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BIOLOGICAL CONTROL OF MICROBIAL CONTAMINATION ASSOCIATED WITH PLANT TISSUE CULTURE OF DATE PALM (*PHOENIX DACTYLIFERA*)

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ABSTRACT

Biotechnology in the agriculture field provides tremendous potential for producing crops with lower costs and increased productivity. This is a very essential choice for introducing the best tools of biotechnology to overcome the challenges in this field.In this studyeight bacteria isolate from the contaminated tissue culture of date palm, all isolates were genetically identified based on the 16SrRNA gene. The bacterial strains were *Enterococcus faecium* strain MK748256 (IW21), *Bacillus cereus* strain B26 (IW22), *Bacillus* sp. strain PK-7 (IW24), *Bacillus cereus* strain JS22 (IW25), *Lysinibacillus* sp. Strain IADCASC12 (IW28), *Lysinibacillus* sp. strain IADCASC11 (IW29), *Bacillus thuringiensis* strain Sol 1 (IW31) and *Bacillus cereus* strain Q1 (IW32).In the current study, the genus *Lysinibacillus* was isolated for the first time from a culture of plant tissues contaminated with microbes.The isolated bacteria were tested with four different types of antibiotics. The results showed all bacterial isolates were sensitive to Amikacin, the highest inhibition zone was 21mm against IW31 *Bacillus thuringiensis* while the lowest was 12mmagainst ýIW22 *Bacillus ýcereus* strain B26, also all isolates were resistant to Ampicillin.Antibacterial assay of extracts amber wasfound the alcoholic extract of orange amber and the DMSO extract of yellow amber best antimicrobial activity against *Enterococcus faecium* strain MK748256, *Bacillus cereus* strain B26 and *Bacillus* sp. strain PK-7.The yellow amber extract showed wide effectiveness against seven out of eight strains, the highest inhibition value was 20 mm and the lowest was 8 mm, while the orange amber extract was less effective against four strains, the highest inhibition was 15 mm and the lowest was 10 mm. Sterilization of the excised plant parts and the control of microbes is an important stage in the success of plant tissue culture. This study demonstrated the possibility of using natural extracts to reduce bacterial contamination without harming cultured plant tissues.

KEY WORDS : Microbial contamination, Micropropagation, Date palm, Tissue culture sterilizing agents

INTRODUCTION

The date palm (*Phoenix dactylifera L.*) is a fruit tree that predominatesin hot arid regions of the Middle East and North Africa and is resilient to adverse climatic conditions (Al-Khayri and Naik, 2017). This species of the Arecaceae family is propagated by seed, which is known as sexual reproduction in addition to many other methods of reproduction. Propagation by seed is one of the oldest methods; the fruits produced are of poor quality and have high contrast. The second method of reproduction is vegetative or asexual reproduction by branches known as offshoots. The resulting palm tree is identical to the original parent species, but this method has some disadvantages, such as the limitation of offshoots that are produced between the first ten and fifteen years after planting (Jain, 2012). As a date producer, Iraq is among the top ten of the world's major date producing zones. Several commercial and government institutions are working on the propagation of date palm cultivars for tissue culture (Abass and Awad, 2019). The date palm is one of the palm family's most significant

