



MOLECULAR DIAGNOSTICS OF *Cuscuta sp* AND ITS ASSOCIATED BACTERIA

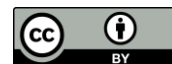
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ABSTRACT

This study was conducted at the Faculty of Agricultural Engineering Sciences/ Plant Protection Department, University of Baghdad – Jadriya, with the aim of isolating and diagnosing bacteria molecules from samples of *Cuscuta sp.* (stems and seeds) intruding on different plants, diagnosing *Cuscuta sp.* molecules and testing the effect of bacteria in the germination of *Ocimum sp.* and *Cuscuta sp.* seeds. The results of isolating and diagnosing bacteria from samples of *Cuscuta sp.* (stems and seeds) intruding on the plants *Conocarpus*, *Chenopodium* and *Cynanchum* showed the obtaining of three types of bacteria B1, B2 and B3 sequentially. The results of testing the efficiency of three types of bacteria that were isolated from the *Cuscuta sp.* (B1, B2 and B3) showed that the isolation of B2 bacteria had a significant effect in raising the germination rate of seeds of *Cuscuta sp.* and *Ocimum sp.* and did not differ significantly with the two comparative coefficients, while it differed significantly with isolates B1 and B3. The results of the nucleotide sequence analysis of the two samples showed that Sample 1 belongs to the type *Cuscuta reflexa* and was recorded in the Gene bank under the serial number (OQ746922) and was recorded for the first time in Iraq. and the results also showed that the sample 6 belongs to *Cuscuta campestris* and was registered in the Gene bank under the serial number (OQ746927). The results of the nucleotide sequence analysis of the isolates of bacteria B1, B2 and B3 showed that they belonged to *Staphylococcus aureus*, *Bacillus subtilis* and *Achromobacter xylosoxidans* sequentially and were deposited in the Gene bank under the accession numbers OQ976982, OQ976981 and OQ976979 On the relay.

Keywords. *Conocarpus*, *Chenopodium*, *Cynanchum*, *Cuscuta sp.*

التشخيص الجزيئي للحامول والبكتريا المرافقة له

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الخلاصة

أجريت هذه الدراسة في كلية علوم الهندسة الزراعية/ قسم وقاية النبات/ جامعة بغداد/ الجادرية، بهدف عزل وتشخيص البكتريا جزيئاً من عينات الحامول (سيقان وبذور) المتطفلة على نباتات المختلفة وتشخيص الحامول جزيئاً واختبار تأثير البكتريا في انبات بذور الريحان والحامول. بينت نتائج عزل وتشخيص البكتريا من عينات الحامول (سيقان وبذور) متطفلة على نباتات كينوكاربس، رغيلة والمديد الحصول على ثلاث انواع من البكتريا هي B1، B2 و B3 على التتابع. بينت نتائج اختبار كفاءة ثلاث انواع من البكتريا التي تم عزلها من الحامول (B1، B2 و B3) في تأثيرها في النسبة المئوية لانبات بذور الريحان والحامول على الوسط الزراعي Nutrient Agar ان عزلة البكتريا B2 اثرت معنوياً في رفع نسبة انبات بذور الحامول والريحان ولم تختلف معنوياً مع معاملتي المقارنة، في حين اختلفت معنوياً مع العزلتين B1 و B3. بينت نتائج تحليل التتابع النيوكلوتيدي للعينتين ان S1 تعود الى النوع *Cuscuta reflexa* وسجل



في بنك الجينات ضمن الرقم التسلسلي (OQ746922) وقد تم تسجيله لأول مرة في العراق كما بينت النتائج ان العينة S6 تعود الى *Cuscuta campestris* وسجل في بنك الجينات ضمن الرقم لتسلسلي (OQ746927). بينت نتائج تحليل التتابع النيوكليوتيدي لعزلات البكتريا B1، B2 و B3 هي عائدة للبكتريا *Staphylococcus aureus* و *Bacillus subtilis* و *Achromobacter xylosoxidans* على التتابع وأودعت في بنك الجينات تحت ارقام انضمام OQ976982، OQ976981 و OQ976979. على التتابع.

الكلمات المفتاحية: كينوكاربس، رغيلة، المديد، حامول.

