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Cultivation and Detection of Unculturable Fungi in Soil Using Soil Infusion Agar(SIA).

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ABSTRACT

Unculturable and non sporulating fungi represent a great challenge in studying biotrophic ,endotrophic and mycorrhizal fungal groups. In this study a novel culture dependent technique complemented with PCR was used to detect and identify of unculturable fungi in soil . To develop techniques for cultivation of unculturable fungi for the purpose of expanding studies on fungal biodiversity in soil , Soil Infusion Agar (SIA) was developed . By this technique, from higher dilutions of soil five tiny colonies (less than 1mm in diameter) had grown on SIA after incubation for 1-3 months at 28^0C , but had not shown growth when replicated on traditional mycological media ,were selected , purified studied culturally and microscopically and identified by molecular methods . Growth of the five clones on PDA, but not Sabouraud Dextrose Agar and Czpak Dox Agar of incubation when a loopful of a colony grown after several successive subculturing on SIA was transferred to PDA. The growth on PDA has been empirically proven due to the inoculums size effect and the period of incubation at 28^0C. Two fungal specific primer sets (EF4F/ITS4r and EF60F/ITS4r) were used to amplify partial sequences of fungal r RNA gene included ITS sequences .The five partial sequences of the five clones were aligned through the BLASTN phylogenetic analysis against NCBI database which revealed higher identities. The blast tree showed that all clones are neighbor joined to *Aspergillus* ssp and *Pichia* sp. These results led us to consider the clones as viable but non culturable fungi (VBNC fungi) like the common phenomenon found in some bacterial species.

Introduction

Unculturable and non-sporulating fungi have been existed a major challenge on studying biotrophic, endophytic and mycorrhizal groups thus, a novel techniques are required when trying to detect fungi in the soil [1]. The gap between the known microbial groups and their culturable is now clearly apparent the diversity of un –or not cultured microbes exists in nature [2].

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Possibility causes for the follow up cultivation include lack of growth of many species in current laboratory rich media, lack of advantage from new media form and optimization and death of individuals in studies of microbial nutrition and physiology.

The challenge lies in transporting these microorganisms into the laboratory for future study [3]. Microorganisms are slow growing during the period of incubation because the use of non-diagnostic or complex culture media and a lack of physical and chemical states of sampling site [2] ,[4],[5].

On the other hand, the failure or disability to grow under the conditions natural habitats as nutritional environment, symbiosis as the pioneering agents responsible for low or lack ability to grow microorganism [6]. The causes of un or non-culturability that inhibits growth of microbes in laboratory Petri dishes include short incubation time, substrate urgent death lack of advanced way, complex nutritional environment, over growth of microbes syntrophic interaction and co-culture, lack of will information and patience and disturbance of inter and intra cellular connection [7].

Culture- independent directly in soil, These techniques are used type analysis DNA-based ways have detected and identified clones of unculturable fungi, to search of microbial groups in soil. Such analysis have been done based on specific primer sets [8], [9].

The fungal diversity reported in environment that determined by culture-dependent techniques represent a small fraction of their diversity detected by molecular techniques [10]. A variety of related factors, such as the limitation of culture methods, complex inter dependence between microbes in soil can be replicated in the laboratory "viable but non culturable" states. However, there are some organisms located between the vegetative and spores . Known as viable but non-culturable (VBNC) , the (VBNC) state are useful to identify organisms that unable to sporolate because they offered greater protection from environment, the vegetative state helping to increase the chance of survival. The organisms are still viable but lose the ability to grow on culture media [11].

This study aims to cultivate those fungi that do not form obvious colonies on ordinary growth media used in isolation of fungi from soil but may remain viable or to cultivate micro colonies that may appeared after long times of incubation by replicating the primary master plates on soil infusion agar (SIA). Then, when the potential viable or micro colony forming fungi grow well on SIA they are grown on the primary ordinary media and identified through amplification of partial r RNA gene sequences including internal transcribed spacers (ITS) and then their sequences used as queries in BLASTN search to test their identity with those sequences in database and their potential affiliation to fungal groups to ascertain the validity of the method .