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economic species, grown primarily for its fruits (dates). The market for date palm fruits around the world is growing nowadays. To satisfy this demand, many methods of propagation have been used, including micropropagation that has been used for large-scale date palm multiplication in Iraq and many other nations to multiplication of Date palm in large-scale (Abass, 2013). In recent years, the technology of micropropagation has expanded in date palm cultivated, due to not controlled to seasonal influences and produces healthy varieties free from diseases and identical to the mother plant in genetic characteristics, as well as producing large numbers of offshoots ina short time (Kriaa *et al*., 2011). The early stages of the initiation of culture are the most important and face challenges in micropropagation. The risk of this stage is microbial contamination (Daud *et al*., 2012; Vujovic *et al*., 2012). Infection or microbial contamination, both endogenous and exogenous, is one of the most common risks that reduce the chances of success of in vitro date palm micropropagation techniques (Emoghene *et al*., 2020; El-DawayatiMaiada *et al*., 2020). Microbial contamination causes a lot of harmful effects on the tissues of cultivated date palms, including the consumption of foodstuffs by microbes and the competition of plants for them, the internal and external enzymes and toxins resulting from the effectiveness of microbes into the medium, whether bacteria or fungi, lead to damage to the affected tissue and change in color. Resulting from these economic losses and wasted time and effort(Hameed and Abass, 2006; Omamor *et al*., 2007). Contamination of date palm tissues with fungi, bacteria, yeast and viruses is one of the most difficult that impedes the reproduction process, which can infect cultures at every micropropagation stage (Odutayo *et al*., 2007; Abass, 2013). Plant tissue cultures may be infected by significant bacterial diversity which leads to contamination, which can decrease shoot and root growth rate, multiplication factor, and sometimes cause plant death. (Kidus and Teka, 2020). The bacterial contamination of date palms may result from plant materials, laboratory environment, tools used, or inefficient sterilization stages, all of this leads to increased mortality and decreased success rates, especially when the infection or contamination is severe with internal bacteria (Mbah and Wakil, 2012). Several species of bacteria were isolated from a culture of contaminated date palm tissue and identified by biochemical tests or by genetic identification depended on 16Sr RNA. The most commonly explored endogenous Gram-positive and Gram-negative bacterial species in date palm tissues are the genus *Bacillus*. It is a predominant group that includes *B. subtilis, B. safensis, B. cereus* and *B. sonorensis*, other species belonging to the Enterobacteriaceae family, some isolates of *Achromobacter*, *Acinetobacter*, *Serratia*and *Pseudomonas*. (Al-Hadethy *et al*., 2007; Al-Mussawi 2010; Al-Dosary*et al*., 2011).The basic procedures in plant tissue culture are sterilization for controlling microbial contamination by using physical, chemical, and filtration methods (Singh *et al*., 2015). The culture media should be sterilized for 15-20 minutes at 121 °C by an autoclave, tools are washing with sodium hypochlorite 5% for 15 minutes (Ikenganyia *et al*., 2017). Disinfecting of glassware in the oven at 180 °C for 2 hours, as well as using flame and methyl alcohol 99% in addition to sterilization of lamina flow table and hands with 70% alcohol (Leelavathy and Sankar 2016).Many compounds are used to control microbial contamination associated with tissue culture, including antibiotics, chemicals such as hydrogen peroxide, and plant extracts. The aim of this study wasthe isolation and identification of bacterial contaminants of ý date palm in *vitro* cultures and to evaluate the efficacy of antibacterial and amber extract ýagents identified bacteria.

MATERIALS AND METHODS

Isolation of bacteria

Contamination of palm tissue culture inPalm Research Center at Basrah university is shown in Figure 1. The contaminating bacteria were isolated ona nutrient agar medium by placing a small amount of sample on the side of the agar plate with inoculum from an inoculating loop. A sterile loop is then used to spread the bacteria out in one direction from the initial site of inoculation. This is done by moving the loop from side to side, passing through the initial site. The loop is then sterilized (by flaming) again and the first streaks are then spread out themselves, this is repeated 2-3 times (Collins and Lyne, 1984). In this method single bacterial cells get isolated by the streaking method. After incubation at 35°C±2 for 24 hours, colonies were observed and pure cultures of these bacteria were obtained by picking up the colonies and streaking them onto the fresh medium. Bacterial isolates were characterized morphologically after staining with Gram stain to distinguish Gram-positive, Gram-

Fig. 1. The appearance of an advanced stage of bacterial contamination associated with the cultivation of date palm tissue, the different color represents the aggravation of bacterial growth.

negative bacteriaand spores forming cells.

