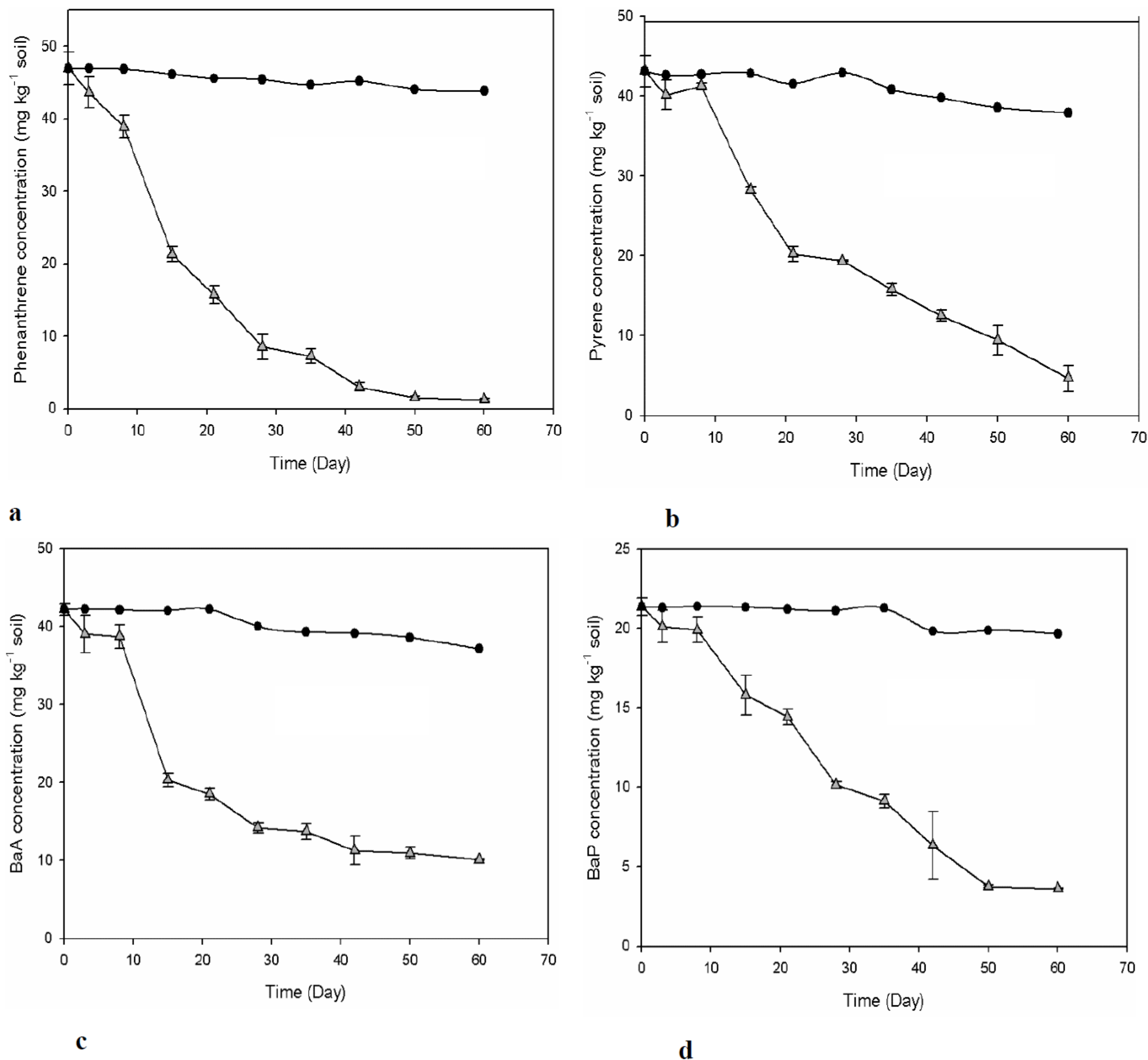

Figure 1. Concentration profile of (a) phenanthrene, (b) pyrene, (c) benz(a)anthracene and (d) benzo(a)pyrene for *Bacillus licheniformis* STK 01 cultures without biosurfactant supplementation. Error bar represents the standard deviation of three replicate determinations. Solid circles – control.

was able to remediate silty soil contaminated with PAHs (Figure 3). A high-remediation capability was observed for all the cultures studied, with the highest being 100% for phenanthrene, 95.32% for pyrene, 82.71% for benz(a)anthracene and 86.17% for benzo(a)pyrene. The results obtained showed that both Gram-positive and negative bacteria used were effective in remediating PAH contaminated soils particularly with enhanced mass transfer rates and bioavailability of the contaminants. Biosurfactant supplementation was found to significantly improve the remediation process of all the PAHs studied. On the other hand, culture supplementation with *B. vulgaris* agrowaste extract as a co-metabolic substrate in *B. licheniformis* STK 01 cultures enhanced removal rates for Phe, B(a)A and B(a)P. First-order reaction rate kinetics was found to fit the kinetic data well and analyses of the rate constant showed that phenanthrene degradation was the fastest both for cultures without biosurfactant (k = 0.0620 day^{-1}) and with biosurfactant (k = 0.0664 day^{-1}), in comparison with the rate constant

