



Detection of Unculturable Fungi in Soil Regions of Aziz Awa in Sulaimani province

Wijdan Ahmed Ali¹, Bahrouz M.M AL-Jaaf², SajidS.Saleam AL-SaeedI¹

¹ Department of Biology, College of Science, University of Anbar, Ramadi, Iraq

² Department of Biology, College of Education, University of Sulaimani, Sulaimaniyah, Iraq

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Corresponding Author:

Name: Wijdan Ahmed Ali

E-mail: wjehmed@yahoo.com

Tel:

Abstract

Unculturable and non-sporulating fungi represent a great challenge in studying biotrophic, endotrophic and mycorrhizal fungal groups. In this study collection of soil sample from region Aziz Awa in sulaimani province and using culture-dependent and culture in-dependent techniques for cultivation of unculturable fungi for the purpose of expanding studies on fungal biodiversity in soil. Sabouraud Dextrose Agar with supplement as pyridoxine by replicating master plate from higher dilution of soil three tinny colonies (less than 1mm in diameter) had grown on medium after incubation for 5-7 weeks at 28⁰C but had not shown growth when replicated on traditional mycological media (PDA, Czapeck Dox Agar and Sabouraud Dextrose Agar) were selected, purified, studied culturally and microscopically and identified by molecular methods. Four specific primer sets (NSIF/ITS4r, NSIF/ LRIF, EF4F/ITS4r and EF60F/ITS4r) were used to amplify partial sequences of fungal r RNA gene included ITS sequences. The partial sequences of three clones were aligned through the BLASTN phylogenetic analysis in NCBI were available at GenBank database and revealed higher scores and identities with *Fusarium* sp, *Penicillium* sp and Uncultured soil fungus clone. These results led us to consider the clones as viable but non culturable (VBNC) soil fungi like the common phenomenon in some bacterial species.

Introduction

Unculturable and non-sporulating Fungi are been existed a major challenge on studying biotrophic, endotrophic and mycorrhizal fungal groups, thus, techniques are required when trying to detect fungi in the soil [1]. The gap between the known microbial group and their culturable is now clearly apparent, diversity of un – or not cultured microbes exists in nature [2]. Possibility causes for follow up cultivation include lack of growth of many species on current laboratory rich media, lack of advantage for new media form and optimization and death of individuals in studies of microbial nutrition and physiology. the challenges in transporting these microorganisms into the laboratory for future study [3]. Slow-growing microorganisms during incubation, 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 are the pioneer 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 strains of unculturable fungi, to search of microbial groups in soil such analysis have been done based on specific primer [8,9]. [10] reported that fungal diversity in environment that determined by culture-dependent techniques represent a small fraction of their diversity that detected by molecular techniques. A variety of

related factors, as the limitation of culture methods, complex inter-dependence between microbes in soil can be replicated in the laboratory them "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 sporulate because they offered greater protection from environment they 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].

Polymerase chain reaction (PCR)

Technique is an important part of fungal molecular identification. PCR is amplification of specific target region using short primers, leading to detectable amounts of DNA from one or a few original sequences [12].

Fungal PCR primers

The primer target for the development of PCR primer for helping Fungal diversity in different types of soil had been the r RNA gene, It's limitations the 18s r RNA gene had been the most widely used [13].

Replica plating techniques:

A method was developed to copy of a pattern of microbial growth one initial agar plate to a series of others with supplements, Replica plating might be useful in the detection of biochemical mutants, classification of fermentation reactions determination of antibiotic sensitivity specters and other required repetitive inoculation of several media contained vitamins and amino acids [14,15].

The second application of replica plating was using a velveteen pad to replicate rapidly from initial plates have large number of distributed of actinomycetes, bacteria and filamentous fungi as *Aspergillus nidulans*. [16,17,18].

The aim of study to cultivate unculturable soil fungi by developing culture dependent methods by replicating master plate on supplemented differential

media to cultivate and detect fungi by using molecular method.

