ISOLATION AND MOLECULAR DETECTION OF ARTHROBACTER SPECIES GROWN ON THE SURFACE OF DATE PALM TISSUE CULTURE MEDIA

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ABSTRACT

This study was aimed to found out the type of bacterial species grown on the surface of date palm tissue culture media. Shoot tips of date palm (Barhi cv.) were cultured on MS media supplemented with various combinations of hormones. During growth an infection was appeared in all cultures with gray colour. Primarily identification, proved a bacterial infection due to its colour and appearance. The genus *Arthrobacter* was isolated Antibiotic sensitivity test showed that the isolate was sensitive to ciprofloxacin,Trimethoprim, and Amikacin and resistant to Clarithromycin,Ceftriaxone and Gentamycin. Sequence analysis of 16 rRNA indicated a new isolates were closely related to the *Glutamicibacter arilaitensis* strain ebst40 and *Arthrobacter arilaitensis* strain L11 with the highest sequence similarity (100%) .

Keywords: shoot tips, *Glutamicibacter arilaitensis* ,polymerase chain reaction, hormons, antibiotic sensitivity.

العزل والتشخيص الجزيئي لبكتريا Arthrobacter النامية على سطح الوسط الزرعي لنسيج نخلة التمر

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المستخلص

زرعت اطراف الافرع لفسائل البرحي في وسط موراشيح وسكوج المجهزبتوليفات مختلفة من الهرمونات . اثناء فترة النمو ظهرت هناك اصابات في المزارع ذات لون رمادي. كان التشخيص الأولي لها على انها اصابات بكتيرية من خلال اللون والمظهر. وقد تم عزل الجنس ارثروباكتر. كما أظهر فحص الحساسية للمضادات الحيوية بانها حساسة للسايبروفلوكساسين والميثبريم والاميكاسين في حين اظهرت مقاومة للجنتامايسين والسيفترازون والكلارثرومايسين . اظهر تحليل التسلسل الجيني 16r-RNA ان العزلة جديدة ومشابهة جدا لعزلة *Glutamicibacter arilaitensis* و *Arthrobacter arilaitensis* بنسبة تشابه تصل الى 100%.

كلمات مفتاحية : اطراف الأفرع للنخيل, تفاعل انزيم البلمرة المتسلسل ,هرمونات , *Glutamicibacter arilaitensis* ,

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INTRODUCTION

In date palm tissue culture, shoot tips cultures exhibit two kinds of contaminants. External contaminants which can be removed by surface sterilization, while internal one (existed in the explants) is hard to eliminate and appear during the course of growth *in vitro*. The genus Arthrobacter, was defined by Conn and Dimmick (6) and emended by Koch *et a*l. (16), belongs to the class Actinobacteria. The genus Arthrobacter comprises of 70 species with validly published names, species of the genus Arthrobacter have been isolated from a variety of environmental sources including soil, air, water, oil brine, plants, biofilms, cyanobacterial mats, sediment, poultry litter, cheese, human clinical specimens and animal specimens (5,11.25). Soil systems contain the greatest diversity of microorganisms on earth, with 5,000–10,000 species of microorganism per gram of soil. *Arthrobacter* spp. strains have a primitive life cycle and are among the most frequently isolated, indigenous soil bacteria, found in common and deep subsurface soils, arctic ice (24). All species in this genus are gram-positive, obligate aerobes , rod shape during exponential growth and members of the genus Arthrobacter are catalase-positive, a sporforming bacteria that display a coryneform morphology and cocci in their stationary phase (15). They have a distinctive cell division called "snapping division" or reversion in which the outer bacterial cell wall ruptures at a joint and can be grown on mineral salts pyridone broth, where colonies have a greenish metallic center when incubated at 20 °C (4). The Immigration of bacteria from soil represents one of the mechanisms to explain the presence of *Arthrobacter* on leaf and stem surfaces of plants (20). Soil particles are common on foliage of plants that are grown outdoors, wind and rain splatter may deliver soil particles to leaf surfaces, especially if leaves are close to the soil surface (21). In a study that compared bacterial diversity of the lettuce phyllosphere to soil in which these plants were grown, it was revealed that many bacterial species were common between the two compartments (26). This was taken as indirect evidence for the movement of soil bacteria to the lettuce canopy, the transport of bacteria by soil particles across larger spatial scales has also been documented (12).