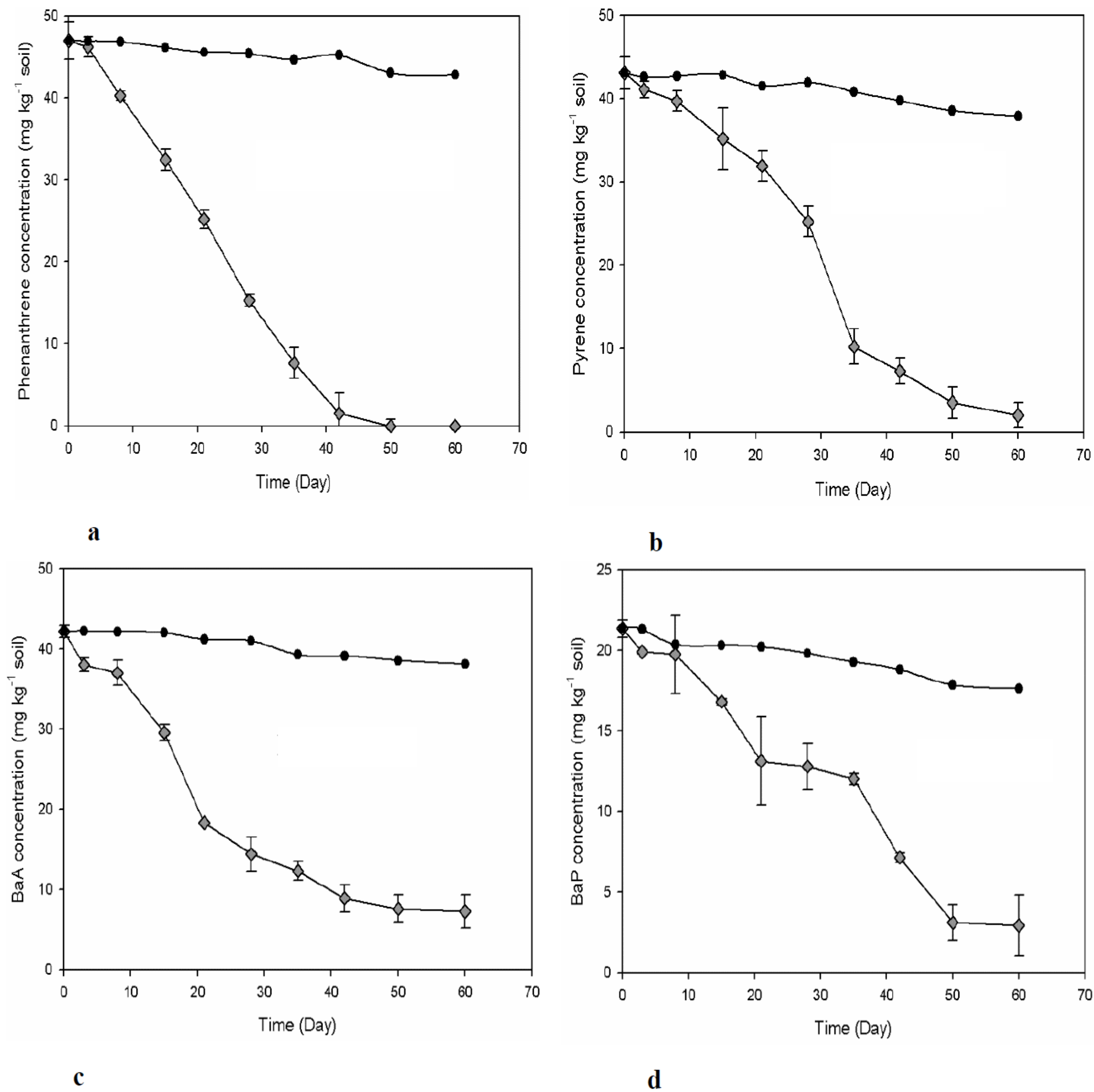

Figure 2. Concentration profile of (a) phenanthrene, (b) pyrene, (c) benz(a)anthracene and (d) benzo(a)pyrene for *Bacillus licheniformis* STK 01 cultures supplemented with a biosurfactant. Error bars represents the standard deviation of triplicate determinations. Solid circles – control.

determined for pyrene, benz(a)anthracene and benzo(a)pyrene. For further investigations, the effects of structural symmetry of PAHs on bioremediation together with the effects of micellar core solubilization and pre-micellar surface activity on the bioremediation kinetics of PAHs is recommended.

Abbreviations

PAHs, Polycyclic aromatic hydrocarbons; **BaP,** 5-benzene ring PAH; **LMW,** low molecular weight; **HMW,** high molecular weight; **rDNA,** ribosomal deoxyribonucleic acid; **SPE,** solid phase extraction; **HEX,** hexane; **DCM,**

Table 3. Summary of studies with significant degradation of PAHs in soil environment.

Microorganisms (with or without surfactant augmentation)	Soil media/initial concentration of PAHs	PAHs removed	Degradation level (%)	Experimental period (days)	References
B. licheniformis STK01	PAH contaminated soil	PHE, PY, BaA, BaP	100, 95.32, 82.71, and 86.17 respectively	60	This study
Peniophora incarnata KUC8836 *Mycoaciella bispora* KUC8201	Creosote-contaminated soil with an initial concentration of 229.49 mg kg^{-1} PAHs	PHE, FLUT, PY ANT	86.5, 77.4, 82.6, and 61.8 respectively	14	Lee et al. (2015)
Pseudomonas aeruginosa SP4	PAH contaminated soil (initial conc. 500 mg/kg soil)	PY	84.6	-	Jorfi et al. (2013)
Sphingobacteria and *Proteobacteria*	Soil contained 9362.1 μg kg^{-1} of USEPA priority PAHs	USEPA priority PAHs	20.2 - 35.8	56	Mao et al. (2012)
White-rot fungi *Trametes versicolor*	Contaminated soil (initial concentration - 1g of total PAHs/kg dry soil)	FLU, PHE, ANT, PY, BaA, CHRY	89	30	Sayara et al. (2011)
Acidovorax and *Sphingomonas* genera	PAH contaminated soil-contained 64% sand, 30% silt and 6% clay	NAPH, ACE, FLU, PHE, ANT, FLUT, PY, BaP, CHRY, BbF, BkF, BaP, DBahA, BghiP	76	140	Singleton et al. (2011)
Anthracophyllum discolor	PAH contaminated soil	PY BaP	60 75	60	Acevedo et al. (2011)
Enterobacteria and *Pseudomonas* genera	PAH contaminated soil	2-, 3- and 4-ring PAH concentrations	98, 97 and 82 respectively	180	Lors et al. (2010)
Pseudomonas fluorescens	soil contaminated with PAHs	PY	98	10	Husain (2008)

NAPH– Naphthalene, ACE– acenaphthene, FLU– fluorene, PHE– phenanthrene, ANT– anthracene, FLUT– fluoranthene, PY– pyrene, BaP benz[a]anthracene, CHRY– chrysene, BbF– benzo[b]fluoranthene, BkF– benzo[k]fluoranthene, BaP– benzo[a]pyrene, DBahA– dibenz[a,h]anthracene, BghiP– benzo[g,h,i]perylene.