Material and Methods

Soil sampling

Soil samples were collected from cultivated location, Aziz Awa. Soil samples taken from the superficial layer, in depth didn't exceed (5-30)cm. Soil was collected in sterile container and placed in sterile polyethylene bags and brought to the Laboratory. In the laboratory the sample was milled and sieved twice to remove various stones and debris to obtain soil sample with small particles [19].

Isolation of soil fungi

Fungi were isolated by serial dilution methods 1g of soil was mixed with 9ml of autoclaved distilled water shaken gently for 5 -10min then 1ml was pipette out from the agitated suspension and added to 9ml sterile distilled water creating a 10^{-1} delution [20] successive dilution of 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} performed one milliliter aliquot of the 10^{-1} to 10^{-6} were placed into respective and spread on media sabouroud Dextrose Agar, an supplemented with 5mg/L streptomycin. vitamin(purchased from sigma, USA) was added to the media as pyridoxine 5mg/ml was filter-sterilized before it was added to the autoclaved media [21].

Replica plating procedure

The general methods of Replica Plating was described by [17,22].

DNA Extraction of fungi

Genomic DNA extraction was achieved depending on the protocol provided by the manufacture (DNA^{fm} fungal/ Bacterial Mini prepkit) four pairs of fungal specific primers that are shown in table(1) were used to amplify partial sequences of fungal r DNA included internal transcribed spacer regions (ITS) sequences. Run was optimized as came in the literatures that used the primers (table1). PCR products were virtualized on 1% a garose gel electrophoresis in 1X TBE buffer (9Mm Trisborate, 0.2Mm EDTA)and staining with ethidium chloride.

Table(1):primers used in PCR analyses

Primers	sequence	target	Reference
NSIF/ITS4r	Forward: GTAGTCATATGCTTGTCTC Reverse: TCCTCCGCTTATTGATATGC	18SrRNA	[25]
NSIF/LRIF	Forward: GTAGTCAGTCATATGCTTGTCTC Reverse: GGTTGGTTCTTTTCCT	18SrRNA	[25]
EF4F/ITS4r	Forward: GGAAGGG[G1A]TGTATTTATTAG Reverse: TCCTCCGCTTATTGATATGC	18SrRNA	[25]
EF60F/ITS4r	Forward: TGTCTAAGTATAAGGAATT Reverse: TCCTCCGCTTATTGATATGC	18SrRNA	[25]

Sequencing and alignment of amplicons:

PCR product (amplicones) were sequenced at macrogen company (Korea) by using Applied Biosystemes 3730 Mxl automated DNA sequencer. Then the sequences were submitted to BLASTN for pair wise alignment against sequences available at Gen Bank database (<http://WWW.ncbi.nlm.nih.gov>) and for phylogenetic analyses.

Results

In the present study, soils are collected from cultivation soil of region Aziz Awa in Sulaimani province. Using serial dilution methods to isolate fungal groups (uncultured fungi) were recovered then through cultural and microscopic characteristics they were identified by molecular techniques .

A few tiny pale white colonies were grown selective media (sabouraud dextrose agar) with supplement pyridoxine (vitamins B6) by using current knowledge it's replica plating. Three colonies were succeeded in

growth when replicated on PDA, they symbolized I₉, I₁₀, I₁₁ the positive control plates showed vigorous fungal growth while negative ones showed no growth (figuer1).

Table(2): Detection of unculturable soil fungi in Aziz Awa by using selective media with Pyridoxine

soil	Master plate Triplatedilution		Media	Replica plating	New colon	Purification	Growth
Aziz Awa	1	10 ⁻³	Sabouraud + pyridoxine	PDA Czapak Sabouraud	I ₉	Sabouraud + pyridoxine	+ve
	2	10 ⁻³	Sabouraud + pyridoxine	PDA Czapak Sabouraud	I ₁₀	Sabouraud + pyridoxine	+ve
	3	10 ⁻⁴	Sabouraud + pyridoxine	PDA Czapak Sabouraud	I ₁₁	Sabouraud + pyridoxine	+ve

pH of soil=8.5

period of growth=(5-7)weeks in 28C°

+ve=positive growth of unculturable soil fungi.