Materials and Methods

Preparation of Media

To prepare Soil Infusion Agar (SIA) new culture media obtained from agriculture soil (5 ,10,20 and 40) g was used as a source. The soil infusion was centrifuged in 15 ml centrifugal tube at 1000 rpm for 10 min. The soil infusion filtrated through whatmann No.4 filter paper and collected as infusion in test tube and preserved in refrigerator for not more than 2 days. The medium (SIA) was prepared by adding 20 g agar to 250 ml of distilled water

Agar dissolved by boiling with shaking, the volume completed to 1L with distilled water and autoclaved at 121^0C at 15 pounds/in^2 for 15 min. The medium maintained melted at 44^0C in shaking water bath, and then 5 mg of streptomycin was added to prevent bacterial growth with existent shaking. Concentrations of soil in medium were prepared by completing (5 ,10 , 20 and 40) ml of soil infusion to 100 ml D.W with melted medium to prepare (0.5, 1, 2 and 4) ml/L and poured in to Petri dishes.

Soil

Agricultural soil samples were collected from a garden in the University of Sulaimani. Samples were taken from the superficial layer within a depth not exceeded 30 cm, mixed thoroughly and collected in sterile containers sealed and carefully placed in a sterile polyethylene bag and brought to the Laboratory. Samples were mixed and sieved twice to remove large stones and debris and tested immediately.

Isolation of Fungi

Serial dilutions till 10^{-6} were done from 10 g of soil in 90 ml of sterilized distilled water [12]. Aliquot of 0.1 ml from the dilutions was spread on SIA plates containing different concentrations of Soil. Cultures were incubated at 28°C for 1 to 3 months and checked every 5 d. The plates used as master plates and replicated on Potato Dextrose Agar Czapk Dox Agar and Sabouraud Dextrose Agar by using an inoculation needle. The replicated plates were incubated again as that of master plates. When tiny colonies of 1 mm in diameter on master plates failed to grow on the replicated plates, incubation of the master plates was extended for 1 week more with adding drops of normal saline to the medium to prevent dryness of the medium. The colonies that failed to grow on replication were purified and subcultured for several times till the size of the colonies reaches an enough size to be succeeded in growth when inoculated on ordinary media. A loopfull from the colonies subcultured on the SIA were inoculated on PDA to examine their growth with larger inoculums' size. Several other subcultures on PDA slants were preserved in refrigerator and repeated every one month as working preserved cultures and to obtain vigorous growth to be used for cultural, morphological and molecular identification.

Identification of the clones

Taxonomic guides and standard procedures [13]-[14] were used to identify the clones culturally and microscopically.

Inoculum Size Effect

The method of Choi *et al*. [15] was used in repetition to obtain colonies originated from single spores. The growth of germinated single spores was tested on SIA and the three ordinary media at 28 °C for three weeks. The ability of the single spore to grow was interpreted as the ability of the germinated single spore to form apparent colony on the agar medium. The clones that fail to grow from single spores were considered as viable but non culturable (VBNC) may be for of the insufficient inoculums size .

Polymerase Chain Reaction (PCR)

Genomic DNA was extracted depending on the protocol provided by the manufacture of the extraction Kit
(Quick- DNA Tm fungal/ Bacterial Miniprep kit) and purified by using Zymo-Spin TM technology of the same company. The amplification of partial genomic sequences of r RNA genes between 18S rRNA and 28S r RNA included internal transcribed spacer regions (ITS) sequences was done using Maxime PreMix Kit (i- Taq). Two pairs of fungal specific primers that are shown in table (1) were used. Run was optimized as came in the literatures that used the primers (table 1). PCR products were visualized on 2 % agarose gel electrophoresis in 1X TBE buffer (9 m MTris-borate, 0.2 mM EDTA) and staining with ethidium bromide.