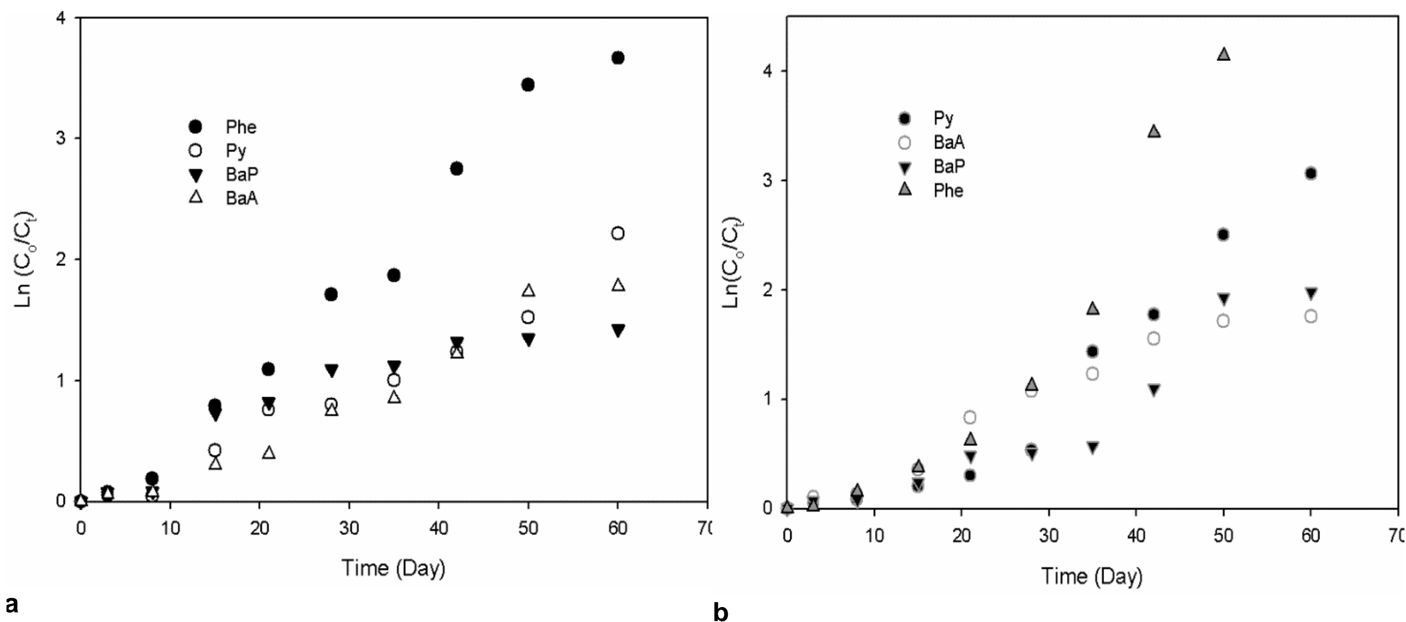

Figure 3. Linearized plot of first-order degradation kinetic model for (a) *B. licheniformis* STK 01 and (b) *B. licheniformis* STK 01 supplemented with biosurfactant.

Table 4. PAH-reduction rate constants and regression determining coefficients.

PAH compounds	*B. licheniformis* STK 01		*B. licheniformis* STK 01 supplemented with biosurfactant	
	k **(day^{-1})**	**R^2**	***k*** **(day^{-1})**	**R^2**
Phenanthrene	0.0620	0.9759	0.0664	0.8382
Pyrene	0.0332	0.9602	0.0432	0.9208
Benz(a)anthracene	0.0290	0.8724	0.0292	0.8647
Benzo(a)pyrene	0.0291	0.9496	0.0272	0.8387

dichloromethane; **GC-FID,** gas chromatography-flame ionisation detector; **NCBI,** National Center for Biotechnology Information.

Conflict of Interests

The authors have not declared any conflict of interest.

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Vol. 15(22), pp. 1069-1077, 1 June, 2016
DOI: 10.5897/AJB2015.15114
Article Number: 37B41C358691
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Temporal profiles of intra- and extracellular laccase isoenzymes from *Pleurotus ostreatus* produced in submerged and solid-state cultures

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Received 20 November, 2015; Accepted 9 May, 2016

Laccases are enzymes that have a great potential for use in breaking down toxic compounds. Fungal laccases show high enzymatic activity, especially those produced by basidiomycetes. Depending on the culture conditions and the strain used, a variety of isoenzymes and/or enzymatic activities can be obtained. In this study, extracellular laccase enzymes produced by *Pleurotus ostreatus* was identified in a submerged culture (SmF), with and without copper sulphate as a chemical inducer, and in a solid-state culture (SSF), using wheat straw as natural inducer. This study was conducted to observe the expression of the enzymes produced under the culture conditions tested and their persistence during the culture, as well as the extracellular activity produced and the correspondence that the isoenzymes presented between the intracellular and extracellular media. A positive effect of the inducers on the specific laccase activity was observed either in SmF with copper sulphate or SSF (41.11 and 40.43 UI/mg protein, respectively), compared with that obtained in SmF without copper sulphate (2.87 UI/mg protein). This effect was different only at the time when the highest activity appeared (360 and 120 h, respectively), showing advantages in SSF. The same three isoenzymes were observed in the three kinds of cultures. The main differences among the laccase profiles reside in the time when they appeared in each culture and only an additional form of lower molecular weight was observed in SSF. The laccase enzymes in the intracellular extracts were equal to those in the extracellular ones. The laccase isoenzymes profiles suggest that the presence of inducers helps in maintaining the activity through the culture time.

Key words: Phenol oxidases, basidiomycete, enzymatic activity, copper, wheat straw, solid-state culture (SSF), submerged culture (SMF).

INTRODUCTION

Pleurotus ostreatus is one of the most widely studied fungal species, because it is edible, has medicinal properties, and can produce enzymes used at experimental and industrial levels in food, medicine, and

recently, in bioremediation processes (Piscitelli et al., 2010). The fungus belongs to the white rot basidiomycetes and some of the enzymes they produce (and of the greatest interest) present laccase activity (Eggert et al., 1997; Baldrian, 2006).

Laccases (benzenediol: oxygen oxide-reductases EC 1.10.3.2) are glycoproteins known as blue multi copper oxidases. In general, they are monomeric enzymes, although some of them are multimeric, intracellular or extracellular, and belong to the family of phenoloxydases, which act on ρ-diphenols (Palmieri et al., 2000). They have a catalytic site, characterized by four copper atoms linked to three REDOX sites (T1, T2, and T3), through which it binds to four electrons to reduce oxygen to water, while oxidizing a wide spectrum of substrates (Kunamneni et al., 2008). The different forms in which they occur are called isoforms or isoenzymes. The term “isoenzyme” has been used as an operational definition that brings together multiple enzyme forms with the same substrate specificity (Marker and Moller, 1959). Nevertheless, the nomenclature established by the IUPAC-IUB (1981) recommends to restrict the use of the term "isoenzyme" to enzymatic products with the same catalytic activity but different due to genetic causes.

