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Original Research Article

Characteristics of Microbial Communities of Medicinal Plants Rhizosphere from Halayeb and Shalateen

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Abstract: Five different soil rhizosphere samples were collected from the following medicinal plants: *Heliotropium pterocarpum, Euphorbia cuneata, Dracaena ombet, Balanites aegyptiaca and Prosopis juliflra* located in Halayeb-Shalateen region, Egypt. Physico-chemical characterization of these soil was performed. Also, six microbial DNA extraction protocols (A-F) including hexadecylmethylammonium bromide (CTAB) and sodium docdeylsulfate (SDS)-based methods were compared for these soil. Moreover, Phospholipid Fatty Acid (PLFA) analysis was applied to characterize the microbial community structure of these samples. Consequently, this work provides rapid microbial DNA extraction and precipitation method and biodiversity evaluation of microbial community structure in the samples.
Keywords: DNA extraction, Hexadecylmethylammonium bromide (CTAB), Microbial Finger Print, Phospholipid Fatty Acid (PLFA), Halayeb and Shalateen.

INTRODUCTION

Rhizosphere is the region of soil surrounding roots of plants. In this region, there are complex interactions between plant roots, soil microorganisms and soil minerals. Plant species and soil type robustly influence the structure and function of microbial populations associated with the rhizosphere [1-3]**.** Soil microorganisms substantially determine soil organic matter decomposition, nutrient cycling, soil degradation and bioremediation of soil pollution [4]. Variations in the structure and composition of the microbial community are strong indicators of soil biological activity, soil quality and plant productivity of terrestrial agro-ecosystems [5].

Recently, different methods have been developed for studying composition of microbial community in soil to overcome the uncultivability of most of these microorganisms (98%) using the traditional cultivation-based methods [6]. Nucleic acid based techniques including direct extraction of total community DNA from soil samples have revealed enormous diversity in the rhizosphere inhabiting microbial flora [7]. DNA isolation represents the limiting step not only in molecular biology for microbial strains, but also for microbial community analyses. There is still great need for optimization of total microbial community DNA isolation protocols in spite of the presence of commercially available genomic kits. Thus, there are still many obstacles in recovering high genomic DNA amounts, especially from soil samples [8].

Moreover, Phospholipid fatty acids (PLFA) analysis is used to measure microbial biomass and community composition in soil. PLFAs are potentially useful signature molecules due to their presence only in living cells. Thus, analysis of microbial populations using PLFA analysis provides direct information for the identification, classification and quantification of microbial community composition [9]. PLFA analysis has provided a fingerprint of community structure, although it does not give precise species composition. PLFA analysis detects global disturbances in microbial communities and has been widely used in soil microbial community studies [10].

In this study, several DNA isolation methods for total microbial community were tested for some medicinal plants rhizosphere in Halayeb and Shalateen regions and PLFA analysis was used to detect the living microbial community and its structure for these rhizosphere samples. Moreover, soil physico-chemical analysis was done to study the relation between microbial community structure and soil structure.

MATERIALS AND METHODS

Soil sampling protocol

Five different soil samples from Halayeb and Shalateen (Egypt) were collected from rhizosphere of medicinal plants in January, 2016 (Table 1). Samples for each plant were collected at a depth of 15 cm using a 2.5 cm diameter soil auger. Soil samples were air-dried and sieved (2 mm) to remove organic debris and larger inorganic fragments and then stored in sealed containers at 4°C before analysis.

Soil physico-chemical characterization

Saturated soil paste extract pH, electrical conductivity (EC) of soluble ions ($ds.m^{-1}$) (Jenway Model 4520 Laboratory Conductivity/TDS Meter), saturation percent (SP), cations (Na^+, K^+, Mg^{2+}, Ca^{2+}) and anions (SO_4^{2-}, Cl^- , HCO^{3-}, CO_3^{2-}) were determined for each sample [11]. Soil calcium carbonate was measured by the calcimeter method [13]. Mechanical analysis was done by the international pipette method [12] and the textural class was found out from SP and confirmed by textural triangle.