Table 1: Primers used in PCR Analyses

primers	Sequences (3'…….5')	target	Reference
EF4F/ITS4r	Forword: GGAAGGG ATGTATT TATTAG Reverse: TCCTCCG CTTATTG ATATGC	18S rRNA 28S rRNA	[16] [17]
EF60F/ITS4r	Forword: TGTCTAA GTATAA GCAATT Reverse: TCCTCC GCTTAT TGATATGC	18S rRNA 28S rRNA	[18] [17]

Sequencing and Alignment of Amplicons

PCR products (amplicons) were sequenced at Macrogen Company (Korea) by using Applied Biosystems 3730 mXL automated DNA sequencer. Then the sequences were submitted to BLASTN for pair wise alignment against sequences available at GenBank database (https://www.ncbi. nlm.nih.gov/) and for phylogenetic analyses. Alignment between the sequences of the clones obtained in this study was also achieved as multiple queries against each others to identify the phylogenetic relatedness between them.

Result

A few tiny white colonies were grown on 1% SIA from the dilution10^{-3} and from 10^{-4} of 2% and 4% SIA (figure 1). The purification of the colonies on 2% and 4% SIA and several sub culturing on the same medium has led to enlarge the size of colonies. Five colonies were succeeded in growth when replicated on PDA they symbolized I_1 , I_2 , I_3 , I_6 and I_{12} The positive control plates showed vigorous fungal growth while negative ones showed no growth.

Table 2: Detection of Unculturable Fungi in the Soil of University of Sulaimani using Soil Infusion Agar (SIA)

dilution	(SIA) 0.1ml/L	(SIA) 1ml/L	(SIA) 2ml/L	(SIA) 4ml/L	Control (PDA)
10^{-3}	-	I_1 Colony white, 0.1 mm	-	-	+
10^{-4}	-	-	I_2,I_3 Colonies white 0.1mm	-	+
10^{-5}	-	-	-	I_6,I_{12} Colonies white 0.1mm	+

- = Negative growth , + = positive growth

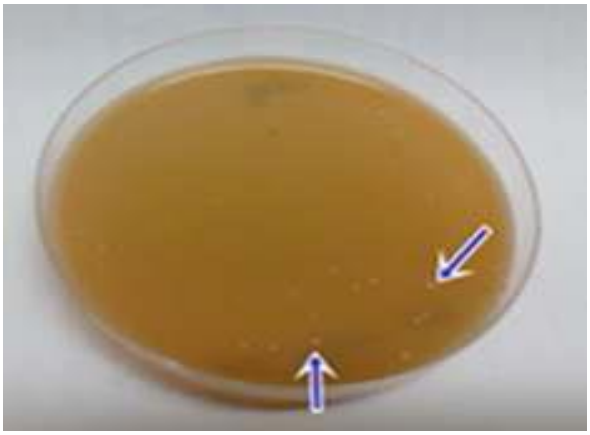

Figure 1. Growth of tiny colonies on 2% SIA medium

The cultural characteristics on PDA and microscopic examinations revealed that isolates are *Aspergillus* spp. And *Pichia sp*. Respectively . (fig. 2). The effect of inoculum size on growth test revealed that the germinated single spores grow only on PDA plates after three weeks of incubation , but growth was failed on Sabouraud Dextrose Agar(SDA), and Czpak Dox Agar(CDA).