The laccase activity and the number of isoenzymes vary depending on the culture conditions (including the use of inducers), the fungus species, and even the specific strain used (D´Souza et al., 1999; Giardina et al., 1999; Mansur et al., 2003; Palmieri et al., 2000, 2003).

Nevertheless, it is difficult to compare the results from different reports because the laccase activity and the number of isoenzymes are measured using different substrates. Additionally, each strain of a particular fungal species has been tested in one specific condition at a time, making difficult to know the difference between enzymatic activities and isoenzyme patterns of the same strain under different culture systems, which would help to improve comparisons and to reach more accurate conclusions.

It has been reported that the *P. ostreatus* genome encodes around eleven genes of laccases, and six different laccases have been isolated and characterized showing that they are encoded by different genes. For this reason, the different forms of the laccase enzymes produced by *P. ostreatus* are referred as isoenzymes in several reports (Pezzella et al., 2013; Velázquez-Cedeño et al., 2007).

On the other hand, it has been reported that laccases have different functions, some of them are carried out in the intracellular environment (defense under stressful condition, formation of conidia) and others extracellularly (lignin degradation, detoxification of pollutants), without making it clear whether the isoenzymes operating in each environment are different or the different functions are performed by the same isoenzymes (Mansur et al., 1997; Galhaup et al., 2002; Velázquez-Cedeño et al., 2007).

P. ostreatus produces higher enzymatic activities during submerged culture (SmF) than those produced during solid-state culture (SSF) using inert supports (e.g., polyurethane foam) (Téllez-Téllez et al., 2008). However, the differences between laccase activities produced during SSF on natural and biodegradable supports, and those produced during SmF are not so evident due to the nature of the systems, the units used to report these activities, the substrate kind and the different parameters used for their evaluation. There are reports showing higher laccase activities in SSF than in SmF (Ramírez et al., 2003; Stajić et al., 2004). In another study (Teixeira et al., 2010), enzymatic extracts (EE) obtained from this species, cultured on waste materials from agricultural processes, yielded better laccase activities when compared with those obtained from commercial enzymes applied to the degradation of AZO pigments, in spite of their lower activity (5.4 U/g of dry substrate (DS)). All of this has increased the interest in the study of SSF with the goal of applying them to these processes at different levels.

Few studies have assessed whether these variations are due mainly to the type of isoenzymes produced in both types of cultures: some studies show more isoenzymes in SmF than in SSF and, in other works, opposite results have been obtained. In the field of gene manipulation, this information is very useful because of the interest in working with those isoenzymes, which express themselves efficiently under particular conditions for a better handling of organisms focusing on improved production. So far, this has not been achieved in existing improved strains (Piscitelli et al., 2010; Record et al., 2002; Rodgers et al., 2010), due to the lack of studies on the regulatory processes of the species used.

The aim of this study was to evaluate changes in the laccase isoenzymes produced by *P. ostreatus* and the time when these isoenzymes appear in three culture conditions: SmF (without and with $CuSO_4$ as a chemical inducer) and SSF (assuming that the lignocellulosic components of wheat straw could act as natural inducers), as well as to determine the correspondence between the intra and extracellular isoenzymes observed.

MATERIALS AND METHODS

Strain and culture conditions

The *P. ostreatus* strain ATCC 32783 was used. The fungus was prepared in a medium of malt agar extract (MAE) (DIBICO, Mexico)

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and incubated at 25°C for 168 h. The mycelium was conserved and refrigerated at 4°C.

Production of laccase enzymes

For SmF, the fungus was inoculated into 125 ml Erlenmeyer flasks, in 50 ml of a medium for laccase production by *P. ostreatus*, containing (in grams per liter): glucose, 10; yeast extract, 5; KH_2PO_4, 0.6; $MgSO_4·7H_2O$, 0.5; K_2HPO_4, 0.4; $FeSO_4·7H_2O$, 0.05; $MnSO_4·H_2O$, 0.05; $ZnSO_4·7H_2O$, 0.001. For SmF with chemical inducer, 0.25 g/L of $CuSO_4·5H_2O$ was added to the aforementioned composition. The pH was adjusted to 6.0 with 0.1 M NaOH (Téllez-Téllez et al., 2008). For SSF, wheat straw was used as substrate and natural inducer. As a pre-treatment, the wheat straw was washed three times with distiller water and then pasteurized and adjusted to 70% humidity. Previously, the laccase activity was tested in the extracts of the pre-treated wheat straw without fungus and laccase activity was not observed. The fungus was inoculated in crystallizers of 9 × 5 cm^2, containing 35 g of pre-treated wheat straw.

The experimental units were inoculated with 2 ml of a suspension of the fungal mycelium (0.05 g approximately), obtained after mechanic homogenization of the mycelium in physiological solution, with the help of an electric mixer (Aerolatte®, aerolatte® ltd, United Kingdom). The experiment was replicated three times and conducted during 20 days, sampling three experimental units from each experimental condition every 24 h.

The enzymatic extract (EE) and biomass were obtained from each experimental unit. In the case of the SmF with and without $CuSO_4$, EE was obtained by centrifugation at 5000 rpm for 10 min and stored at -20°C.

For the SSF on wheat straw, the EE was recovered through lixiviation of extracellular products with 75 ml of distilled water and orbital shaking at 300 rpm for 30 min and subsequent extrusion of the substrate with the mycelium through a gauze. This straw residue with mycelium was used for the extraction of the intracellular enzymatic extract (IEE).

The IEE was obtained through maceration of the frozen straw residue with mycelium with liquid nitrogen and lixiviation with 50 ml of 0.1 M phosphates buffer, pH 6.5, and recovered by centrifugation at 10000 rpm for 10 min.