Isolation of microbial DNA from soil

Six microbial DNA extraction protocols (A-F); one standard and five modified methods were investigated for each soil sample. The volume and composition of extraction buffer (CTAB, SDS, CTAB and SDS) was different in each method (Table 1). Each rhizosphere sample (0.5 g) was homogenized on a vortex mixer (2200 rpm, 5 min) with DNA extraction buffer, Phenol: Chloroform: Isoamyl alcohol (25:24:1 v/v) and 0.5 g sterile glass beads (0.5 mm diameter) and centrifuged, followed by the same procedure in all methods as follows: The upper layer (supernatant) and mixed with an equal volume of chloroformisoamyl alcohol (24:1 v/v) [14]. The aqueous phase was recovered by centrifugation and precipitated with three different methods.

Table 1: Experimental design of microbial DNA extraction from soil samples

DNA extraction	SDS protocol	CTAB protocol	DNA extraction buffer	P: C: I (25:24:1 v/v)	Reference
Method A	+	-	600µl of extraction buffer (50 mM Na-phosphate buffer [pH 8], 50 mM NaCl, 500 mM Tris-HCl [pH 8], and 5% SDS).	300µl	Tanasea *et al*., [8]
Method B	-.	+	500µl of of extraction buffer (50 mM Na-phosphate buffer [pH 8], 50 mM NaCl, 500 mM Tris-HCl [pH 8], and 1% CTAB).	500µl	This study modified from Zhou *et al*., [14].
Method C	-	+	500 µl of extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB).	500µl	
Method D	+	+	500 µl of extraction buffer of method A, adding 1% CTAB (1:1).	500µl	This study modified from Tanasea *et al*., [8]
Method E	+	+	600 µl of extraction buffer of method C adding 5% SDS (1:1).	300µl	This study modified from Zhou *et al*., [14].
Method F	-	+	600 µl of extraction buffer of method B.	300µl	

P: C: I : Phenol: Chloroform: Isoamyl alcohol.

DNA precipitation methods

Three different precipitation methods were investigated for the best DNA isolation method resulted from the previous experiment.

Isopropanol: DNA volume, 2 v 7.5 M ammonium acetate or 2 v 5 M sodium acetate and 1 v isopropanol, incubated (RT, 10 min), centrifuged (14.500 ×g, 10 min), washed with 100 µl of 70 % ethanol. Then, vortexed for 1 min and centrifuged (14.500 ×g, 10 min) [15].

Polyethylene glycol (PEG): The supernatant was mixed with 2 v of 30% PEG 6000 (w/v) and 1.6 M NaCl (2 h, RT), followed by centrifugation (18,000 × g, 10 min, 4 ^{O}C). All liquid was discard, then 500 µl of 70% ethanol was added, centrifuged for 1 min, the cap was opened for air dry for 1 h [16].

Ethanol: One tenth v 3 M Sodium acetate or 5 M ammonium acetate (pH 5.2), mixed well with 2- 2.5 v 100% cold ethanol, and placed in ice for 20 min, then centrifuged (14.500 ×g, 15 min). The supernatant was decanted and 70% ethanol was added and mixed well [17].

The DNA resulted from the previous methods was allowed to dry and resuspended in 50 µl Tris-EDTA (T.E) buffer for the evaluation of DNA extraction and precipitation methods.

Evaluation of DNA extraction: DNA was quantified after large-scale purification by determining fluorescence intensities of extracts in agarose gel bands in scanned Polaroid photographs. DNA extracts were subjected to electrophoresis in Tris-acetate-EDTA (TAE) buffer containing ethidium bromide (0.5 mg/ ml) in 0.7% agarose gels. Gel photographs were scanned with a Hewlett-Packard ScanJetIIc scanner [18].