MATERIALS AND METHODS

Tissue culture work

Offshoots of date palm (Barhi cv.) were detached from the mother tree and brought to the laboratory for dissecting. Each offshoot was dissected and leaves were removed acropetally. Shoot tips (explants) were removed from the shoot terminals after all mature leaves were pealed away. All shoot tips then were stored in cold anti-oxidant solution (100mg/l ascorbic acid and 150mg/l citric acid) in a refrigerator until surface sterilization. Under a laminar air flow cabinet. Tips were surface sterilized with Mercury Chlorid 0.1%($HgCl_2$) and few drops of Tween 20 for 15 minutes. Then rinsed three times in double steriled distilled water. Explants were removed and transferred aseptically to sterile petri dish. After that explants were cultured in jars containing MS medium (22), except hormone which were modified. Cultures were incubated under specific conditions for each stage. In the multiplication and rooting stages, gray colour appeared (Fig.1), causing explants death (Fig.2).

Isolation and identification

Twenty samples of palm tree tissue culture were brought to the department of microbiology in Veterinary Medicine College, University of Baghdad to investigate the bacterial infection which caused the death of cultured explants. Bacterial species were isolated from the agar surface after incubation at 37ºC for 48 h using trypticase soya agar. Identification was done by biochemical tests such as Catalase, Gelatin Hydrolysis, Citrate utilization, motility, starch haemolysis test, Indole production and Urease test.

Figure 1. Apperance of bacterial infection with gray colour

Figure 2. Bacterial infection led to the death of the plantlet

Antibiotic sensitivity test

Antibiotic sensitivity test was performed using a disc diffusion method on Mueller-Hinton Agar according to Bauer-Kirby (1). The discs of antibiotic which were used Amikacin 30mg , amoxacillin 20mg, Clavulanic acid 10mg , Ciprofloxacin 5mg, Trimethoprim sulfamethoxazole 10mg, Clarithromycin 15mg , ceftriaxone30mg and Gentamycin 10mg. Colonies of suspected bacterial isolate were transfer from pure culture, to Muller-Hinton agar. The whole surface of the agar was covered by streaking. The antibiotic discs placed on the surface of the medium by using sterilized forceps pressed firmly to ensure contact with the agar, then the plates inverted and incubated aerobically at 37˚C for 24 hours. Recorded as inhibition zones were measured by millimeter (mm) using calipers, and the isolates were interpreted either susceptible, intermediate or resistant to a particular antibiotic (2).

DNA extraction

Genomic DNA was extracted using awizard genomic DNA extraction kit supplied by Promega used as follows: Strains were grown at 37˚C for 24h in a flask containing 500 ml BHI broth. Cultures were harvested at 8000 g for 10 min and washed twice with 50 mM Tris/HCl (pH 8). 15–50 ml (depending on the pellet size) of a solution containing Tris/ HCl (50 mM), pH 8, sucrose (100 mM) and 0.5% (v/v) Triton X-100 was added, mixed and incubated overnight at 37˚C. Subsequently, 250 mg of lysozyme and 375µl mutanolysin (6µ/ml) were added, mixed and incubated for 1 h at 37C˚. Thereafter, 3.75 ml proteinase K (20 mg/ ml) and 3.7 ml 10% Sarkosyl were added, mixed and incubated for 24 h at 37˚C. Finally, 3.75µl 25% SDS was added, mixed and incubated for 1 h at 55˚C.DNA was purified according to (3).

