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# Physiological and Molecular Assessment of *Sesbania* Root Nodules Bacteria from Different Iraqi Areas for Salt Tolerance

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**Thirty two isolates of aerobic gram-negative bacteria associated with *Sesbania sesban* grown in different saline Iraqi soils was identified according to morphological and physiological characteristics, cultured on yeast-mannitol agar medium (YEMA) supplemented with different NaCl concentrations. It was indicated that 53.12% of isolates were highly tolerant to salinity, tolerated from 4.0 to 5.0 w/v NaCl. All thirty two Rhizobia isolates performed positive strong reaction to Catalase enzyme except for three were negative to this enzyme. Concerning exo-polysaccharide (EPS) production the isolates displayed a significant difference between them and that salt tolerance isolates gave a high amount of EPS production in compare to the sensitive ones. As for antibiotic sensitivity of *Sesbania* isolates data revealed that 83% of isolates were highly resistant to Ampicilinat 50 $\mu$gml$^{-1}$, the cluster analysis based on all phenotypical and physiological characters divided the isolates into two major groups, the first group included one isolate Ses10, which was salt moderate tolerant. The second group included the rest of isolates which splits into two subgroups with 6% similarity, the first subgroup comprised all sensitive isolates plus one salt moderate tolerant isolate (Ses9).The assumption that district environmental conditions plays a vital role on field survival of bacteria, give rise to the use of PCR methods to identify Rhizobia. In this study the genetic divergence of fast nodulating bacteria connected with *Sesbania* in Iraq was examined. A selection of Rhizobia isolates were characterized by RAPD –PCR. Amplification of genomic DNA using three random primers (RAPD) gave variousbands, the results revealed that most efficient and highest discrimintorypower primer was 35.4% and 37% respectively for primer OPA-10. The cluster analysis based on RAPD-PCR amplification results showed two divergent groups with 15% similarity, the first group included two salt sensitive Ses17 and Ses28, and the second major group comprised all salt moderate and tolerant isolates.**

**Keywords:** Physiological, Molecular, Root nodules, Salt tolerance, *Sesbania*.

Nitrogen fixation using biological organisms is a powerful source of nitrogen in the biosphere (200-300 kg N/ha/year), it plays a significant role in nitrogen enhancement of the earth. Leguminous plants by their symbiotic association with certain gram-negative soil bacteria, commonly known as Rhizobia, promote to fix atmospheric nitrogen. Most of rhizobial strains isolated from wild legumes were classified as members of the genera Rhizobium[1].

In corporation with rhizobia, *Sesbania sesban* is a plant growing in various environments, even in salinity areas, drought and arid infertility[2]. Salinity influence the survival and multiplication

of Rhizobium spp. In rhizosphere and soil, as well as reducing plant growth, photosynthesis, yet rhizobial populations are known to differ in their tolerance to important environment factors[3]. In 2009 Ali and his coworker[4] indicated that inoculation with salt tolerant strains would improve nitrogen fixing ability and nodulation of the leguminous plants subjected to saline conditions. Also in 2017 Yanand his coworker[5] reported that bacteria associated with *Sesbania cannabina* could grow weakly in the presence of 5.0% w/v NaCl. Ali[4] revealed that rhizobia isolated from tree legumes *Leucaenaleucocephala* were tolerant up to 2.5-3.5% salinity.

Many Iraqi areas had under gone harmful environmental conditions in the last decades, such like salinity and drought due to miss usage of efficient irrigation systems and low rainfalls. In 2013 Sharma and his coworker[6] pointed that the naturally occurring soil rhizobia nodulating legumes plants lived in the desert areas are expected to have higher tolerance to common adverse conditions such as salt stress. While N-fixing legumes tolerant of environmental stresses represent an important procedure to improve agricultural productivity, Rhizobia with genetic potentiality for stress tolerance are evenly vital for efficient nodulation and increase productivity of the host plants[1].