DNA extraction and PCR amplification

Genomic DNA was extracted from 12 isolates using Promega, USA extraction kit according to the manufacture's protocol. Extracted DNAwas detected by 1% agarose gel electrophoresis. The bacterial 16SrDNA was amplified using the universal bacteria-specific primers, 27 F (52-AGAGTTTGATCCTGGCTCAG-32) and 1492R (5' CGGCTACCTTGTTACGACTT-3') (Lane, 1991). The PCR reactingsystem consists of green mastermix 25 µl mixed with 2 µl of each forward and reverse primers (10 pmol/ µl), 5 µl of template DNA and equal volume to 50 µl by added nuclease-free water. The thermal cycling conditions were as follows: initial denaturation for 2 min at 94 °C, 35 cycles of 40 sec. of denaturation at 94 °C, 30 sec. of annealing at 55°C, and 1 min of elongation at 72 °C. Cycling was completed by a final elongation step at 72 °C for 10 min. Amplified fragments were approximately 1,500 bp (Liu *et al*. 2007). Gene products (16S rRNA) about ý1500 bpý were detected by electrophoresis 1.5% agarose with TBE buffer in conjunction with DNA ladder (100 base pair) from Thermo Fisher Scientificý Company, USA.

A sequence of PCR products

ýthe 16S rDNA genes products from Amplified DNA were sequencedýýat Macrogen company laboratories /Korea. 16S rDNA of each isolate was purified and sequencing then aligned with known 16S rDNA sequences Gen bank using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information ý(http//www.ncbi.nlm.nih.gov/BLAST) (Katoh *et al*., 2002).

Preparation of DMSO and alcohol extraction of amber

Three types of amber were used in the present study. The necklaces of orange, brown and yellow Baltic amber were obtained from the local market. after Amber crushed by using a ceramic mortar, dissolved in Quantity information of Dimethyl Sulfoxide (DMSO) to obtain the required concentrations of 250 mg/ml. five grams of orang amber powder was dissolved in 100 ml of ethanol to prepare alcohol extract, the mixtureleft for 24 hr. with continuous stirring on the magnetic stirrer, then the solution filtered and dried in a Petri dish at room temperature. The dry orange amber was collected as a powder, a certain weight dissolves with DMSO to obtain the required concentrations of 250 mg/ml.

Determination of antibiotic-resistant and amber activity

Susceptibility of 12 isolatesto 5 types of antibiotics was achieved by the disc diffusion method (Van Dijký *et al*., 2014). Commercially available antibiotics disc (Bioanalysis company, Turkey) were used containing Nitro furantoin 300 µg, Chloramphenicol 30µg, Ampicillin 10µg, Norfloxacin 10 µg and Amikacin10 µg.0.1 ml inoculum of bacteria was added to 100 ml of nutrient broth and then incubated at 30 °C for 24h and then dilution of bacterial solution withphysiological normal saline compared with the standard test tube McFarland for 10^8 cells/ml of stuck bacterial and inoculated into nutrient agar, using L-shape to spread bacteria on Muller Hinton Agarmedia, put the antibiotic disc and incubated dishes in the incubator 37 °C for 24 h. Inhibition zone diameters were measured inclusive of the diameter of the discs. The Results were expressed as sensitive, S (≥ 21 mm); intermediate, I (16-20 mm) andresistant, R (≤ 15 mm), respectively according to that described by Vlková *et al*., (2006). Alcohol and DMSO extractsof amber efficacy were tested for antimicrobial activity against isolates were achieved on Mueller Hinton agar medium by using the diffusion method (Gandhimathi *et al*., 2008). The plates were incubated at 37 °C for 24 h. the diameters of the inhibition zones were measured.