Amplification of 16S rRNA gene

Amplification of 16S rRNA gene was performed using GoTaq® Green Master Mix (Promega, USA) according to the manufacturer's recommendations. The synthesized primers were used for amplification. Temperature–time profile of PCR was as follows:

Amplification was performed using chromosomal DNA as a template and oligonucleotides Ribo-For (5′ AGTTTGATCCTGGCTCAG-3′; and Ribo-Rev (5′ CCTACGTATTACCGCGGC-3′. Those two oligonucleotides were designed to amplify a 540 bp DNA fragmend., (15). The nucleotide sequences were used for the analysis of sequence similarity through Blast (https://blast.ncbi.nlm.nih. gov/Blast.cgi). The percentage differences of the resultant partial 16S rRNA gene sequences among different species in the same group/genus of species were described previously (26). Thirty cycles at 95 °C for 0.5 min, 65 °C for 2 min and 72 °C for 2 min, all steps at maximal ramp rate (approximately 3 °C s–1 in heating mode and 1.5 °C s–1 in cooling mode).Analysis of PCR products was performed using electrophoresis in 2% gel, containing ethidium bromide. A

marker contains DNA fragments of known size were used to know the expected product size. Then gel was visualized under ultraviolet (UV) light.

RESULTS AND DISUSSION

Isolation and identification of *Arthrobacter*:
Tissues were cultured in nutrient agar in septic jar and incubated for seven months at 25˚C (re cultures were included). During the incubation period it was noticed that the color of the medium turned from greenish to yellowish and then death of all plantlets. Twenty samples from the surface of infected media were cultured and incubated over night. The results gave one isolate, it was positive to gram-stain, aerobic, none motile, rod-shaped, spore forming. The bacterial isolated from agar found to exhibit many characteristics of the genus *Arthrobacter*. Biochemical tests were done to complete diagnosis of the *Arthrobacter* isolate, the results are shown in (Table 1).

Table 1. Biochemical test for Arthrobacter isolate

Biochemical tests	results
Catalase	+ve
Gelatinase	+ve
Citrate utilization	+ve
Motility	+ve
Spore formation	+ve
Starch hydrolysis	+ve
Oxidase	Variable
Indole	-ve
Urase	-ve
Pigment	-ve
Rod shape	+ve

Antibiotic sensitivity test

The pattern of antibiotic sensitivity to *Arthrobacter* species were determined using disc diffusion method according to the guidelines recommended by the National Committee for Clinical Laboratory Standards as shown in (Table 2).

Table 2. Antibiotic sensitivity test of Arthrobacter isolate

Antibiotic (µg)	Susceptibility Results
Amoxacillin 20 +Clavulanic acid 10mcg(AMC30)	sensitive (++)
Amikacin (AK30)	Sensitive (+++)
Trimethoprim+sulfamethoxazole (SXT25)	sensitive (++++)
Ciprofloxacin (CIP5)	Sensitive(++++)
Clarithromycin (CLR 15)	Resistant
Ceftriaxone (CRO30)	Resistant
Gentamycin (CN10)	Resistant

The results in Table- 2 shows exhibits the antimicrobial susceptibility patterns of the isolate studied, the markedly better sensitive were Ciprofloxacin Trimethoprim+ sulfamethoxazole then Amikacin and Amoxacillin 20 +Clavulanic acid 10mcg. While the bacterial isolates were resistant to Clarithromycin, Ceftriaxone and Gentamycin.

Amplification of 16S rRNA gene and DNA sequences

Based on 16S rRNA gene sequence similarity for this isolate was related to species of the genus *Arthrobacter arilaitensis* strain L11(100% similarity), *Glutamicibacter arilaitensis* ebst40(100%), *Arthrobacter nicotianae* strain (100%), and *Glutamicibacter nicotianae* (100 %) as shown in Table- 3) according to the descriptions sequences producing significant alignments from NCBI Blast:9-16srRNA

Table 3 . 16s rRNA gene sequences producing significant alignments (NCBI: 9_16sr RNA-R)