## MATERIALS AND METHODS

Different geographical agricultural field sites of Iraqi regions of host plant Sesbania nodule sample were collected. Thirty two isolates were obtained from these regions, the isolates were isolated from nodules after surface sterilization according to Vincent[7], and cultured on yeast extract mannitol agar (YEMA) media containing Congo-red dye, petri dish plates were incubated at 28°C in the dark. The bacterial isolates were examined for morphological characteristics and gram-staining reaction as described by Somasegaran and Hoben[8]. Rhizobia authenticity for host specificity was studied as demonstrated by Engelkeand his coworker[9]. The effect of salt on isolates were

**Table 1.** RAPD primers and PCR reaction conditions

| Primer | Sequence from 5' to 3' end |
|---|---|
| OPA-10 | GTGATCGCAG |
| OPC-16 | CACACTCCAG |
| OPN-16 | AAGCGACCTG |
| PCR reaction condition | |
| Initial denaturizing 5 min , 94$^{O}$C | |
| Denaturizing 1 min , 94 $^{O}$C—— | |
| Annealing 1 min , 32 $^{O}$C \| 34cycle | |
| Extension 1 min , 72 $^{O}$C—— | |
| Final extension 1 min , 72 $^{O}$C | |

**Table 2.** Nomination, Geographical origin and soil description of *Sesbania* isolates

| Isolate Name | Geographical Origin | EC | Soil description | Isolate Name | Geographical Origin | EC | Soil description |
|---|---|---|---|---|---|---|---|
| Ses1 | Amryia-Fallujah 1 | 5.3 | Arid | Ses17 | Mustansiryia,1 Baghdad | 2.2 | g. irrigate |
| Ses 2 | Amryia-Fallujah 2 | 4.6 | Arid | Ses18 | Mustansiryia 2, Baghdad | 1.7 | g. irrigate |
| Ses 3 | Abu-ghreb1 | 7.7 | Arid | Ses19 | Rashdiya, Baghdad | 1.2 | g. irrigate |
| Ses 4 | Abu-ghreb2 | 7.1 | Arid | Ses20 | Zafrania 1, Baghdad | 6.1 | arid |
| Ses 5 | Abu-ghreb3 | 5.8 | Semiarid | Ses21 | Zafrania 2, Baghdad | 5.7 | arid |
| Ses 6 | Khaluss 1, Diyala | 5.0 | Semiarid | Ses22 | Nasir, Nasiriyah | 3.0 | g. irrigate |
| Ses 7 | Khaluss 2, Diyala | 4.6 | g. irrigate | Ses23 | Fajir 1, Nasiriyah | 4.9 | Semiarid |
| Ses 8 | Khaluss 3, Diyala | 4.5 | g. irrigate | Ses24 | Fajir 2,Nasiriyah | 5.4 | Semiarid |
| Ses 9 | Baquba 1,Diyala | 3.8 | g. irrigate | Ses25 | Mahmodea1, Baghdad | 5.2 | Semiarid |
| Ses 10 | Baquba 2,Diyala | 3.0 | g. irrigate | Ses26 | Mahmodea2, Baghdad | 4.7 | Semiarid |
| Ses 11 | Baquba 3, Diyala | 4.3 | Semiarid | Ses27 | Saydia 1, Baghdad | 3.4 | g. irrigate |
| Ses 12 | Balad 1, Diyala | 5.1 | Semiarid | Ses28 | Saydia 2, Baghdad | 3.2 | g. irrigate |
| Ses 13 | Balad 2, Diyala | 5.4 | Semiarid | Ses29 | Saydia 3, Baghdad | 4.0 | g. irrigate |
| Ses 14 | Seqlawea 1, Baghdad | 1.3 | v.g.irrigate | Ses30 | Mahawel 1, Babel | 5.6 | Semiarid |
| Ses 15 | Seqlawea 2, Baghdad | 1.7 | v.g.irrigate | Ses31 | Mahawel 2, Babel | 5.7 | Semiarid |
| Ses 16 | Seqlawea 3, Baghdad | 2.0 | v.g.irrigate | Ses32 | Mahawel 3, Babel | 6.0 | Semiarid |