Determination of laccase enzymatic activity

The enzymatic activities of extracellular laccases in the EE were evaluated through absorbance changes at λ=468 nm, after 1 min of the reaction at 39°C. The reaction mixture contained 950 µl of 2 mM 2, 6-dimethoxy phenol (DMP) (ε_{DMP}= 49,600/M cm), as a substrate in 0.1 M phosphates buffer, at pH 6.5, and 50 µl of EE. One enzymatic international unit of laccase was defined as the amount of enzyme which catalyzes the conversion of 1 µM of substrate to product per minute, in the reaction mixture. The activity was expressed as international units per liter in SmF and as units per kilogram of dry substrate (DS) in SSF on wheat straw. The specific activity was reported as international units per milligram of extracellular protein calculated according to the relationship between the maximum activity of the laccase obtained and the extracellular protein produced at the same culture time. The extracellular protein was determined in the EE through the Bradford method using bovine serum albumin as standard (Bradford, 1976).

Laccase isoenzymes profiles

The profiles of isoenzymes with laccase activities were evaluated based on both the EE (for the extracellular forms) and the IEE (for the intracellular forms), through the modified zymography method of electrophoresis in polyacrylamide gels with sodium dodecyl sulfate (SDS-PAGE) (Laemmli, 1970), following the procedure using electrophoresis in semi-denaturalizing polyacrylamide gels (Téllez-Téllez et al., 2005). The activity was detected with 50 ml of 2 mM of DMP as substrate in 0.1 M phosphate buffer, at pH of 6.5, and using the Precision Plus Protein Standard (BIO-RAD. Hercules, CA) marker for the partial characterization of the size of the bands observed. Finally, the size of the isoenzymes was estimated using the band analysis software Quantity One (BioRad).

To compare the maximum specific laccase activities found among the three kinds of cultures (SmF with and without $CuSO_4$ and SSF), the one-way analysis of variance (ANOVA) test was applied. The differences among the treatments were determined by the pos-hoc Tukey test.

RESULTS

Laccase enzymatic activity

Enzymatic activities were higher in SmF using $CuSO_4$ as inducer (9000 ± 622 UI/L) compared to the activities of SmF without $CuSO_4$ (498 ± 35 UI/L); these peak activities were observed at 360 and 264 h, respectively. Furthermore, the maximal enzymatic activity observed in SSF was 4821 ± 30.41 UI/kg DS at 120 h of culture (Figure 1A and B), a shorter time than those in SmF with or without $CuSO_4$. To assess the effect of the induction on both cultures, laccase specific activities relative to the amount of protein produced was compared: the effect of the inducer yielded around 14 times more activity (41.11 ± 1.00 and 40.43 ± 2.21 UI/mg protein for SmF and SSF, respectively) compared to that obtained without induction in SmF (2.87 ± 0.23 UI/mg protein). The activities are shown in Table 1.

Significantly, one of the most important differences is the time when the maximum specific activities were achieved, with the shortest time to obtain the maximum specific activity in SSF compared to SmF.

Variance analysis revealed highly significant differences among treatments ($F_{(2,6)} = 723.9$; $p < 0.001$). Tukey test indicated differences in the maximum specific laccase activities between SmF and both SSF and SmF without $CuSO_4$ ($p < 0.001$) but not between the last two. However, the times when maximum activities were attained, were different from one treatment to another.

Intracellular and extracellular laccase isoenzymes profiles

In the three conditions for growing this *P. ostreatus* strain, three bands corresponding to isoenzymes were observed with laccase activity over DMP. These three isoenzymes were observed in both EE and IEE. However, there were differences in the profile for each culture condition and in the time elapsed. The isoenzymes size was estimated by the bands analyzer software, considering the sizes of the band of weight marker as referring. Figures 2 and 3 show