RESULTS

Isolation and genetic identification

The results of bacterial isolation from tissue culture

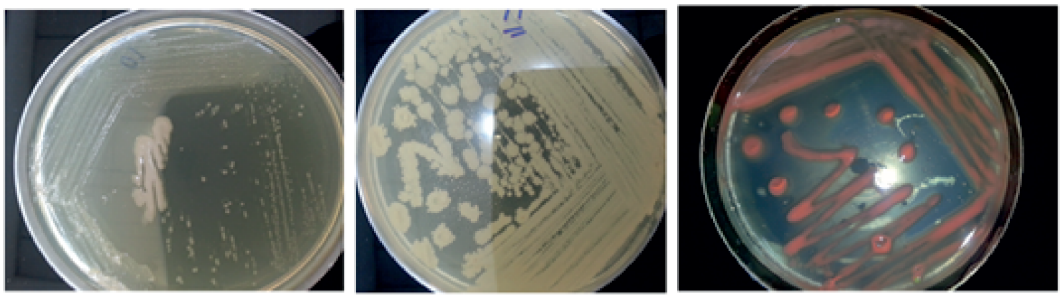

Fig. 2. Colony grown of some bacterial isolates on nutrient agar medium after 24 hr. of incubation at ý3ýþ5þ ý°C±2ý.

of (*Phoenix dactylifera*) date palm on ýnutrient ager during isolation technique were showed that most isolates were detected as ýGram positive, while only one isolates was Gram negative ýFigure 2.

The genetic identification of bacterial isolates was showed that 8 strains identified according to the partial sequence obtained from their 16S rRNA gene, after compared the sequences with those described in databases the partial sequence of 16S rRNA genes. The results showed that 5 strains belong to *Bacillus* genus, 2 isolates identified as *Lysinbacillus* while one strain was *Enterococcus* as described in Table 1.

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Antibiotic sensitivity and amber activity

Results of the sensitivity studies of the isolatestested against 5 types of antimicrobialagents are shown in Table 2 and Figure 3. All isolates were sensitive to Amikacin**,** the highest inhibition zone was 21 against isolate IW31 *Bacillus thuringiensis* while the lowest was 12 of isolate IW22 *Bacillus cereus* strain B26. In addition, all isolates were also resistant toAmpicillin, some strains include *Bacillus cereus* strain B26, *Bacillus cereus* strain JS22 and *Bacillus thuringiensis* strain Sol 1 were highly sensitive against Norfloxacin, the inhibition zone 31, 27 and 23 respectively. other isolates showed different susceptibilities to the following antibiotics

Table 1. The BLAST results of the16SrRNA gene sequences of the bacteria contaminated plant tissue cultures

Isolates code	Identified strains	Accession number	Length pb
IW21	*Enterococcus faecium* strain MK748256	MW078406.1	510
IW 22	*Bacillus cereus* strain B26	JN624919.1	515
IW 24	*Bacillus* sp. strain PK-7	EU685824.1	536
IW 25	*Bacillus cereus* strain JS22	MT102926.1	940
IW 28	L*ysinibacillus* sp. Strain IADCASC12	MH973472.1	504
IW 29	*Lysinibacillus* sp. strain IADCASC11	MH973471.1	515
IW 31	*Bacillus thuringiensis* strain Sol 1	KF812554.1	513
IW 32	*Bacillus cereus* strain Q1	KU977289.1	676

Table 2. Assessment of bacterial strains against 5 types of antibiotics

Sample	Nitrofurantoin 300 µg	Chloramphenicol 30µg	Ampicillin 10 µg	Norfloxacin 10 µg	Amikacin 10µg(s)
	Inhibition zone (mm)				
Control	≤14	≥18	≥ 15	≥ 17	e" 17
IW21	7(R)	0(R)	0(R)	0(R)	17(S)
IW22	13(R)	16(I)	0(R)	31(S)	12(R)
IW24	0(R)	0(R)	0(R)	0(R)	17(S)
IW25	0(R)	0(R)	0(R)	27(S)	15(I)
IW28	15(I)	0(R)	0(R)	0(R)	19(S)
IW29	0(R)	0(R)	0(R)	0(R)	17(S)
IW31	19(S)	0(R)	0(R)	23(S)	21 (S)
IW32	15(I)	25(S)	0(R)	0(R)	18 (S)

R: Resent, S:Sensitinve, I:Intermidite.