studied by inoculating one loopfull of each isolate on YEMA media in triplicate plates containing 1, 2, 3, 4, 5 w/v% NaCl, growth was compared with control(no NaCl) and evaluated qualitatively according to Somasegaran and Hoben[8]. Enzymes activity were examined for Catalase, Urease and Gelatinase enzymes as displayed by Ronald and his coworkers[10], appearance of gas bubble indicate the presence of Catalase enzyme. Intrinsic antibiotics resistance (IAR) were examined by taking freshly prepared, filter sterilized (0.22 µm) solution of antibiotics and added to cooled, molten YEMA media to give the following concentration µg/ml: 10, 25 and 50 of Ampicillin, Erythromycin and Kanamycin. The control treatment was consisted of YEMA plates without antibiotics. Isolates showing growth were scored as positive. Triplicate plates for each antibiotic were incubated at 28 R"C for 7 days, and scored for growth. For the evaluation of exo polysaccharide production (EPS), a loop full of each *Sesbania* isolates were inoculated into conical flasks containing 100 ml of yeast extract mannitol broth media. The flasks were incubated at 28 R"C on revolving shaker at 200 rpm for 72h. After incubation, the broth was centrifuged 3500xg and the supernatant was mixed with two volumes of chilled acetone. The crude polysaccharide developed was collected by centrifugation at 3500 xg for 30 min. EPS then Was washed with distilled water and acetone alternatively, then transferred to a filter paper and weighed after overnight drying at 105°C.

**Table 3.** Salinity tolerating levels of *Sesbania* isolates

| Isolate. No | Highest NaCl con. tolerated (%) |
|---|---|
| Ses1 | 4 |
| Ses 2 | 5 |
| Ses 3 | 4 |
| Ses 4 | 5 |
| Ses 5 | 5 |
| Ses 6 | 4 |
| Ses 7 | 3 |
| Ses 8 | 3 |
| Ses 9 | 2 |
| Ses 10 | 2 |
| Ses 11 | 4 |
| Ses 12 | 4 |
| Ses 13 | 5 |
| Ses 14 | 2 |
| Ses 15 | 1 |
| Ses16 | 1 |
| Ses17 | 1 |
| Ses18 | 3 |
| Ses19 | 1 |
| Ses20 | 5 |
| Ses21 | 5 |
| Ses22 | 2 |
| Ses23 | 4 |
| Ses24 | 4 |
| Ses25 | 5 |
| Ses26 | 4 |
| Ses27 | 3 |
| Ses28 | 1 |
| Ses29 | 1 |
| Ses30 | 4 |
| Ses31 | 4 |
| Ses32 | 2 |

DNA Extraction of *Sesbania* isolates was done using Wizard genomic DNA purification kit (promega), DNA purity was identified using Shimadzu spectrophotometer, DNA concentration was calculated using the following equation:

ds DNA con.= O.D 260 nm x dilution factor x 50µg /ml (Sambrook*et al*., 1989). DNA fingerprinting of *Sesbania* isolates by RAPD-PCR was applied using Master Mix reaction kit and three random primers from BioneerCoporation (South Korea). Sequence of the primers and the reaction conditions were described in table-1. Amplification products were separated and electrophoresed on 1.5% w/v agarose gel. Total band number were calculated according to Sahiand his coworkers[11].
Primer efficiency and primer discriminatory power was measured using the formulas:
Primer efficiency=total number of bands amplified by a primer / total number of bands amplified by all primers x 100.
Primer discriminatory power= the total of polymorphic bands amplified by a primer / total number of polymorphic bands amplified by all primers x 100 [(12)].

Statistical analysis for EPS production was done using ANOVA. Significant differences were identified by the least significant difference (L.S.D) multiple mean comparison test at p d"0.05 (Genestat program software, 2008, VSN International Ltd), as for physiological traits, comparison was performed quantitavelly on the basis of growth + or no growth – for each isolate.

PCR fingerprints pattern were converted into a two-dimensional binary matrix (1, presence of a band, 0, absence of a band) and analyzed using statistic software package (version 1.92; past software, Ohammer, 2009).