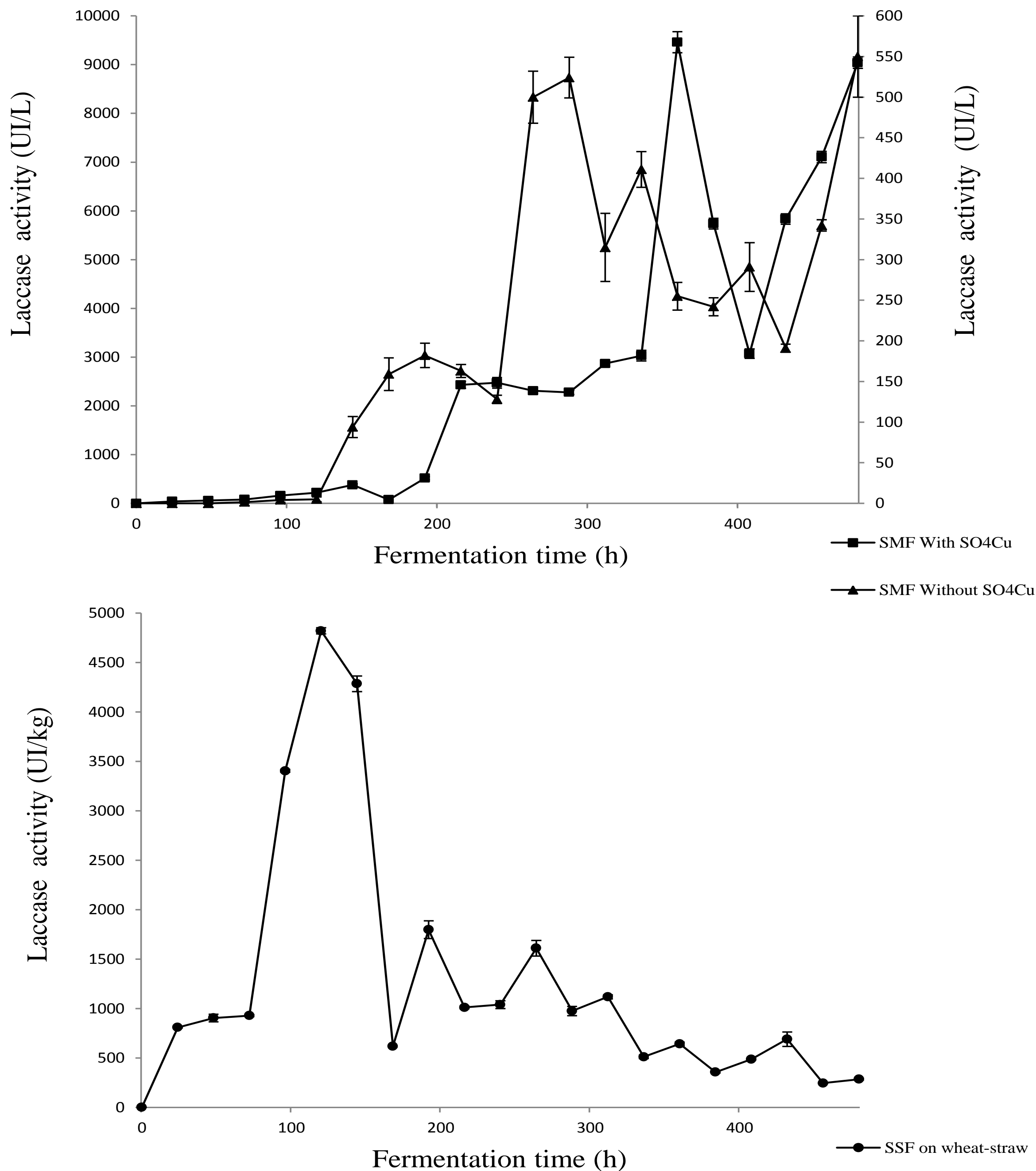

Figure 1. Laccase production by *P. ostreatus* (A) in SMF with $CuSO_4$ (rhombus, left axis), without $CuSO_4$ (triangle, right axis), and (B) in SSF (circle). The error bars represent the standard deviation of three separated replicates of each experiment.

Table 1. Activities of extracellular laccase produced in SMF with and without $CuSO_4$ and in SSF.

Type of fermentation	Maximum enzymatic activity (UI/L y UI/kg DS)	Specific activity (UI/mg protein)
SMF without $CuSO_4$	498 ± 35 (264 h)*	2.87 ± 0.23
SMF with $CuSO_4$	9000 ± 622 (360 h)*	41.11 ± 1.00
SSF	4821 ± 30.41 (120 h)*	40.43 ± 2.21

*Time of culture when the maximum values of enzymatic activity were obtained.

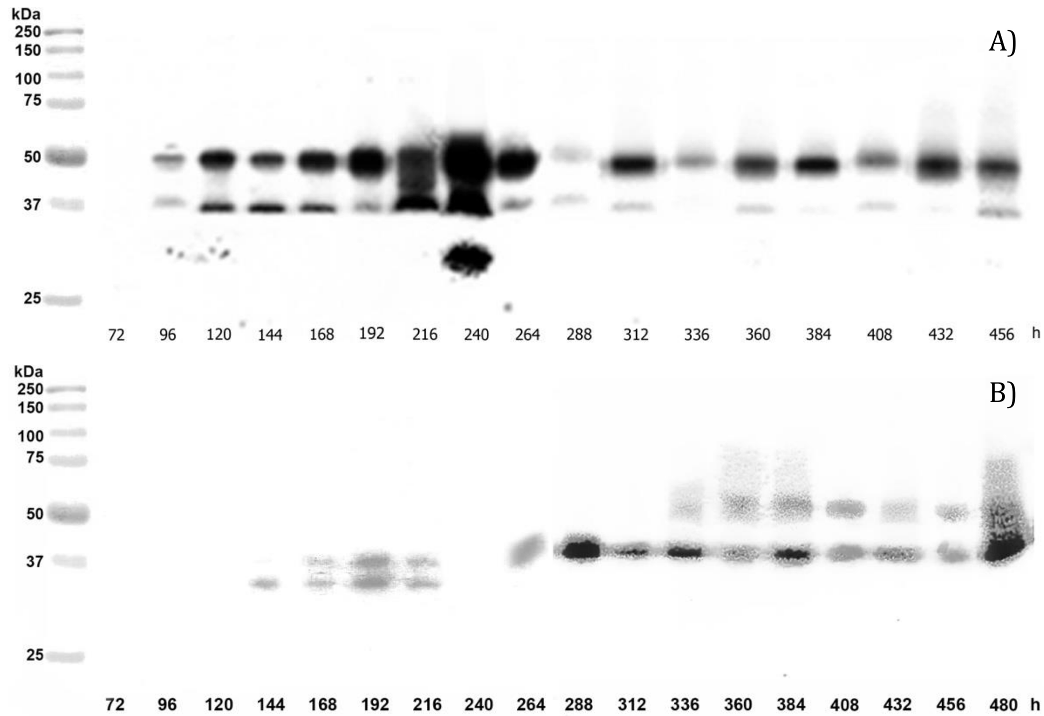

Figure 2. Profiles of laccase isoenzymes produced by *P. ostreatus* in SMF without $CuSO_4$, A) intracellular and B) extracellular.

the profiles of laccase isoenzymes produced by *P. ostreatus* in SmF without and with $CuSO_4$, respectively.

For the two conditions of liquid culture, the isoenzyme with constant activity is found around 50 kDa molecular weight markers, and it is present intracellularly during the culture period. For the intracellular profile, the intensity of the bands is higher between 96 and 200 h in the culture without $CuSO_4$, while in the culture with $CuSO_4$, the higher intensity of the bands is found between 240 and 456 h. This same isoenzyme is present in the extracellular extracts but it can be seen only from the 336 h of culture, not showing differences in the intensity of the bands due to the presence of the chemical inducer. This isoenzyme was followed by the presence of another isoenzyme around 37 kDa molecular weight markers. For the intracellular profile of the culture without $CuSO_4$, its activity begins at 96 h, but it decreased from the 264 h. In the case of the culture with $CuSO_4$, its activity begins at 72 h and shows a slight increase in intensity until the end of the culture. For the extracellular profile, this isoenzyme showed that the activity beginning at 168 and 192 h for the cultures with and without $CuSO_4$, respectively. In this isoenzyme, no differences were found in the intensity of the bands due to the presence of chemical inducer.

The largest difference between the two conditions of SmF was found in the isoenzyme with estimated size of 34 kDa, showing activity only at 240 h, in the intracellular profile for the culture without $CuSO_4$. Nevertheless, for the culture with $CuSO_4$, the isoenzyme showed activity beginning at 72 h and its activity increased gradually until the end of the culture. For the extracellular profile, in the culture without $CuSO_4$, this isoenzyme showed activity only between 144 and 216 h, while for the culture with $CuSO_4$, its activity appeared at 192 h and shows an increase in intensity of the band, until the end of the culture. Both profiles suggest a positive effect of the chemical inducer.