Fig. 3. Antibiotics testes for bacterial isolates from tissue culture of date palm

Nitrofurantoin, Chloramphenicol and Norfloxacin.

The result of antibacterial activity of extracts amber was shown in Table 3 and Figure 4. According to the data included in Table 2, it was found that the alcoholic extract of orange amber and the DMSO extract of yellow amber showed the best antimicrobial activity. The maximum value of bacterial inhibition was 20 mm from both extracts against isolate *Bacillus* sp. strain PK-7. The values varied between 8 to 12 mm for the alcoholic extract of orange amber and 10 to 16 mm for the DMSO extract of yellow amber towards most of the bacterial isolates. Whereas, the DMSO extract of brown amber showed moderate efficacy towards four bacterial isolates, ranging from10 to 12 mm. While the lowest efficacy showed by the DMSO extract of orange amber reached 12 and 15 mm only against *Bacillus cereus* strain B26 and *Bacillus* sp. strain PK-7, respectively.

Table 3. Antimicrobial activity of DMSO and alcohol extract of amber against bacterial strain

Isolates	DMSO extract of amber		Alcohol extract	
	Orange	Brown	Yellow	Orange amber
WI 21	0	11	16	12
WI 22	12	10	15	10
WI 24	15	12	20	20
WI 25	0	0	15	0
WI 28	0	0	0	0
WI 29	0	10	0	8
WI 31	0	0	10	0
WI 32	0	0	15	8

DISCUSSION

From the results in Figure 1 the striking plating is a technique used to isolate pure isolates from a single colony of bacteria. the colonies can be grown on a new plate so that the bacteria can be identified, studied, or tested, rapid and ideally by a simple process of dilution methods. The technique is done by diluting a comparatively large concentration of bacteria to a smaller concentration. The decrease of bacteria should show that are sufficiently spread apart to affect the separation of the different types of bacteria. The genetic identification showed that different species of bacteria were isolated from tissue culture after cultivated on an agar medium. The most common species were the *Bacillus*. This genus of Gram-positive bacteria possesses the ability to withstand extreme environmental conditions by producing strong, protective endospores (Nakanand Zuber, 1998). ýTherefore, it is found in different environments such as soil, air, and human. *Bacillus* is one of the most common bacterial species that was isolated from a date palm culture contaminated with microbes(Al-Dosary *et al*. 2011). While *Lysinibacillus* sp. is rare in plant tissue, they may be isolated for the first time in this study from contaminated date palm tissues. There is no study currently indicating the isolation of species of this genus from such environments. In a study by Ndiaye*et al*. (2019), three novel bacterial species were isolated from human skin, one of these was *Lysinibacillus timonensis*.

The majority of bacterial species that have been

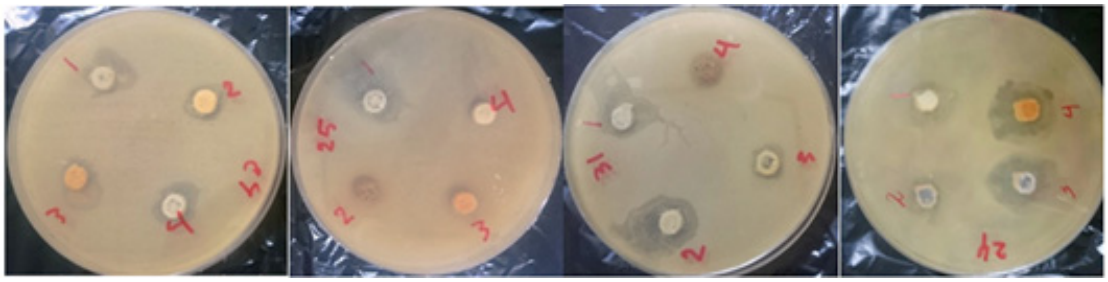

Fig. 4. Antimicrobial efficacy of different amber extract against bacterial strains