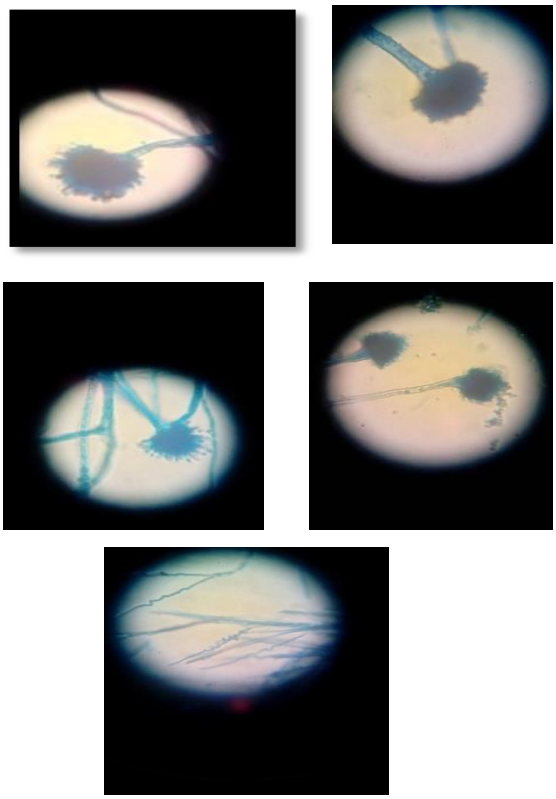

Figure 2. Microscopic feature of (from left to right) I_1 , I_2 , I_3 , I_6 and I_{12} clones on PDA respectivel

The PCR targeted the sequences between 18S r RNA and 28S r RNA gene sequences as expected. Clones I_1 , I_2 , I_3 , I_6 and I_{12} revealed length more than 1000 bp .

The sequencing of amplicons revealed that the amplicon that amplified with EF4F / ITS4r is 1250 bp (Figure 3) and that with EF60F / ITS4r is 1200bp .(figure 4).

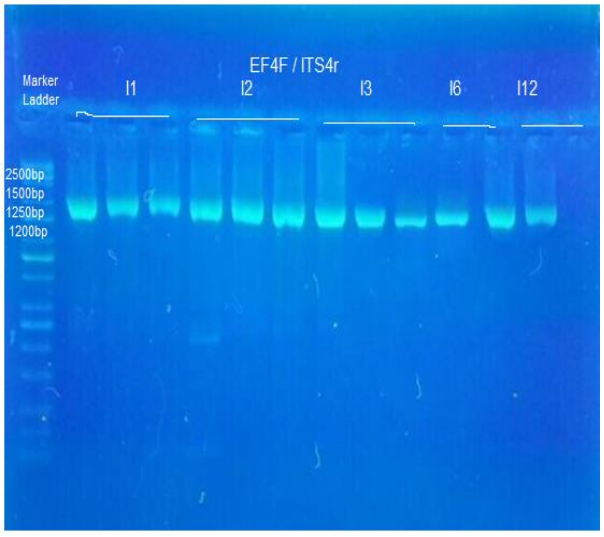

Figure (3) PCR product EF4F / ITS4r , the band size 1250 bp . Electrophoresis was achieved on 2 % Agrose at 5 volt /cm^2 .1× TBE buffer for 1:30 hours. DNA ladder (100) , lane (I_1,…I_{12}) PCR product visualized under U.V light.

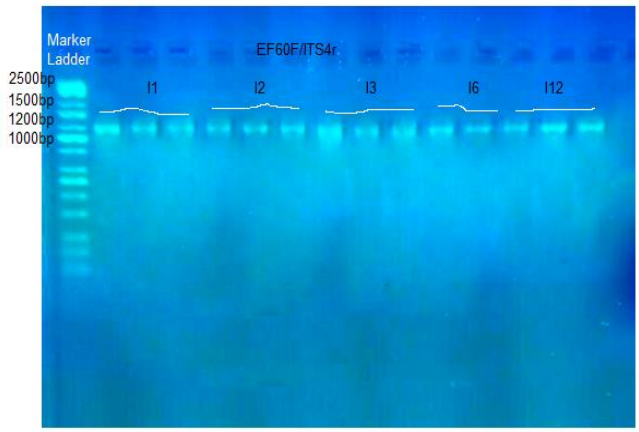

Figure (4) PCR product EF60F / ITS4r , the band size 1200 bp. Electrophoresis was achieved on 2 % Agrose at 5 volt/cm^2.1× TBE buffer for 1:30 hours DNA ladder (100) , lane (I_1,…I_{12}) PCR product visualized under U.V light .