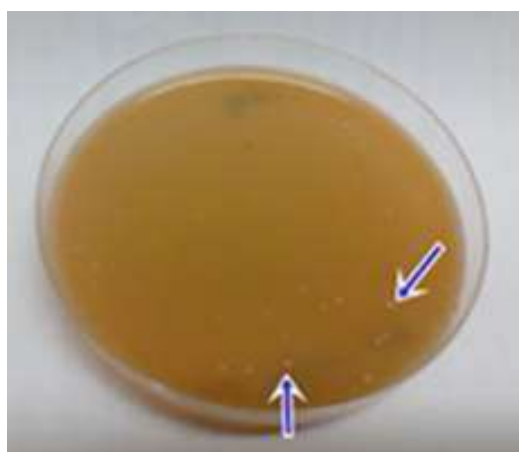


Figure1:Growth of tiny pale white colonies on selective media with pyridoxine

The cultural characteristics and microscopic examination revealed that colonies are *Fusarium sp*,

Penicillium sp, Uncultured soil fungus clone respectively (Figure 2).



Figure 2:The microscopical observation of unculturable soil fungi(*Fusarium*(I₉), *Penicillium*(I₁₀), Uncultured soil fungus clone (I₁₁). respectively.

The PCR targeted the sequences between 18S rRNA and 28S rRNA gene sequences as expected. The bands of three clones I₉, I₁₀, I₁₁ revealed length more than 1000 bp. The sequences of the amplicons with

NSIF/ITS4r is 1500bp, NSIF/LRIF is 650 bp, EF4F/ITS4r is 1250bp and EF60F/ITS4r is 1200bp (Figure3).

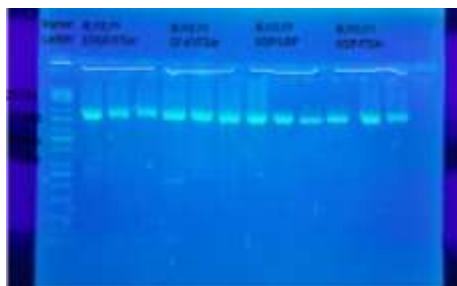


Figure 3: Amplification products of rRNA gene fragments for the fungal clones using four primers pairs for each of fungal clones

When the sequences of the clones were used as BLASTN queries against the GenBank database (Figure 4), the NSIF/ITS4r amplicone of I₉ showed higher identity (99%) with the partial rRNA sequence of *Fusarium oxysporum* isolate K5 (acc.no. JF807399.1) the amplicon of NSIF/LR1r of I₉ showed higher identity (97%) with the partial rRNA sequence of *Fusarium oxysporum* str. 150403 – 45 - m07 - lonsi - abl (acc. no. KX384665.1). The EF4F/ITS4r amplicone of I₉ showed higher identity (98%) with the partial rRNA sequence of *Gibberella fujikuroi* (acc.no.HM165488.1) and the EF60F/ITS4r amplicone of I₉ showed higher identity (97%) with the partial rRNA sequence of *Fusarium sacchari*