Figure 4A and B shows the profiles of laccase isoenzymes produced by *P. ostreatus* in the SSF on wheat straw, (A) intracellular and (B) extracellular. First, in the case of the isoenzyme of size of 50 kDa approximately, its activity is not evident from the beginning of the culture as in the SmF, it can be seen from 144 and 192 h in the intra and extracellular profiles, respectively. The isoenzyme of size of 37 kDa approximately begins its activity at 240 h in both profiles, but only showed activity until 312 and 336 h in the extra and intracellular profiles, respectively. Finally, the isoenzyme of estimated size of 34 kDa, showed activity between 120 and 240 h, in the intra and extracellular profiles, respectively. In addition to these three isoenzymes, the same isoenzymes observed in the SmF with and without $CuSO_4$, another isoenzyme with lower molecular weight, estimated in 30 kDa, was identified in both intra and extracellular profiles for the SSF; its enzymatic activity is low in the beginning, but it remains

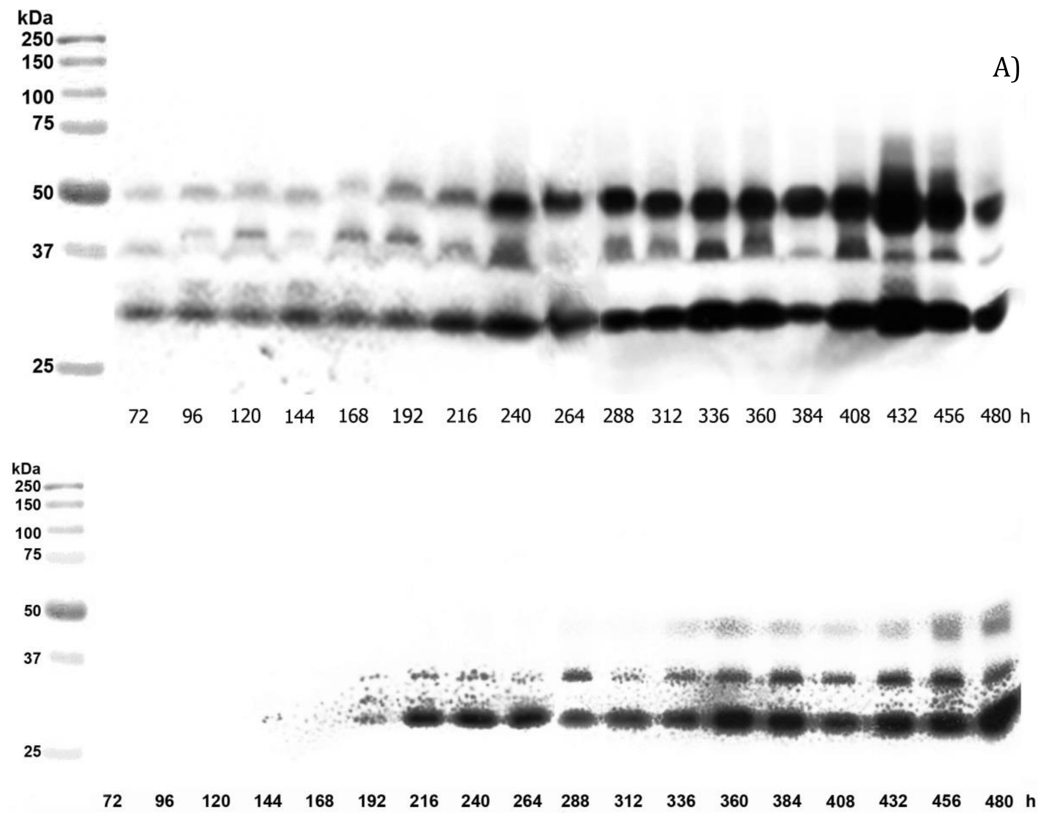

Figure 3. Profiles of laccase isoenzymes produced by *P. ostreatus* in SMF with $CuSO_4$. A) intracellular and B) extracellular.

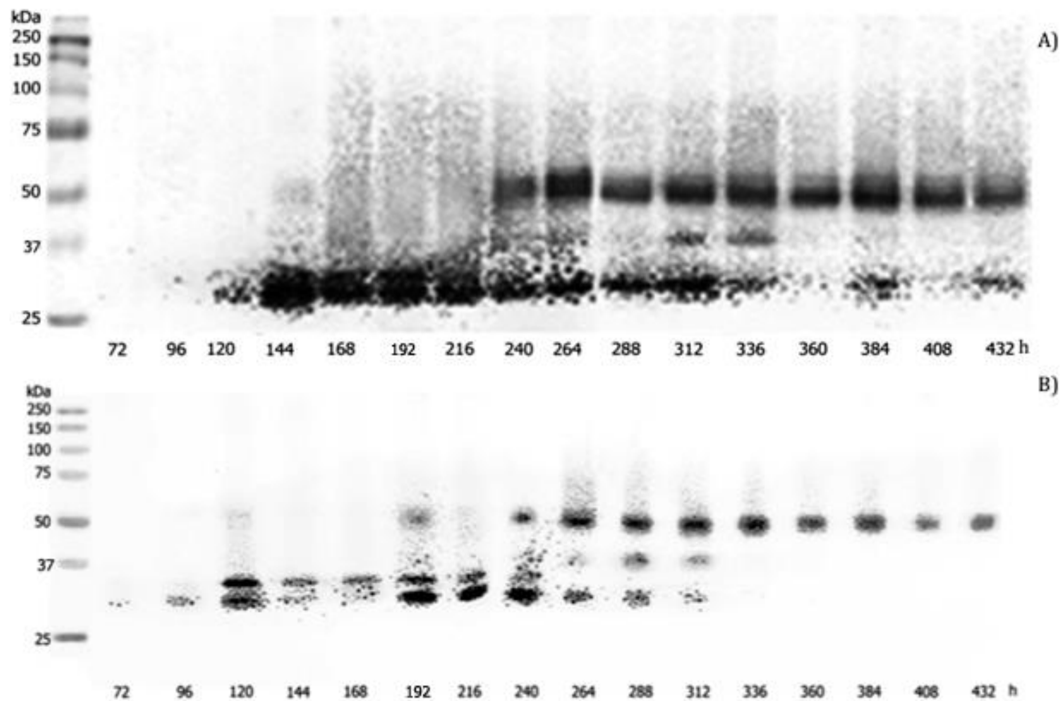

Figure 4. Profiles of laccase isoenzymes produced by *P. ostreatus* in SSF over wheat straw, A) intracellular and B) extracellular.

throughout the whole culture in the intracellular profile, while it only presents activity until 312 h in the extra-cellular profile.