## RESULTS AND DISCUSSION

### Morphological characterization

All thirty two *Sesbania* isolates were comparable in form with translucent gummy glistening, entire margin and circular rounded with diameter of 2.5-3.5mm, except of the isolates( Ses4, Ses5, Ses30 and Ses23) which were little translucent, milky and about 1-2mm in diameter after two days growth. The isolates were tested on Congo red as indicator incorporated with YEMA media, the data showed that all isolates did not absorb Congo red under dark conditions except Ses8, Ses9and Ses19 that appeared to be pink as a result of taking Congo red. Table- 2 shows the nomination and geographical origin and soil description. All isolates were examined under microscope and it revealed that the isolates were rod, motile and gram-negative cells.

**Table 4.** Enzymes reaction pattern of *Sesbania* isolates

| Isolate no. | Catalase | Urease | Gelatinase | Isolate. No | Catalase | Urease | Gelatinase |
|---|---|---|---|---|---|---|---|
| Ses1 | ++ | - | - | Ses17 | + | - | + |
| Ses 2 | ++ | - | + | Ses18 | ++ | + | - |
| Ses 3 | ++ | - | - | Ses19 | + | + | - |
| Ses 4 | ++ | + | - | Ses20 | + | - | - |
| Ses 5 | ++ | - | - | Ses21 | ++ | - | - |
| Ses 6 | ++ | - | + | Ses22 | + | - | - |
| Ses 7 | ++ | + | - | Ses23 | + | - | + |
| Ses 8 | ++ | - | - | Ses24 | ++ | - | - |
| Ses 9 | + | - | - | Ses25 | + | - | - |
| Ses 10 | + | - | - | Ses26 | + | - | + |
| Ses 11 | ++ | - | - | Ses27 | ++ | - | + |
| Ses 12 | ++ | - | - | Ses28 | - | + | + |
| Ses 13 | ++ | - | + | Ses29 | + | + | + |
| Ses 14 | + | + | - | Ses30 | + | + | - |
| Ses 15 | - | - | + | Ses31 | + | - | - |
| Ses16 | - | - | + | Ses32 | + | - | + |

### Physiological characterization

### Salt stress response

Viability of Rhizobia isolates grown under various salt concentration using NaCl was measured. Table-3 displayed a high level of variety between isolates, data showed that 53.12% of

**Table 5.** Antibiotic resistance of thirty two *Sesbania* isolates

| Antibiotic | % Resistance of isolates | | |
|---|---|---|---|
| | 10µgmL$^{-1}$ | 25µgmL$^{-1}$ | 50µgmL$^{-1}$ |
| Erthromycin | 71 | 42 | 33 |
| Kanamycin | 56 | 44 | 24 |
| Ampicilin | 86 | 85 | 83 |

**Table 6.** EPS production in *Sesbania* isolates(mg/g)

| Isolate | EPSmg/g | Isolate | EPS mg/g |
|---|---|---|---|
| Ses1 | 900.1 | Ses17 | 296.6 |
| Ses 2 | 678.3 | Ses18 | 746.3 |
| Ses 3 | 720.8 | Ses19 | 361.0 |
| Ses 4 | 923.6 | Ses20 | 867.3 |
| Ses 5 | 892.6 | Ses21 | 981.2 |
| Ses 6 | 763.3 | Ses22 | 324.6 |
| Ses 7 | 522.1 | Ses23 | 656.8 |
| Ses 8 | 564,9 | Ses24 | 654.0 |
| Ses 9 | 497.9 | Ses25 | 566.3 |
| Ses 10 | 488.9 | Ses26 | 786.2 |
| Ses 11 | 656.6 | Ses27 | 678.2 |
| Ses 12 | 745.6 | Ses28 | 401.0 |
| Ses 13 | 824.2 | Ses29 | 325.3 |
| Ses 14 | 325.6 | Ses30 | 697.4 |
| Ses 15 | 433.3 | Ses31 | 548.9 |
| Ses16 | 312.9 | Ses32 | 300.4 |