contaminated withdate palm due to Dates are a good source of energy and rich in nutrients. Date fruits consist of 70% carbohydrates; mostly as sugars, and 15-30% water. Dates are also a source of different mineralsincluding iron, potassium and calcium, with low levels of sodium and fat (Abass, 2013). Our antibiotic sensitivity testing revealed Amikacinas the most effective antibiotic against the contaminating bacteria Table 1 and Figure 2 inhibited the bacterial growth due to most bacteria produce a cell wall that is composed partly of a macromolecule called peptidoglycan, itself made up of amino sugars and short peptides. Amikacinprevents the final cross-linking step, or transpeptidation, in the assembly of this macromolecule and inhibits bacterial growth by stopping protein synthesis through binding to the 30S subunit of bacterial ribosome (Ramirez *et al*., 2017). Both bacteria and humans carry out protein synthesis on structures called ribosomes. Another antibiotic such as Norfloxacin accumulated in high concentrations in the cytoplasm after crossing ýthrough membranes of bacteria.ýThe target enzymes of these antibiotics are DNAgyrase and topoisomerase IV for inhibiting replication of DNA and other function. Norfloxacinis a broad spectrum used against both G+ve and G-ve to treat bacterial infection (Defant *et al*., 2019).

In plant tissue culture research and applications, bacterial contamination represents a ýserious problem. if the presence of bacteria is ignored bacterial interference with normal ýplant physiology and morphology generating misleading conclusions (Moreno-Vázquez *et al*., 2014). To eliminate bacterial contaminants serious attempts ýhave been performed to support sterile procedures to reduce the microbial contamination of tissue culture, including controlling environmental ýand nutritional factors and treatment with antibiotics (Abass, 2013).

During the past decades, antibiotic-resistant bacteria have proliferated widely and at alarming rates due to a combination of different factors. There are strenuous efforts by scientists to find new materials to control intrinsic and acquired drug resistance, including natural active products. The continued use of antibiotics in the medium or repeated treatments with a single antibiotic may lead to bacterial resistance (Kneifel and Leonhardt. 1992). Also,antibiotics have been shown to restrict rooting, general growth and multiplication in plant cultures (Leifert *et al*., 1994). antibiotics may be harmful to plants because of their phytotoxicity. So in our study, we used a Baltic amber extract as an antibacterial, amber has no toxicity because it's a natural product the results showed that three types of amber has activity against isolates of bacteria that contaminated plant tissue culture. Many compounds present in Baltic amber show antimicrobial properties against bacteria, fungi and viruses (Tumi³owicz*et al*., 2016).

Baltic Amber is a resin that contains several natural organic biomolecules and can be used against microbial infections, and protects the tree from disease and injury infected by insects and fungi (Langenheim, 1969). The results in the present study are compatible with the study of Carpenter *et al*. (2012) who showed that some compounds of amber such asabietic acid, á-pinene and camphene are effective antibacterial agents. Also, the results agreed with Tumi³owicz1 *et al*. (2016) that showed Baltic amber has potentýantibacterial and antifungal properties against pathogenic ýmicroorganisms that existing on human skin.Al- Tamimi *et al*. (2016) reported the potential activity of Baltic amber on clinical ýbacteria so it's can be used as an alternative chemotherapeutic agent. Since alcohol ýextract of orang amber was more effective than DMSO extract, our study recommends the use of amber alcohol extracts to control bacteria associated with plant tissue culture.

CONCLUSION

Controlling microbial contamination is one of the most important priorities for plant tissue culture. In this study, different sterilization methods are chosen in terms of efficiency and safety. Some bacterial species associated with date palm plant tissue contamination were isolated, identified andtested for sensitivityto four types of antibiotics. This study also determined the efficiency of using natural agents such as amber extracts, in controlling microbial contamination related to tissue culture.

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