Test for Co-Extraction of Contaminants: The quality and quantity of DNA were estimated using a Spectrophotometer (UV-1601, Shimadzu) by calculating the A_{260}/A_{230} and A_{260}/A_{280} ratios. This method was based on the principle that co-extracted humic acids, phenol, and other aromatic compounds are absorbed at 230 nm whereas DNA and protein absorbed at 260 and 280 nm respectively; high A_{260}/A_{230} and A_{260}/ A_{280} ratios are indicative of purity of DNA [19].

Phospholipid fatty acid (PLFA): Lipids were extracted from soil using single-phase mixture of chloroform, methanol and aqueous citrate buffer ([20]. Briefly, fatty acids were extracted from 10 g of soil sample into a 1:2:0.8 chloroform ($CHCl_3$): methanol (MeOH): phosphate buffer through centrifugation (2500 rpm 10 min, 30 min). Additional $CHCl_3$ and phosphate buffer (12 ml each) were added and phases were allowed to separate. The $CHCl_3$ phase was removed, evaporated under nitrogen (N_2). **Lipid Fractionation:** The organic phase containing the lipids was separated on silicic acid columns into neutral-, glyco-, and phospholipid fatty acids by eluting with chloroform, acetone and methanol respectively [21]. **Alkaline Methanolysis:** The phospholipids were then converted to their methyl-esters by alkaline methanolysis phospholipids in the polar fraction were treated by mild alkaline methanolysis [22]. The resulting fatty acid methyl esters (FAME) were extracted with a hexane/chloroform (4:1) mixture and dried under a stream of N_2. Samples were dissolved in heptane and transferred to GC vials. The separation and quantification of FAME was performed on a gas chromatograph with flame ionization detector (GC-FID) on a Hewlett Packard 6890 instrument equipped with a HP-1MS capillary column (30m x 250µm x 0.1µm, cross linked methyl siloxane). However, a different temperature program was established: after two minutes at the injection temperature of 50°C, the oven temperature was increased at 30°C/min to 110°C, then at 3°C/min to 220°C and finally at 10°C/min to 300°C where the temperature was maintained for ten minutes. Total amounts of the different PLFA biomarkers were used to represent the different groups of soil micro-organisms. The following combinations of PLFA biomarkers were considered to represent the bacterial origin: (15:0, i15:0, a15:0, i16:0, 16:1ω7, 16:1ω9, 17:0, i17:0, a17:0, 18:1ω7, i19:0, and cy19:0) were chosen as indicators of bacterial PLFA [20]. Fungal PLFA was represented by 18:2ω6 [23]; anaerobic bacterial PLFA was represented by cy17:0 and cy19:0 [25]; aerobic bacterial PLFA was represented by 16:1ω7, 16:1ω7t, and 18:1ω7; Gram negative bacterial PLFA and monounsaturated fatty acids were represented by cy17:0 and cy19:0 [24]; Gram-positive bacterial PLFAs were represented by five fatty acids (i15:0, a15:0, i16:0, i17:0, and a17:0) [21].

RESULTS

Soil physico-chemical characterization

The physico-chemical properties of the five different soils according to their cations, anions and calcium carbonate were different among samples (Table 2). On the other hand, pH of soil samples ranged from neutral to slightly alkaline (6.50- 8.0), EC values varied among the soil samples ranged from 1.64 to 3.68 dS/ m. In addition, soil water contents were significantly increased with increases of clay percentage in soil textures. Soils classified according to its texture as clay soil (Table 2).