When the sequences of the ten amplicons of the five clones were used as BLASTN queries against the GenBank database .The EF4F/ITS4r amplicon of I_1 showed higher identity (99%) with partial rRNA sequence of *Aspergillus flavus* (acce.No.EU263602.1) .The amplicon of I_2 showed higher identity (94%) with partial r RNA sequence of *Aspergillus* sp.(acce.No.KF562826.1) , the amplicon of I_3 showed higher identity (97%) with partial rRNA sequence of *Aspergillus ustus* (acce.No.KY233193.1) , the amplicone of I_6 showed higher identity (99%) with partial r RNA sequence of *Aspergillus ustus* (acce.No.KY233193.1) and the amplicon of I_{12} showed higher identity (98%) with partial rRNA sequence of *uncultured Pichia* isolate. LZZ40 (acce.No. LC050437.1) while the amplicon of EF60F/ITS4r of I_1 showed higher identity (89%) with partial r RNA sequence of *Aspergillus* sp strain X22 (acce. No.KU350749.1), the amplicon of I_2 showed higher identity (99%) with partial rRNA sequence of *Aspergillus fischeri* (acce.No. GU733354.1) .The

amplicon of I_3 showed higher identity (95%) with partial rRNA sequence of *Aspergillus flavus* .strain DAOM 225949 (acce.No. JN938987.1) . The amplicon of I_6 showed higher identity (96%) with partial rRNA sequence of *Trichoderma reesei* (acce.No. KY100257.1) and the amplicon of I_{12} showed higher identity (93%) with partial rRNA sequence of *Cyberlindneya fabianii* (acce.No. EF550459.1).

Table 2: The BLASTN matching result of rDNA fragment sequences of fungal clones against analogues sequences available in Genbank database.

Source	clone	Media	Amplicon of	Species	Accession number	ID
soil	I_1	SIA	EF4F/ITS4r	*Aspergillus flavus*	EU263602.1	99%
			EF60F/ITs4r	*Aspergillus s. sp.* Strain X22	KU350749.1	89%
	I_2	SIA	EF4F/ITS4r	*Aspergillus s.* Sp	KF562826.1	94%
			EF60F/ITs4r	*Aspergillus s fischeri*	GU733354.1	99%
soil	I_3	SIA	EF4F/ITS4r	*Aspergillus s ustus*	Ky233193.1	97%
			EF60F/ITs4r	*Aspergillus s flavusst* DAOM 225949	JN938987.1	95%
	I_6	SIA	EF4F/ITS4r	*Aspergillus s ustus*	Ky233193.1	99%
			EF60F/ITs4r	*Trichoderma reesei*	Ky100257.1	96%
	I_{12}	SIA	EF4F/ITS4r	*Unculture d pichia* isolate. LZZ40	LC050437.1	98%
			EF60F/ITs4r	*Cyberlindneya fabianii*	EF550459.1	93%

The clone I_{12} is recorded on the NCBI with (accession No. nuccore/MF614964).

Fungal clone sp Suly Ag1

Discussion

The study introduced a method pertaining to the diversity of unculturable soil fungi to detect and identify these fungi from of soil such as: (university of Sulaimni) , during the period from (2016-2017) in Sulaimani province using culture dependent and culture-independent techniques to detect and identify of unculturable fungi [19]. Isolation and morphological identification of unculturable soil fungi are difficulties at both the cultured and molecular techniques. Moreover clones were difficult to detect by using media (PDA, CDA and SDA). Therefore , the aim of this study was to use new media as Soil Infusion Agar (SIA), in order to isolate unculturable soil fungi which was detected by molecular technique (PCR) with specific primers sets [20],[21]. These techniques are of great benefit affecting into the laboratory have allowed the cultivation of clones, increased the rate of isolation of unculturable fungi grown in the laboratory and increased the ability to culture fungi [22]. The study improved that select culture media supporting growth of many unculturable fungi using developed methods to detect and identify a very tiny-small size colony as (viable but non-culturable soil fungi [23], and ability to grow on PDA media by new technique for making single spore isolation to get a pure culture of fungi [24]. Result of current study was showed that several effective approaches for growing unculturable fungi, which suggested to use in media such as Soil Infusion Agar has allowed the clone of cultivated and increased the rate of isolation of micro (tiny) colonies appeared on media (Figure 1), because of the importance of medium choice the grouping of the nutritional requirements of the soil to produce bioactive compounds suitable for growing fungi [25]. Inoculum size and incubation time on cultivation the period between (1-3) months in 28˚C, incubation period is significant of isolation these fungi because of these fungi (unculturable) are slow growing and their pure cultures of these fungi and took longer time three weeks to produce visible colonies. However, colonies which are able to grow more rapidly in media (PDA). In general , they will not grow in media if optimized the growth conditions. As result, these are viable but non culturable fungi [26], [27].