INTRODUCTION

The *Ocimum sp.* plant (Osimum) belongs to the oral family (Lamiaceae) and is one of the aromatic and economic medicinal plants grown in almost most regions of the world, it is a multi-varietal plant capable of withstanding the diversity in climate (Fatope *et al.*, 2008), the economic importance of the *Ocimum sp.* plant is increasing with its widespread use in various medical fields and the preparation of medicines (Liber *et al.*, 2011; Hussein, 2021). The *Ocimum sp.* plant is exposed to a number of pathogens, including floral parasites, which damage its leaves and reduce their value marketing, from these parasites the *Cuscuta sp.*, as its threads wrap around the plants and suck out the juice from it and eventually lead to the death of the plant (Weerakoonand *et al.*, 2011; Al-Dae., 2018). *Cuscuta* dodders (*Cuscuta spp*) is a fully parasitized floral plant belonging to the Convolvulaceae family, formerly classified to the Cuscutaceae family, parasitizes on the stems and leaves of economic plants, including grasses, field crops, ornamental plants and vegetables in countries of the world, including *Ocimum sp.* Plants (Abdurrahman, 2008; El-Yazal & IHH., 2019), the parasitization process is cuscuted out by sending special pipettes called Haustorium along the host plant stems to the host plant tissues and reach the *Cuscuta sp.* vessels to obtain water and nutrients (Kim *et al.*, 2014; Yahya *et al.*, 2016), infection of the host leads to a reduction in the quantity of the product and affects its quality and causes economic losses ranging from (Agios., 2005; Elsayhookie *et al.*, 2014). There are approximately 170 species widely distributed in temperate and subtropical regions of the world (Noureen *et al.*, 2019). Due to the importance and danger of *Cuscuta sp.*, it has received the attention of many researchers, as a number of scientific studies and research have been conducted, especially with regard to chemical pesticides in combating it and reducing its spread and because of the difficulty of finding selective pesticides in combating it without harming the host plant, especially if it is an economic plant (Hoseyni *et al.*, 2018), as well as the risks of using manufactured chemical pesticides in agricultural pest control and increasing the costs of the control process and increasing resistance cases, as well as environmental damage as a result of the repeated use of these pesticides (Al-Gburi *et al.*, 2019; Hussein & Juber, 2015). Methods and Means were used in the control of pathogens, the most focused of which was to find methods Alternative methods of chemical control include the use of plant extracts (Hadithi *et al.*, 2007; Abdurrahman., 2008), and microbiology was also used in biological control programs, including fungi, as there were facilities for cuscutes, they were isolated and diagnosed, and a number of them were used as biocides (Fayad *et al.*, 1990; al-Hattar, 2003; Saleh *et al.*, 2016; Lateef *et al.*, 2022).

Due to the spread of pregnancy to multiple plant families and the economic damage it causes to families, this study included:

- Isolation of bacteria from the parasite of some plant families and their molecular diagnosis



- Testing its effect on the percentage of germination of *Ocimum sp.* and *Cuscuta sp.* seeds
- Identification of a *Cuscuta sp.* that is parasitized on some plants molecularly

MATERIALS AND METHODS

Collection of *Cuscuta sp.* samples

Samples of *Cuscuta sp.* (stems and seeds) parasitized on different plant families (*Conocarpus*, *Chenopodium*, *Cynanchum*) were collected from several locations from the provinces of Diyala, Baghdad (Table 1) and kept in polyethylene bags and written on the name of the collection area and the date of collection and the samples were transferred to the laboratory and kept in a refrigerator at a temperature of 4 M until the start of isolation.

Table (1): Spatial and temporal distribution of *Cuscuta sp.* Samples.

Host	Location	Collection date
Conocarpus	Baghdad / University of Baghdad	2021/ 5 / 21
Chenopodium	Diyala / Baqubah	2021 / 3 / 19
Cynanchum	Diyala / Khan Bani Saad	2021 / 5 / 17

Isolation of *Cuscuta sp.* associated bacteria

Samples were collected from the stems and seeds of the *Cuscuta sp.* collected from different sites (the stems were cut into small pieces 1-0.5 cm long and the pieces and seeds were sterilized superficially by immersing them in a solution of sodium hypochlorate (free chlorine) for two minutes, washed with sterile distilled water, dried with sterile filter paper of the Whatman No. 1 Type .The cuttings were sown with four pieces and five seeds per petri dish with a diameter of 9 cm containing 15-20 cm³ of sterile harvested potato Dextrose Agar agricultural medium (PDA) at a temperature of 121 m and a pressure of 1.5 kg.Cm⁻² for 15 minutes and the antibiotic Amphotericin in the amount of 200 mg is added to it.L-1 to reduce fungal contamination, the dishes were incubated in the incubator at a temperature of 28 ±2 for 48 hours, the bacteria were purified by taking from the bacterial growth with a needle with a sterile knot and placed in a petri dish containing sterile nutrient Agar (NA) culture medium, the dishes were incubated in the incubator at a temperature of 28 ±2 for two days, the Test tubes containing nutrient broth (NB) culture medium, incubated for one day and then kept in the refrigerator at a temperature of 4 meh.