Table 2: Location and physico-chemical properties of rhizosphere samples

Plant rhizoshere	*H. pterocarpum*	*E. cuneata*	*D. ombet*	*B. aegyptiaca*	*P. juliflra*
Latitude	N 22^{O} 16.003	N 22^{O} 12.250	N 22^{O} 12.575	N 22^{O} 13.794	N 22^{O} 14.887
Longitude	E 36° 22.508	E 36^{O} 23.503	E 036^{O} 20.050	E 036^{O} 20.093	E 036^{O} 34.898
Texture	Clay	Clay	Clay	Clay	Clay
pH	6.50	7.87	7.94	8.0	7.90
SP	45.0	55.0	60.0	45.0	38.0
EC (dS/ m)	2.36	1.64	1.69	3.68	3.11
$CaCO_3$ %	4.2	2.1	1.4	0.7	0.5
Cations (mm/l)					
K+	0.2	0.1	0.1	0.3	0.3
Na+	12.2	7.9	7.3	16.5	17.0
Mg^{2+}	2.6	2.7	2.6	6.2	4.1
Ca^{2+}	5.0	6.3	4.0	9.0	8.6
Anions (mm/l)					
SO_4^{2-}	2.6	0.84	0.75	4.5	3.8
Cl-	15.5	13.54	14.71	25.3	24.2
HCO_3^{2-}	1.9	0.62	0.54	2.2	2.0
CO_3^{2-}	0	0	0	0	0

SP: Saturation percent, EC: Electric conductivity.

Isolation of microbial DNA from soil

In this study the best results obtained by using protocol E which combined both detergents SDS and CTAB. Briefly, 0.5 g of soil sample was homogenized on vortex mixer (2200 rpm, 5 min) with 600 µl of extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB), adding 5% SDS (1:1)), 300 µl of Phenol: Chloroform: Isoamyl alcohol (25: 24: 1 v/v) and 0.5 g sterile glass beads (0.5 mm) (This study modified from 14). The upper layer (supernatant) and mixed with an equal volume of chloroformisoamyl alcohol (24:1 v/v). The aqueous phase was recovered by centrifugation and precipitated with the efficient method.

DNA precipitation methods

Different methods were investigated for DNA isolated from rhizoshere samples. The precipitation of DNA in the five samples by ethanol was higher in DNA quantity and less time consuming (15 min) than isopropanol (30 min). While, PEG method was time consuming (190 min) and not efficient (Table 3).

Table 3: Time consumed for different DNA extraction and precipitation methods for rhizosphere samples

DNA extraction method	DNA isolation time (min)	DNA precipitation time (min)		
		PEG	Ethanol	Isopropanol
A, D	17	190	15	30
B, C, E and F	15			

Test for Co-extraction of contaminants

Co-extracted humic acids and proteins are the major contaminant when DNA is extracted from soil. The absorbance of humic acids, DNA and protein is at 230, 260 and 280 nm respectively.

Table 4: DNA concentration and purity using different DNA extraction protocols

DNA extraction method	Mean DNA/ humic acids A^O 260/ 230 nm	Mean DNA/ protein A^O 260/ 280 nm	No. of total DNA extraction from soil sample
A	0.83 ± 0.03	1.31 ± 0.03	3 + 2 nd*
B	1.06 ± 0.20	1.17 ± 0.05	3 + 2 nd
C	1.56 ± 0.03	1.14 ±0.03	4 +1 nd
D	1.06 ± 0.03	1.10 ± 0.005	2+ 3 nd
E	2.83 ± 0.02	1.93 ± 0.07	5
F	0.50 ± 0.10	1.10 ± 0.02	4 +1 nd

nd*: not determined

Spectrophotometrical determinations for the six DNA extraction methods using ethanol for DNA precipitation in all samples (Table 4). Results showed that method E gave mean 260/ 230 (2.83) and 260/ 280 (1.93) ratios indicates high purity and quantity of DNA and low humic acids and protein respectively. Besides, time consumed using ethanol of DNA extracted by method E is 30 min and it was efficient in all soil samples.

Phospholipid fatty acid (PLFA) analysis

Upon testing the living microbial content using PLFA method, marked variation in microbial percent and structure distribution was observed across tested soil profile samples (Figure 1). In case of *H. pterocarpum* rhizosphere PLFA study profile demonstrated high relative dominance of gram-negative over gram-positive bacterial PLFAs (28.5 and 7.1% respectively) whereas the ratio of Gram positive to Gram negative (G+/G-) bacterial PLFA was 0.25 (Figure 1, 2). It showed also the presence of anaerobes and high level of *Lactobacilli* gram positive bacteria (14.2 and 21.4 %) respectively (Figure 1). Fungal bacterial (F/B) biomass ratio revealed high presence of fungal population indicated by ratio of fungi to bacteria was 0.4 (Figure 2).