(acce.no.KX683426.1). The NSIF/ITS4r amplicone of I₁₀ showed higher identity (99%) with the partial rRNA sequence of *Penicillium polonicum* str. DAOM 216709 (acc. no. JN938978.1) the NSIF / LR1r amplicon of I₁₀ showed identity (98%) of *Penicillium polonicum* str. cBs112560 (acce. no. JN9392254.1). The EF4F/ITS4r amplicon of I₁₀ showed identity (98%) with partial rRNA sequence of *Penicillium* sp (acce.no.KY368614.1) and the EF60F/ITS4r amplicon of I₁₀ showed identity (97%) with the partial rRNA sequence of *Penicillium* sp.str. EGa (acce.no. KX 457676.1) the NSIF/ITS4r amplicone of I₁₁ showed higher identity (98%) with partial rRNA sequence of *Fusarium* sp 16003 (acce.no. Eu 710823.1) the NSIF/LR1r amplicon of I₁₁ showed identity (99%) with partial rRNA sequence of Uncultured ascomycete clone. Ap13-99 (AB 074659.1) the EF4F/ITS4r amplicon of I₁₁ showed identity (97%) with partial rRNA sequence of Uncultured soil fungus clone 5252 (acce.no.GU568145.1) and the EF60F/ITS4r amplicon of I₁₁ showed identity (98%) with partial rRNA sequence of Fungal sp. Nuss - 174 (acce. no. KT 714175.1).

Table(3): The BLASTN matching result of rDNA fragment sequences of fungal clones against analogous sequences available in

GenBank database					
Clone	source	amplicon of	species	Acce.no	identity
I ₉	Soil	EF4F/ITS4r	<i>Gibberella fujikuroi</i>	HM165488.1	99%
		EF60F/ITS4r	<i>Fusarium sacchari</i>	KX683426.1	97%
		NS1F/ITS4r	<i>Fusarium oxysporum</i> isolate K5	JF807399.1	98%
		NS1F/LR1r	<i>Fusarium oxysporum</i> str.150403-45-M07-10 NSi-ab1	KX384665.1	97%
I ₁₀	Soil	EF4F/ITS4r	<i>Penicillium</i> sp strain	Ky368614.1	99%
		EF60F/ITS4r	<i>Penicillium</i> sp. Strain EGa	KX457676.1	98%
		NS1F/ITS4r	<i>Penicillium polonicum</i> strain DAoM216709	JN938978.1	98%
		NS1F/LR1r	<i>Penicillium polonicum</i> strain cBs 112560	JN9392254.1	97%
I ₁₁	soil	EF4F/ITS4r	Uncultured soil fungus clone 5252	GU568145.1	98%
		EF60F/ITS4r	Fungal sp.nuss-174	KT714175.1	99%
		NS1F/ITS4r	<i>Fusarium</i> sp. 16003	Eu710823.1	97%
		NS1F/LR1r	Uncultured Ascomycete clone .Ap13-99	ABo74659.1	98%

The clone I₁₁ was recorded in NCBI (acce.No.MF668636) , Fungal sp. clone Suly Ag2.

Discussion

In this study, the diversity of unculturable soil fungi to detect and identify in region of Aziz Awa. During the period from (2016-2017) in Sulaimani province by using culture dependent and culture-independent techniques. Morphological identification of unculturable soil fungi were difficult to detect by using selective media for isolation unculturable soil

fungi and detect that by molecular technique (PCR) with specific primers [23,24,25] that increasing the rate of isolation of unculturable fungi grow in the laboratory and the ability to culture fungi [26]. Through study improved that select culture media that able to grow many unculturable fungi by having developed methods to detect and identify about very tiny size colony (viable but non-culturable) soil fungi.

The main purpose of the present study as Table (2) show was to investigate and discuss the activity of sabouraud dextrose agar with pyridoxine is the best media for growth of unculturable fungi and purification clone by using replica plating technique [16]. The addition of Pyridoxine (vitamin B6) into selective culture medium increased significantly the mycelium production and pyridoxine is known to be increased cellular permeability and increased bioactivity among the organisms to grow when it mixed with media which led to the best growth [27,28]. As well as accompanied with another compounds in soil played the possible role to grow of unculturable fungi because replica plating is the main advantages method to declaration the copying of type of microbial growth (colonies) from master plate to a series of others and allowed a sensible number of colonies to appears.