The effect of the efficiency of bacteria associated *Cuscuta sp.* on the percentage of germination of seeds of *Cuscuta sp.* and *Ocmium sp.*

The efficiency of the bacteria associated the *Cuscuta sp.*, namely (B1, B2 and B3), was tested on the culture medium (NA) Nutrient Agar, which was isolated from the parasite *Cuscuta sp.* on cynocarpus, ragweed and extended, taking 1 ml of each bacterium stuck both individually and growing on the culture medium liquid Nutrient Broth (NB) and added to petri dishes diameter 9 cm container on the sterile culture medium (NA) before hardening with to distribute the vaccine homogeneously and leave the dishes to harden, the seeds of the 90% concentrated sulfuric acid treatment were sown for 3 minutes to break the dormancy phase (Zakie *et al.*, 1998) , and the *Ocimum sp.* seeds were sown after Surface sterilization with



hypochlorite solution The results of the experiment were taken 7 days after the seeds were planted by calculating the germination percentage of each of the planted dishes according to the following equation:

$$\text{Percentage of germination} = (\text{developing seed number} / \text{Total seed number}) \times 100$$

Molecular diagnostics of the *Cuscuta* sp. using the polymerase chain reaction (PCR) Technique

DNA extraction

DNA was extracted from two samples of *Cuscuta* sp. (S1 and S6) the *Cuscuta* sp. samples were cut into small pieces, ground into a fine powder, placed in sterile plastic tubes individually and kept in the freezer until the DNA was extracted and the DNA extraction process was *Cuscuta* sp. out at Jisr Al-Musaib company using a standard kit using a standard kit (Presto™ Mini gDNA Yes Kit) produced by the Korean company Geneaid .

Polymerase Chain Reaction (PCR)

The doubling process was performed using an initiator that targets the specific sequence of the interstitial region (ITS) of the ribosomal rRNA gene to perform the chain amplification reaction (PCR) of ribosomal RNA and to perform the chain amplification reaction (PCR) I used the initiator of its rRNA and is1, 5.8 S rRNA

To perform the chain amplification reaction (PCR), use the initiator ITS1 and ITS2 manufactured by the American company Integrated DNA Technology as shown in Table (2)

Table (2): The sequence of bases in the prefixes used to amplify pieces of DNA DNA.

Name initiator	Relay initiator	Tm (C)	GC (%)	Product size
ITS1 Primer Forward	5 -TCCGTAGGTGAACCTGCGG-3	56.7	48	600-650 bp
ITS2 Primer Reverse	5 -TCCTCCGCTTATTGATATGC-3	42.7	39	

Preparation of the Master Mix

The polymerase chain reaction mixture was prepared by adding all the components needed for the reaction according to the instructions of the Korean company Intron Biotechnology in PCR tubes with a final volume of 25 microliters.

Electrophoresis on agarose gel for DNA

Electrophoresis was performed to detect the result of the PCR reaction in the presence of standard DNA to characterize the packet size .According to (Sambrook *et al.*, 1989), agarose gel was prepared with a condensation of 1.5% by dissolving 2 g of agarose in 100 ml of a solution of TBE (Tris-Borate EDTA) previously made, let the agarose boil in the oven and then add 2 ml Ethidium Bromide and leave to cool at a temperature of (45 - 50 m), then pour the gel into a tray until the gel solidifies support the agarose after installing the comb to make holes that will hold the samples and carefully pour the gel so as not to make air bubbles and leave for 30 minutes to cool .The comb was gently removed from the solid agarose and the gel was immersed with a TBE emitter after solidification in the basin of the fully electric Relay



device, after which the PCR products were injected into the pits of the submerged gel plate at 5 microliters per hole as well as the presence of the standard solution Ladder and electrically traveled (70 V / H) for 1-2 hours, the DNA pieces dyed with the gel documentation device were examined under UV radiation.