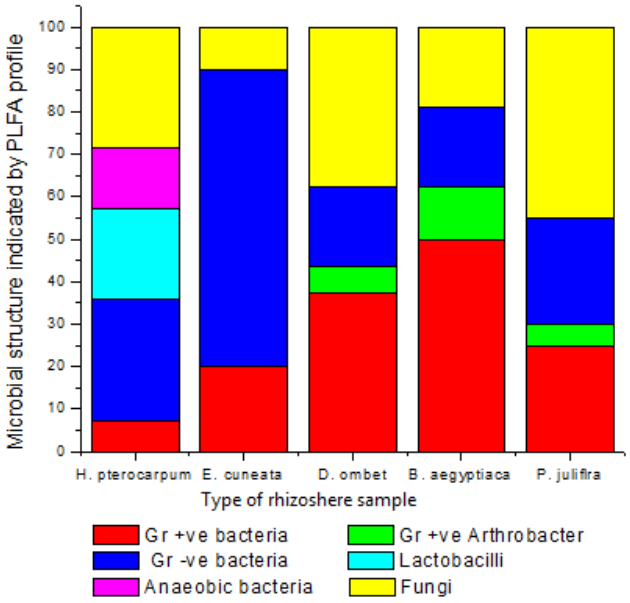

Fig-1: Microbial community structure in rhizosphere samples

In case of *E. cuneata* rhizosphere, PLFA study profile showed high relative dominance of gram-negative with concomitant decrease in gram-positive bacterial PLFAs (68 and 21% respectively) where the ratio of bacterial G+/G- PLFAs was 0.28 (Figure 1, 2). Also, it revealed the lowest level of fungal PLFA distribution (9.8%) and absence of anaerobic bacteria in this soil (Figure 1). F/B biomass ratio revealed low presence of fungal population as the ratio of fungi to bacteria with 0.28 (Figure 2).

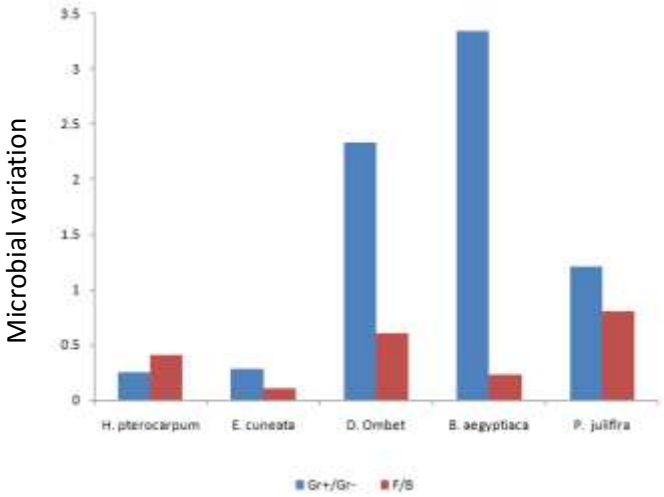

Fig-2: Ratio of microbial community structure according to PLFA profile in different rhizoshere samples
F/B: **Fungal: bacterial biomass PLFA ratio.** G+**:** Gram positive bacteria, G-**:** Gram negative bacteria, F/B : ratio of fungal to bacterial PLFA ratio

On the other hand, the study profile of *D. ombet* rhizosphere PLFA revealed the high relative dominance of gram-positive with moderate level of gram-negative (37.5 and 18.75% respectively) bacterial PLFAs where the ratio of bacterial G+/G- was 2.33 % (Figure 1 and 2). Also, it revealed the high level of fungal distribution (37.5%). Furthermore, low level of *Arthrobacter* gram positive bacteria was detected (6.25%). F/B biomass ratio revealed the high presence of fungal population appeared as the ratio of F/B was 0.6 (Figure 2).