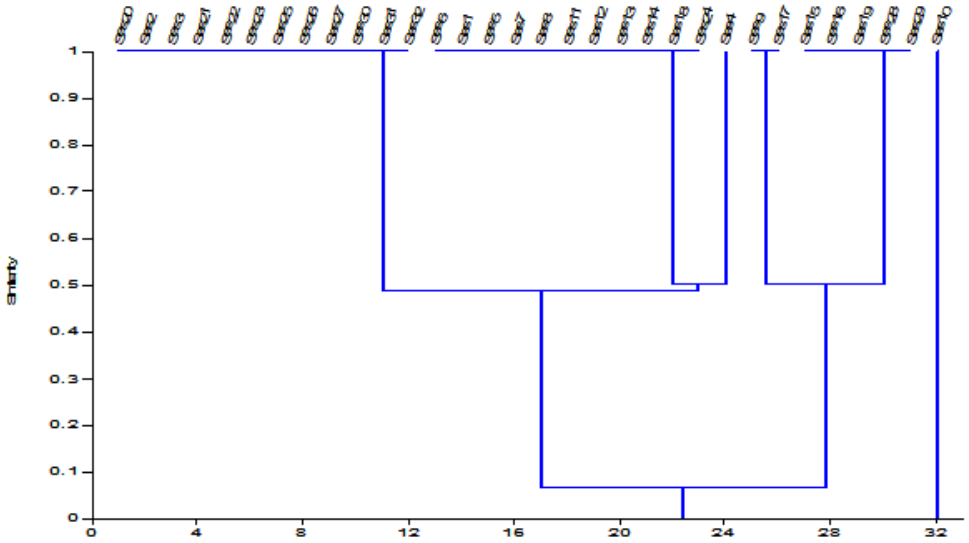


**Fig. 1.** Dendrogram showing similarity levels between Sesbania isolates based on phenotypical and physiological characterization

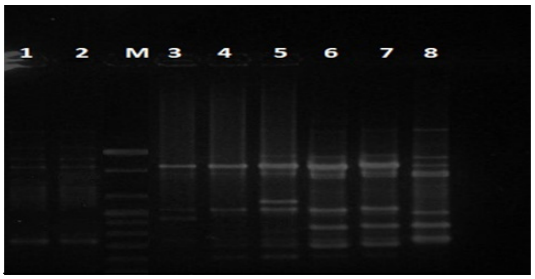


**Fig. 2.** RAPD fingerprint of *Sesbania* isolates generated by primer OPA-10. 1= Ses17; 2-Ses28; M= 1kb DNA ladder; 3=Ses7; 4=Ses18; 5=Ses32; 6=Ses2; 7=Ses13; and 8= Ses20

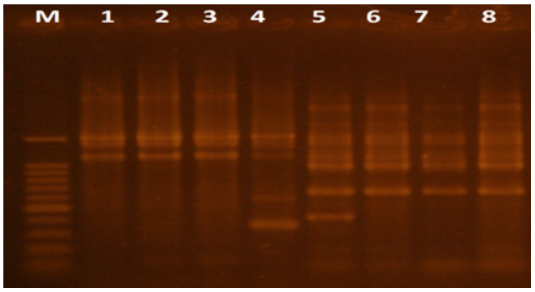


**Fig. 3.** RAPD fingerprint of *Sesbania* isolates generated by primer OPC-16. M= 1kb DNA ladder 1= Ses2; 2-Ses13; 3=Ses20; 4=Ses17; 5=Ses28; 6=Ses7; 7=Ses18; and 8= Ses32

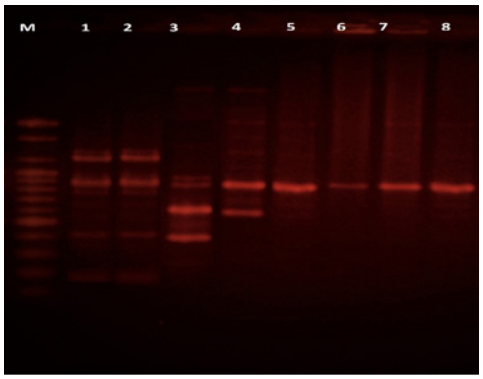


**Fig. 4.** RAPD fingerprint of *Sesbania* isolates generated by primer OPN-16. M= 1kb DNA ladder 1= Ses17; 2-Ses28; 3=Ses7; 4=Ses18; 5=Ses32; 6=Ses13; 7=Ses20; and 8= Ses2

*Sesbania* isolates were highly tolerant to salinity, tolerated from 4-5%w/v NaCl and that 18.75% of isolates were salt sensitive, tolerated up to 1.0%NaCl.