Molecular diagnostics of bacterial isolates using polymerase chain reaction (PCR) technology

DNA extraction of bacterial isolates from the *Cuscuta sp.*

Molecular Diagnostics was performed for three isolates of bacteria (B1, B2 and 3B) that were isolated from the parasite on *Conocarpus*, *Chenopodium* and *Cynanchum*. The bacteria were purified to obtain pure colonies individually by taking a swab with a loop inoculation needle. The individual colonies were selected for bacterial isolates and replanted in a planned manner to ensure the purity of the isolates. Molecular Diagnostics was performed based on the 16SrRNA gene in the PCR polymerase chain reaction, using the standard G-Spin DNA Extraction Kit equipped by Intron Biotechnology/Korea.

Polymerase chain reaction (PCR)

To perform the chain amplification reaction (PCR), I used the specialized initiator Specific primer of gene 16srna manufactured by the company (Integrated DNA Technologies company, USA). As shown in Table (3)

Table (3): Initiators used in the polymerase chain reaction PCR for the identification of bacterial isolates.

Name initiator	Relay initiator	Tm (C)	GC (%)	Product size
Forward	5'- AGAGTTTGATCCTGGCTCAG- 3'	54.3	50.0	1250 – 1500 base pair
Reverse	5'- GGTTACCTTGTTACGACTT- 3'	49.4	42.1	

Preparation of the Master Mix

The polymerase chain reaction mixture was prepared by adding all the components needed for the reaction and according to the instructions of the company, intron was equipped in PCR tubes with a final volume of 25 microliters.

After completing the preparation of the mixture, the tubes were closed and the contents of the mixture were mixed with the Vortex device for 10 seconds and the tubes were transferred to the PCR Thermocycler Conditions and the DNA chain amplification reaction of the samples was *Cuscuta sp.* out according to the program shown in Table (4).

Table (4): Polymerization program for amplifying DNA pieces of bacterial isolates.

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	94°C	3 min.	1 cycle
2-	Denaturation -2	94°C	45sec	35 cycle
3-	Annealing	56°C	1 min.	
4-	Extension-1	72°C	45sec	
5-	Extension -2	72°C	7 min.	1 cycle

Electrophoresis on agarose gel for DNA

Electrophoresis was performed to detect the result of the PCR reaction in the presence of standard DNA to characterize the packet size. According to the (Sambrook et al., 1989).

Results and discussion

Isolation and Identification of bacteria on the *Cuscuta* sp.

The results of the isolation and identification of bacteria from the samples of cuscutes (stems and seeds) intruding on different plants are *Conocarpus*, *Chenopodium* and *Cynanchum* (Table 5) showed the obtaining of three types of bacteria are B1, B2 and B3 (Figure 1).

Table (5): Bacteria associated the *Cuscuta* sp.

The name of isolation	Name of the region	Host	The part from which the insulation was made
B1	Baghdad / University of Baghdad	<i>Conocarpus</i>	Stems
B2	Diyala / Baqubah	<i>Chenopodium</i>	Seeds
B3	Diyala / Khan Bani Saad	<i>Cynanchum</i>	Seeds



Figure (1): Colonies of bacteria B1, B2 and B3 developing on nutrient Agar culture medium.



The effect of the efficiency of bacteria associated *Cuscuta sp.* on the percentage of germination of seeds of *Cuscuta sp.* and *Ocimum sp.*

The results of this study showed Table (6) that the isolation of B2 bacteria had a significant effect in raising the germination rate of seeds of *Cuscuta sp.* and *Ocimum sp.*, reaching 90.66 and 92.33% sequentially, and did not differ significantly with the two comparative coefficients, reaching 88.00 and 92.66% sequentially, while it differed morally with isolates B1 and B3, the percentage of plants in their coefficients was 48.66, 50.00 and 76.00 and 79.33% sequentially.

Table (6): The effect of the efficiency of bacteria associated the *Cuscuta sp.* in the germination of seeds of *Cuscuta sp.* and *Ocimum sp.*

The name of isolation	Percentage of germination	
	<i>Cuscuta sp.</i> %	<i>Ocimum sp.</i> %
B1	48.66	50.00
B2	90.66	92.33
B3	76.00	79.33
Control	88.00	92.66
LSD 5%	7.68**	6.73**

Molecular diagnostics of pregnant women using polymerase chain reaction (PCR) technology

The results of molecular diagnostics using PCR technology to amplify the DNA DNA chain of two *Cuscuta sp.* samples showed that S1 belongs to *Cuscuta reflexa* and S6 belongs to *Cuscuta campestris* the ability of the diagnostic initiator pair (Reverse, Forward) to diagnose *Cuscuta sp.* isolates, as the results of electrophoresis on agarose gel showed the presence of a pair of bands with a molecular size (600-650 bp) as in Figure (2).