Description	Max	Total	Query	E	Ident	Accssion
Arthrobacter arilaitensis strain L11 16S ribosomal RNAgene partial sequence	793	793	100%	0.0	100%	KT834847.1
Arthrobacter sp.RMR 28 16S ribosomal RNA gene partial sequences	793	793	100%	0.0	100%	KT387998.1
Arthrobacter sp.ARUP UnID 131 16S ribosomal RNA gene partial sequences	793	793	100%	0.0	100%	JQ259327.1
Arthrobacter sp. VTT E-073079 16S ribosomal RNA gene partial sequences	793	793	100%	0.0	100%	EU438937.1
Arthrobacter sp.EP_S_54 16S ribosomal RNA gene partial sequences	793	793	100%	0.0	100%	KJ642536.1
Arthrobacter sp.BF-2-2 16S ribosomal RNA gene partial sequences	793	793	100%	0.0	100%	EU668003.1
Arthrobacter nicotianae strain .184 B 16S ribosomal RNA gene partial sequences	787	787	100%	0.0	99%	KF254746.1

The genus *Arthrobacter* was defined by Conn and Dimmik, (6) and belongs to the class *Actinobacteria*. The bacterial genera that show up frequently in culture-independent surveys is *Arthrobacter* (high %GC gram-positive, family Micrococcaceae, order Actinomycetales, phylum Actinobacteria (16). Members of this genus *Arthrobacter* are gram-positive, catalase-positive, aerobic and asporogenous bacteria that display a coryneform morphology (15). This genus is phenotypically heterogeneous and over 35 species are currently recognized (7, 23). The phenotypic identification of *Arthrobacter* spp. is made particularly difficult by the fact that the description of almost every defined *Arthrobacter* species is based on a single strain (15), therefore, it might be necessary at present to supplement the identification of *Arthrobacter* spp. by peptidoglycan and/or molecular genetics methods, although the acknowledge these methods are reserved for the reference laboratories. It is emphasized that members of the genus *Brevibacterium* may be readily confused with *Arthrobacter* species. Strains of both genera show a marked rod-coccus cycle when grown on suitable medium (14). However, gram stains *Arthrobacter* strains may partially present as jointed rods which are not seen in true *Brevibacterium* strains. In our experience jointed rods are rather rarely seen in clinical *Arthrobacter* isolates cultured on SBA. *Arthrobacter* strains may be motile, whereas *Brevibacterium* strains are invariably nonmotile. Many *Brevibacterium* Strains display a typical cheese-like odor which is not noted for *Arthrobacter* strains (9). *Arthrobacter arilaitensis* is one of the major microorganisms found in plant tissue cultures, soil and at the surface of cheeses, remarkably in smear-ripened cheeses, where it is assumed to be responsible for yellow pigmentation of the cheese's rind because of its characteristic overall color and its involvement at the different stages of cheese ripening (8,10,19). The *Arthrobacter* strains that were isolated from agar of plant tissue culture were spores originally recovered from soil ground and when there found a sutiable inviroment (20). It is evident from the phenotypic and molecular genetic data that this strain is representative to the three new *Arthrobacter* species. Analysis of the 16S rRNA gene of this isolate sequence (1482 bp) indicated that it belongs to the genus *Arthrobacter* according to the EzTaxon-e server.The most closely related strains to the strain *Glutamicibacter arilaitensis* ebst40 (100% similarity) were *Arthrobacter arilaitensis* strain L11 (100% similarity to Jensen,(13), *Arthrobacter nicotianae* strain 184B (99% similarity to Kotouckova *et al*, (18). The phylogenetic analysis demonstrated close relationships between this isolate and the members of rRNA cluster 2 (including *Arthrobacter arilaitensis*, *Arthrobacter nicotianae*, and *Glutamicibacter arilaitensis*) (17). Therefore, it might be

necessary at present to supplement the identification of *Arthrobacter* spp. by peptidoglycan and/or molecular genetics methods, although we acknowledge that these methods are reserved for the reference laboratories. It is concluded that the *Arthrobacter* strains isolated from date palm tissue culture were spores originally recovered from soil ground and thrived on the surface of the medium.

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