Another study is to show the relation among DNA extraction, PCR technique, alignment and sequencing, taxonomic and phylogenetic tree to identify clones Table (3). The selection of PCR target and specific primers for the required taxonomic of clones and soils were important [29,30]. PCR- based methods targeting the ribosomal RNA gene which had been in a wide range of use to discuss fungal species diversity, could be perfected using the nuclear

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small (18s r RNA sub unit-ssu) to amplificate the DNA of clones [31]. The sequence supplied information for identifying soil fungal species and they had used the root phylogenetic tree to limit the evolutionary relation between taxonomic group [32,25].

A large sequence difference is required to identify fungal at the species, strain the large internal spacer regions (ITS1 To ITS4) which a large sequence and different size had been useful as appropriate DNA barcode Marker for the identification of fungal species, In addition, the ITS region amplified and supplied enough variation to identify species [33]. When query in the Gen Bank database in (NCBI) to identify uncultured clones to alignment to another fungal species and the taxa report is to identify uncultured clones to phylum order, family to genus [34]. The root of phylogenetic tree of result the alignment of ITS and sequence as a general indicator for the study to compare between fungal species from the analysed Neighbor-joining. It showed the unknown clones with isolated from soil [35]. In addition, sequence obtained from Gen Bank the alignment ID that had been ($\leq 97\%$ – $\geq 99\%$) in the BLASTN search.

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الكشف عن الفطريات غير المنزوعة في منطقة عزيز اوا في محافظة السليمانية

وجدان احمد علي¹ ، بهروز محمود امين الجاف² ، ساجد صلاح الدين سليم¹¹قسم علوم الحياة ، كلية العلوم ، جامعة الانبار ، الرمادي ، العراق²قسم علوم الحياة ، كلية التربية ، جامعة السليمانية ، السليمانية ، العراق

الملخص

واجهت الفطريات غير المنزوعة تحدي كبير عند دراسة مجاميع الفطريات حيوية التغذية، داخلية التغذية و تعايشية التغذية، تم جمع عينة التربة من منطقة عزيز اوا في محافظة السليمانية والتي استخدمت للدراسة بواسطة تقنية التشخيص الزراعي والتشخيص الجزيئي لزراعة الفطريات غير المنزوعة والكشف عنها بالطرق الجزيئية لغرض التوسع في دراسة التنوع الحيوي للفطريات في التربة، بواسطة استخدام وسط السبرويد دكستروز اكار مع بعض المكملات كفيتامين (ب6) مع التخفيف الاعلى للتربة وعن طريق طبع الطبق الاصلي تم الحصول على ثلاث عزلات صغيرة جدا قطرها اقل من 1 ملم بعد فترة الحضان من 5-7 اسابيع بدرجة حرارة 28م، ولكنها لاتظهر نموا عند تكرارها على بقية الاوساط الفطرية التقليدية (مستخلص اكار البطاطا، جابيك دوكس اكار، والسبرويد دكستروز اكار) عند اختبارها، دراسة نقاوتها الزرعية، تشخيصها وكشفها بالطرق الجزيئية. استخدمت هذه الدراسة اربع مجاميع من البرايمرات النوعية لتفاعل البلمرة المتسلسل لتضخيم جين الفطريات r RNA و المتضمن منطقة (ITS) . وتم مقارنة نتائج ثلاث عزلات فطرية ومطابقتها من خلال التحليل التطوري للعزلات على BLASTN ومطابقتها في قاعدة البيانات NCBI وفق المصادر المحفوظة في بنك الجينات Gen bank ، اذ اظهرت جميع العزلات انها تعود الى الانواع الفطرية *Fusarium sp*, *Penicillium sp* and Uncultured soil fungus clone على التوالي ، وهذه النتائج ادت بنا الى النظر في هذه العزلات واعتبارها فطريات قابلة للحياة لكن غير منزوعة (viable but non-culturable)، مشابهه للظاهرة الشائعة التي وجدت في بعض انواع البكتيريا .