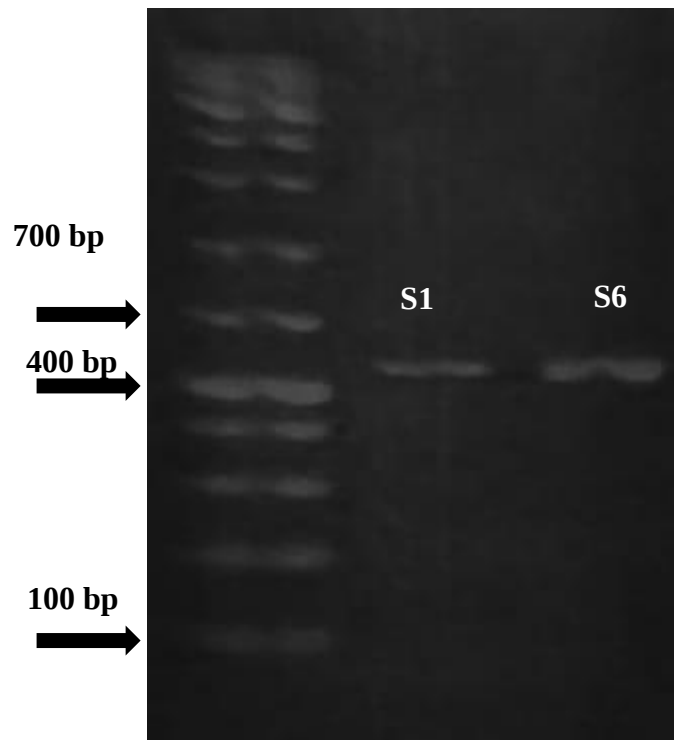


Figure (2): Electrophoresis product of DNA doubling products of *Cuscuta sp.* samples

Nucleotide sequence analysis

The results of the nucleotide sequence analysis, as shown in Figure (3) of the *Cuscuta sp.* that were multiplied for ITS area using the diagnostic initiator (ITS2 and ITS1), showed the presence of two types of *Cuscuta sp.*, namely *Cuscuta reflexa* with an accession number (OQ746922), which was diagnosed for the first time in Iraq and compared with the samples of the Gene bank and found a high 99% match rate with the sample of the USA with an accession number (OL830723) it was also compared with samples from Saudi Arabia, Britain, Kuwait and the Philippines. While the type was recorded, the *Cuscuta campestris* with an accession number (OQ746927) was compared with the samples and the 100% match rate was with the Australian sample with an accession number (MG669314) and also compared with other Canadian, Australian and Turkish samples with accession numbers (KT383193, KT383146, KT383116, MG669315, MG669316, KT383151, MG669318, MW036525, KU725869, KT383145, KT383163, MW036635, KT383162). These two types of *Ocimum sp.* parasite have been diagnosed and recorded for the first time in Iraq, noting that this type of parasite (*Cuscuta campestris*) was recorded on eggplant, potatoes and tomatoes by (Al-Gburi et al., 2018) with accession numbers (MG669313, MG669314, MG669315, MG669316, MG669317, MG669318, MG669319).