In addition, the study profile of *B. aegyptiaca* rhizosphere PLFA demonstrated the highest relative dominance of gram-positive over gram-negative bacterial PLFAs (50 and 18.75%) where the ratio of bacterial G+/G- was 2.33 (Figure 1 and 2). In this soil, highest level of *Arthrobacter* gram positive bacteria PLFA compared to that of other samples and high level of fungal distribution (12.5 and 18.75% respectively) (Figure 1). F/B biomass ratio revealed the moderate presence of fungal population (0.23) (Figure 2).

The study profile of *P. juliflra* rhizosphere PLFA revealed the highest relative dominance of gram-positive bacteria with high level in gram-negative bacteria PLFAs where the ratio of bacterial G+/G- was 1.2. Also, it revealed the highest level of fungal distribution, also, in this soil *Arthrobacter* gram positive bacteria slightly appear with low level (5%) (Figure 1). F/B ratio showed the prevalence of fungal population (0.8) (Figure 2).

DISCUSSION

There are several factors affecting rhizosphere microbial community composition including plant species and soil type [26] Plant species-specific relationships are not universally found for soil microbial communities and studies performed comparing microbial populations for plant species-specificity are rare [27]. Microbial population was significantly affected by particle size, yielding higher bacterial population in finer soil texture, as well microbial population strongly correlated with clay concentration. Soil chemistry also influences soil microbial community composition, diversity and activity [28]. Soils classified according to its texture as clay soil as the clay soil has SP ranging from 35- 50 [29].

The most important step in DNA isolation procedures is represented by the cell lysis that can be obtained with various treatments of soil samples. However, recent studied proved that extraction yields of DNA are higher than with widely used commercial kits ,indicating an advantage to optimizing extraction procedures to match specific sample characteristics [16]. To evaluate the purity of the extracted DNA, absorbance ratios at 260/ 230 nm (DNA/ humic acids) and 260/ 280 nm (DNA/ protein) were determined [18]. Results showed that method E gave mean 260/ 230 (2.83) (> 1.7) and 260/ 280 (1.93) ratios indicates high purity and quantity of DNA and low humic acids and protein respectively [30].

The analysis of phospholipid fatty acids (PLFA) and community-level physiological profiles have been utilized in an attempt to access a greater proportion of the soil microbial community. In microorganisms, phospholipids are found exclusively in cell membranes and not in other parts of the cell as storage products. This is important because cell membranes are rapidly degraded and the component PLFAs are rapidly metabolized following cell death. Consequently, phospholipids can serve as important indicators of active microbial biomass [31]. Moreover, PLFA is a robust and reproducible technique for studying the structure of living microbial community structure across different soil samples.

In case of *H. pterocarpum* rhizosphere PLFA study profile demonstrated high relative dominance of gram-negative over gram-positive bacterial PLFAs (28.5 and 7.1% respectively) whereas the ratio of Gram positive to Gram negative (G+/G-) bacterial PLFA was 0.25 [32]. It showed also the presence of anaerobes (14.2 %) which is indicated by high level of dimethyl acetal (DMA) PLFAs [33]. The importance of F/B ratio was reported as a potential tool to discriminate the disturbed from undisturbed soil system [34]. The disturbed ecosystems have lower F/B ratio, whereas the organically managed soil systems have increased F/B ratio than conventional system [35]. Low variety in microbial population of *E. cuneata* rhizosphere was observed which is may be due to the antimicrobial activities of *E. cuneata* which were observed against different microorganisms [36]. However, after reviewing the research articles, this is the first study performed on the soil microbial community structure for these medicinal plants rhizosphere in Halayeb-Shalateen region.

CONCLUSION

Out of six DNA extraction methods, method E was efficient for total DNA extraction from all rhizosphere samples used in this study with high DNA purityand less time consuming upon using ethanol for DNA precipitation. Moreover, PLFA is a robust and reproducible technique for studying the structure of living microbial community structure across different soil samples.

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