rRNA gene sequences are used frequently in phylogenetic studies as a part of environmental biodiversity screening [28], [29]. The current study revealed that *Aspergillus* spp and *Pichia* sp were isolated as the isolation, alignment, and the phylogenetic tree ensured. In fact, *Aspergillus* spp. are common fungal flora in soil [30]. The four clones were with higher identity with *Aspergillus sp.* but they not originated from a same individual strain because that their sequences that were amplified by using the same primers were not fully identical. The cultural and microscopically characteristics of the four clones ensure their return to *Aspergillus sp* in the view of the molecular identification

CONCLUSION

This study reports the possibility of using low concentrations of Soil in a culture medium (SIA) to isolate viable but non culturable (VBNC), within 1-3 months of incubation at 28˚C that increases the hopes to characterize fungi in screening of fungi in biodiversity studies as well as it opens up new avenues for research in the development of cultural methods for the diagnosis of unculturable fungi.

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زراعة الفطريات غير القابلة للتنمية في التربة والكشف عنها باستخدام اكار منقوع التربة

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الخلاصة:

واجهت الفطريات غير القابلة للتنمية تحدي عند دراسة الفطريات داخلية التغذية ، حيوية التغذية وتعايشية التغذية , في هذه الدراسة تم استخدام تقنيات جديدة تعتمد على التشخيص الزرعي وتاكيدها والمستكملة مع تفاعل البلمرة المتسلسل للكشف عن الفطريات غير القابلة للتنمية وتحديدها في التربة . لتطوير تقنيات زراعة الفطريات غير القابلة للتنمية لغرض توسيع الدراسات حول التنوع الحيوي للفطريات في التربة . فقد تم تطوير اكار منقوع التربة لهذا الغرض ومن خلال هذه التقنية ومن التخفيفات الاعلى للتربة تم ملاحظة نمو خمس مستعمرات صغيرة قطرها اقل من 1ملم في وسط اكار منقوع التربة المرشح والمعقم بعد فترة حضانة من1–3 اشهر عند درجة حرارة 28م ولكنها لم تظهر نموا عند تكرارها على الاوساط الفطرية التقليدية . تم اختيارها , تنقيتها , ودراستها زرعيا ومجهريا وتشخيصها بالطرق الجزيئية .نمو خمس مستعمرات على وسط مستخلص اكار التربة وليس على وسط السبروید دكستروز اكار وجابيك دوكس اكار , لكن بعد حضنها نمت المستعمرات وبعد تكرارها عدة مرات متتالية على اكار منقوع التربة تم نقلها الى وسط مستخلص اكار البطاطا .وقد ثبت تجريبيا نمو العزلات على وسط مستخلص اكار البطاطا بسبب تاثير حجم اللقاح ومدة الحضن عند درجة حرارة 28م° م مما يعني ان المستعمرات حية لكن غير قابلة للتنمية (viable but non–culturable)

استخدم في هذه الدراسة برايمرين نوعيين لتفاعل البلمرة المتسلسل لتضخيم جين الفطريات rRNA و المتضمن منطقة (ITS) وتم مقارنة تتابع 5 عزلات فطرية ومطابقتها من خلال التحليل التطوري للعزلات على BLASTNومطابقتها بالقيم المحفوظة في NCBI وفق المصادر المحفوظة في بنك الجيناتGen bank .

اظهرت الشجرة التطورية لجميع العزلات المجاورة انها تعود الى *Pichia sp.* , *Aspergillus sp.*, وهذه النتائج ادت بنا الى النظر في هذه العزلات واعتبارها فطريات حية لكن غير قابلة للتنمية (viable but non–culturable) ,مشابهه للظاهرة الشائعة التي وجدت في بعض انواع البكتيريا .