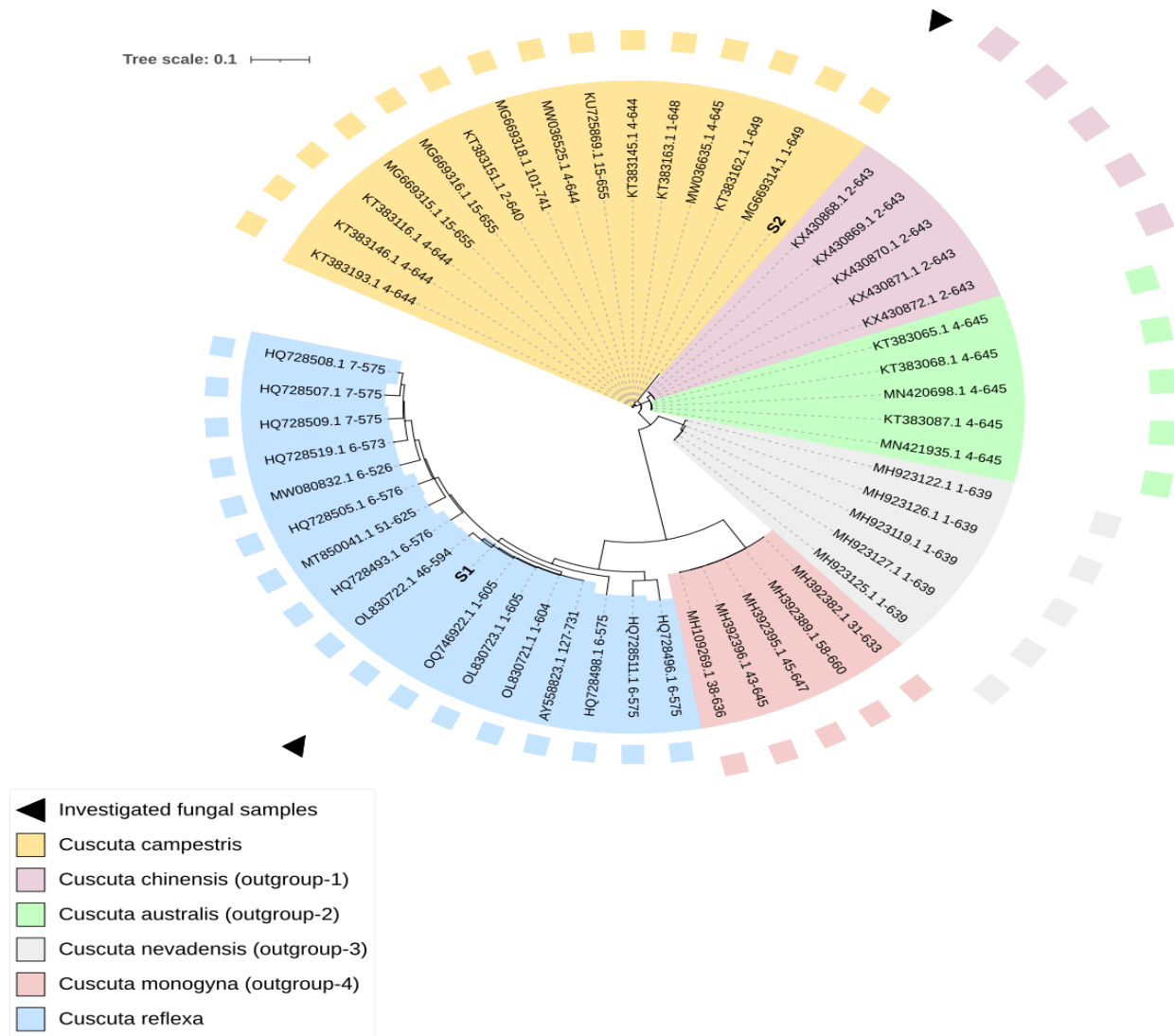


Figure (3): Tree of genetic origins of *Cuscuta reflexa* and *Cuscuta campestris* Porter specimens and their counterparts retrieved from the gene bank.

Molecular diagnostics of isolated bacteria using polymerase chain reaction (PCR) technology

The results of the DNA chain amplification polymerase chain reaction showed that B1 belongs to *Staphylococcus aureus*, B2 belongs to *Bacillus subtilis* and B3 belongs to *Achromobacter xylosoxidans*, the use of the diagnostic initiator 16SRNA led to the appearance of three packages in the chain amplification reaction with a size of 1250-1500 bp as shown in Figure (4).