DISCUSSION

In the SmF, with and without copper sulphate, the enzymatic activities obtained depicted the same behavior reported by others authors in previous papers (D´Souza et al., 1999; Palmieri et al., 2000) where the influence of Cu as the inducer increased the activity around 20 times.

However, the enzymatic activities produced here (9,000 UI/L) were lower than those reported previously in similar conditions (13,000 U/L) (Téllez-Téllez et al., 2008); this may be due to the way the inoculum was prepared, probably because of the extrusion of the agar pellets from the mycelium and the homogenization before the inoculation. This could have promoted the destruction of some cells and perhaps the mycelium had an additional growth recovery process, resulting in a decrease of enzymatic activity.

The modification in the preparation of the inoculum was performed to remove the mycelial agar pellets, because there are reports indicating that the composition of the medium influences the production of laccase isoenzymes (Giardina et al., 1999; Palmieri et al., 2000, 2003; Mansur et al., 2003). In this study, we did not want to interfere in the propagation medium in order to assess the actual effect of the tested substrates. Similarly, attempts were made to provide a homogeneous inoculum to the substrate to allow the mycelium to extend its contact area, primarily in the SSF on wheat straw, so it could grow in all areas of the substrate simultaneously. This cannot be achieved when complete pellets are punctually inoculated.

Moreover, no reports were found evaluating enzymatic activity of laccases produced by *P. ostreatus* on wheat straw using DMP as a substrate. However, there are reports of laccase activity produced on other solid substrates using 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as an enzyme substrate (Márquez-Araque et al., 2007; Sathishkumar et al., 2010).

In the present study, the maximal enzymatic activity observed in the SSF was around 5000 UI/kg of DS, using DMP as substrate, this is comparable to the activity obtained in *P. ostreatus* var*. florida* using banana peel, which was 5400 UI/kg of DS, with ABTS as substrate (Sathishkumar et al., 2010). The activity observed in the present study was lower when compared to that obtained by *P. ostreatus* grown on bagasse where it was observed a maximum activity evaluated on ABTS, of 15,540 UI/kg of DS of an EE (Márquez-Araque et al., 2007).

These differences may be due to the composition of the waste of sugar bagasse since it is richer and more easily assimilated as compared to the wheat straw or banana residue.

Regarding the comparison of cultures, as mentioned earlier, it is difficult to make comparisons based only on the activity of the EE of the obtained laccase due to the nature of the culture systems.

However, a comparison can be made based on the specific activity of laccase; it was observed that in both media, the SmF supplemented with $CuSO_4$ and the SSF using wheat straw as a natural inducer, were able to increase the activity up to 14-fold relative to the activity obtained without addition of $CuSO_4$ in the SmF.

In this comparison, the SSF maximum activity was reached in less time (120 h). Comparing SmF without copper, a significant increase in the activity of laccase was observed in the SmF with $CuSO_4$; however, this was achieved at a longer culture time (360 h). These results could indicate one advantage of the SSF in the production of laccases.

In the three conditions of growing this strain of *P. ostreatus,* three isoenzymes, apparently the same isoenzymes, were observed with laccase activity evaluated with DMP. Taking into account the two conditions of the liquid culture, the isoenzymes profiles suggest a positive effect of $CuSO_4$ on the isoenzyme activity of less molecular weight, which maintain its activity throughout the culture time in the intracellular extracts and for the extracellular profile, they showed activity from the192 h until the end of the culture.

In SSF, the profile was different regarding the time; these isoenzymes were active, but only during the intermediate culture times, when the vigorous growth of the mycelium began. In addition, a new isoenzyme of approximately 30 kDa was active during almost all the culture time in IEE and until 312 h in EE. The isoenzyme profile of 34 kDa approximately, is similar to the profile observed in that of 30 kDa approximately, in the first half of the culture time. It is necessary to perform other studies to determine whether these isoforms are isoenzymes or they are the same isoenzyme but with different glycosylation level. However, the isoform lower size could be related to the fungus response to the growth on a complex carbon source (lignin) because it only appears in SSF. The interplay of the other isoenzymes starts when the concentration of the less complex sugars increases, and finally keeps the isoenzyme of the highest molecular size with constant activity during the rest of the culture.

In the zymograms, one additional band was observed in the SSF with respect to SmF. These results are different, considering a previous report (Téllez-Téllez et al., 2008); this difference can be due to the fact that the solid support is inert and does not produce any inducer effect on the fungus. However, regarding the isoenzymes profile found in the SmF, the differences can be due to the influence of the residual culture medium of the agar pellets on the propagation of the inoculum producing an additional band. The results obtained in the SSF concur with other report, where three isoenzymes were also

observed with sizes of 52, 46, and 30 kDa (Ramirez et al., 2003).

With respect to size, it is not possible to arrive at a conclusion because the sizes of the isoenzymes in the present study were only estimated considering a molecular weight marker. It is worth mentioning, that the sizes of the laccase isoenzymes seem to be lesser (30 to 50 kDa) than those reported for this fungal species (60 to 80 kDa) (Palmieri et al., 2000, 2003; Tlecuitl-Beristain et al., 2008).

It has been observed that at least three of the isoenzymes produced in SmF are present in the stationary phase after 408 and 456 h, whereas just one of them was observed throughout the whole culture (Tlecuitl-Beristain et al., 2008). This study demonstrated that in the SmF intracellular activities of the three isoenzymes are produced from the very beginning of the culture. In the case of the SSF, however, the largest isoenzymes were observed only after the adaptation phase of the fungus.

These results suggest that the changing conditions of the culture regulate the activity of the laccase isoenzymes produced by this *P. ostreatus*, irrespective to the kind of isoenzymes being produced. Only in the case of the exclusive SSF, isoenzyme is possible to suggest its additional participation and differential action on the oxidation of the lignin of the wheat straw.

The number and apparent size of the laccase isoenzymes observed in each condition, for both intracellular and extracellular extracts were similar with differences in their expression along the culture time. These results did not show evidence of the presence of specific isoenzymes for intracellular environment, instead they appear to be the same which are secreted extracellularly according to the needs of the fungus.

Conflict of interest

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The present work was supported by the Programa de Mejoramiento del Profesorado (PROMEP) through the project UATX-EXB-197, as well as by the Consejo Nacional de Ciencia y Tecnología (CONACyT) through the approved project N° 61796. A. Tizapantzi and E. Cuatecontzi were supported through a scholarship of CONACyT, Mexico.

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