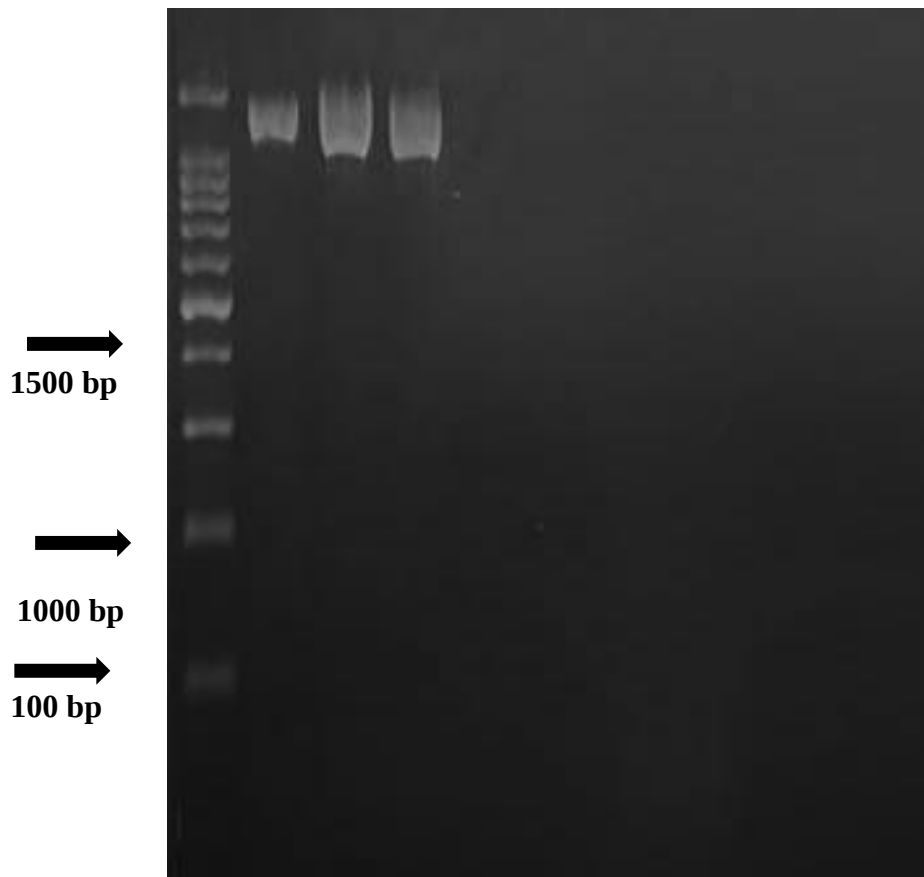


Figure (4): The product of the electrophoresis of the results of the DNA polymerase chain reaction on the agarose gel medium of the bacteria *Staphylococcus aureus*, *Bacillus subtilis* and *Achromobacter xylosoxidans*.

Nucleotide sequence analysis

The tree of the genetic origins of the bacterium (Figure, 5) shows that the *Staphylococcus aureus* bacteria isolated from the stem of the *Conocarpus* parasite with the accession number OQ976979 matched the isolates of the same species in Nigeria and isolated by Pakistan by 100% with the accession numbers MZ185318.1 and MT994845.1 sequentially and matched with the isolates of South Korea with the accession number CP050690.1 by 99% and with the isolations of Poland and Egypt by 97% with accession numbers MZ853764.1 and LC1099444.1 respectively . As for the isolation of the bacterium *Bacillus subtilis* isolated from the seeds of *Chenopodium* parasitizing on ragweed with the accession number OQ976981, it matched the isolates of India and China with the accession numbers OL958544.1 and MH187652.1 by 99%, it matched the isolates of the United States of America with the accession number KY623003.1 by 98%, and it matched the isolates of Pakistan and Iran with accession numbers KY820935.1 and MN153050 by 97%. While the bacteria *Achromobacter xylosoxidans* isolated from the seeds of *Cynanchum* parasitized on the extended period with the accession number OQ976982 was found, it matched the isolates of Pakistan, India, China



and Brazil with the accession numbers KX185939.1, MW624699.1, MZ703196.1 and MT052658.1 with a percentage of 98% and matched the isolates of Japan with the accession number LC506135.1 by 97%.

Staphylococcus aureus has been excluded because it causes infections in children and adults (Orfali *et al.*, 2015; Sheet *et al.*, 2021) and also *Achromobacter xylosoxidans* because it is pathogenic to humans (Holmes *et al.*, 1977). *Bacillus species* are capable of forming stress-tolerant spores and are a type of plant growth-stimulating bacteria (Radhakrishnan *et al.*, 2017; Hamza *et al.*, 2022) and have been used to combat and stimulate resistance to a number of plant pathogens (AL-Juboory *et al.*, 2018 ; Abd & AL-Juboory., 2020). These bacteria are used to enhance crop yields and are substitutes for chemical fertilizers and pesticides (AL-Qathee & AL-Kennany., 2013; Radhakrishnan *et al.*, 2017).

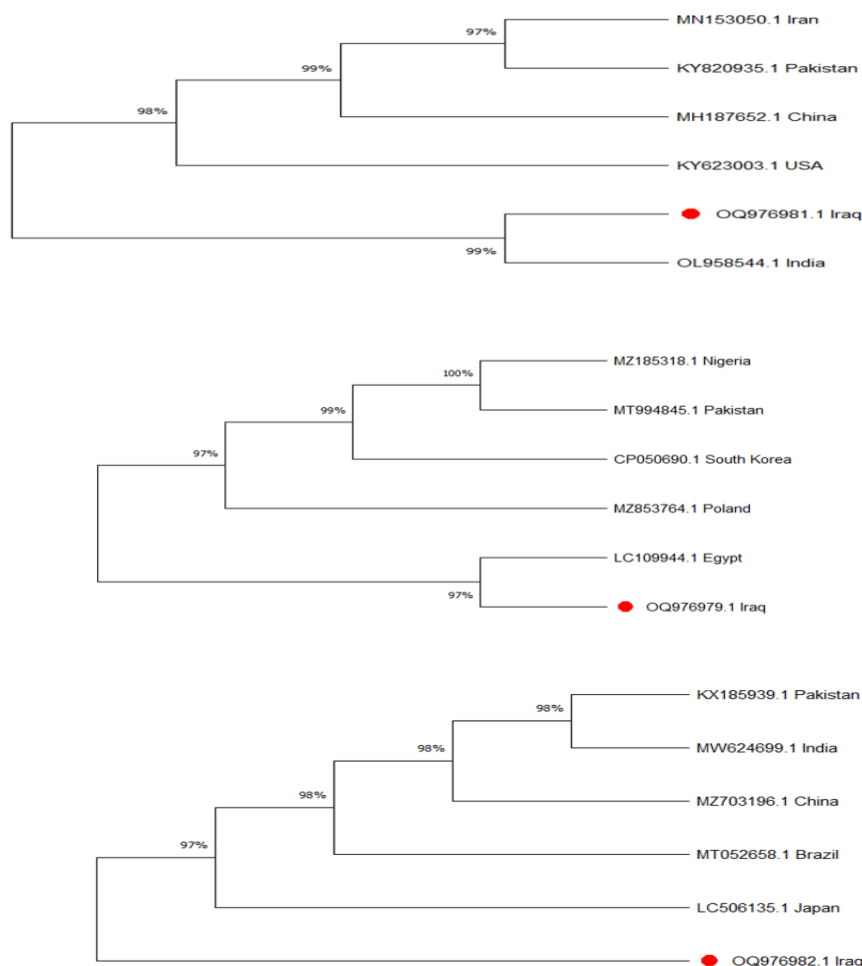


Figure (5): The genetic relationship of *Staphylococcus aureus*, *Bacillus subtilis* and *Achromobacter xylosoxidans*.



CONCLUSION

The results of testing the efficiency of three types of bacteria isolated from *cuacuta* (B1, B2 and B3) in their effect on the percentage of *Ocimum sp.* and *Cuscuta sp.* seeds on the nutrient agar planting medium showed that the isolation of B2 bacteria significantly affected the percentage of germination of *Cuscuta sp.* and *Ocimum sp.* seeds and did not differ significantly with the two comparative coefficients, while it differed significantly with isolates B1 and B3. The results of electrophoresis of two samples from the *Cuscuta sp.*, S1 and S6 on the agarose gel showed. The presence of a pair of bands with a molecular size (600-650 bp). The results of the nucleotide sequence analysis of the two samples showed that S1 belongs to the type *Cuscuta reflexa* and was recorded in the Gene bank within the serial number (OQ746922) and was recorded for the first time in Iraq and compared with the Gene bank samples and found a high 99% match rate with the sample of the United States of America and samples of Saudi Arabia, Britain, Kuwait and the Philippines. The results also showed that the sample S6 belongs to *Cuscuta campestris* and was registered in the Gene bank under the serial number (OQ746927) was compared with the samples and the percentage of correspondence was 100% with the sample of Australia and compared with the samples and the percentage of correspondence was 100% with the sample of Australia with samples of Canada, Australia and Turkey. The results of the nucleotide sequence analysis showed that the isolates of bacteria B1, B2 and B3 belong to *Staphylococcus aureus*, *Bacillus subtilis* and *Achromobacter xylosoxidans* sequentially and were deposited in the Gene bank under the accession numbers OQ976982, OQ976981 and OQ976979 on the relay. *Staphylococcus aureus* and *Achromobacter xylosoxidans* were excluded from subsequent experiments because they are pathogenic to humans.

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