**Enzymes activity**

Data in table-4 showed that all isolates gave strong positive reaction to Catalase enzyme except for Ses19, Ses28 and Ses29 were negative for Catalase test. As for Urease the table cleared that 88.23% of salt tolerant isolates were negative for this enzyme comparing to 50% of salt sensitive isolates. In regards to Gelatinase enzyme, results indicated that 70.58% of salt tolerant isolates were negative for Gelatinase production.

**Antibiotic sensitivity**

All thirty two isolates were tested against

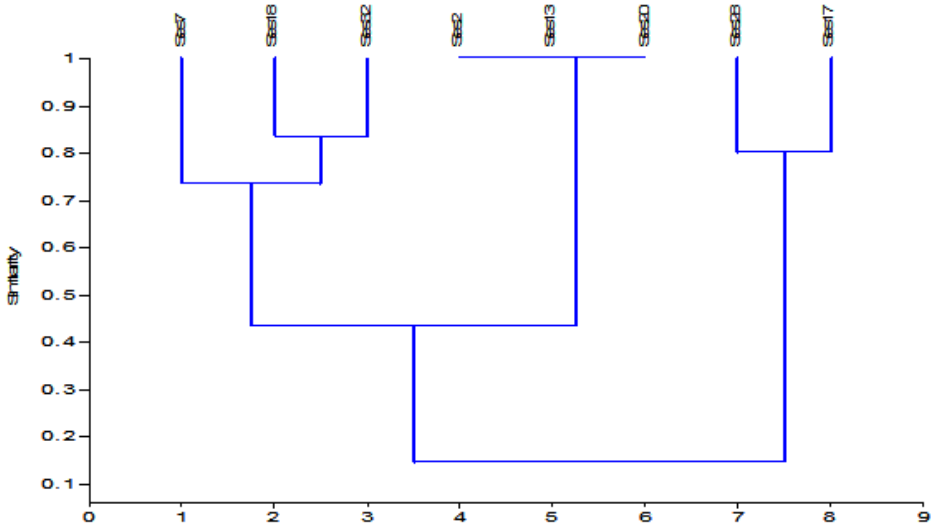


**Fig. 5.** Dendrogram of *Sesbania* isolates derived from RAPD fingerprints generated using three different primers (OPA-10, OPC-16, and OPN-16)

**Table 7.** Fragments amplified by three random primers in eight Sesbania isolates and the efficiency and discriminatory power of each primer

| Primer | No. of bands amplified in all isolates | | Primer efficiency (%) | Primer discriminatory power (%) |
|---|---|---|---|---|
| | Total | Polymorphic | | |
| OPA-10 | 11 | 10 | 35.4 | 37.0 |
| OPC-16 | 10 | 8 | 32.2 | 29.6 |
| OPN-16 | 10 | 9 | 32.2 | 33.3 |
| Total | 31 | 27 | - | - |

three kinds of antibiotics in 10, 25, 50 µgml$^{-1}$ concentration. Table-5 showed that all isolates were highly resistant to Ampicilin at 50 µgml$^{-1}$concentration.

**Exopolysaccharide (EPS) production**

Table-6 showed a significant difference between *Sesbania* isolates regarding EPS production, the results revealed that salt tolerant isolates gave higher amount of EPS production in compared to sensitive isolates, this is agreed withFreitasand his coworkers as well as Saritha and her coworkers in addition to Alroomi[13-16]. The highest production of EPS was recorded for the isolate Ses21 producing 981.2 mg/g EPS, and the least production was found in Ses17 producing 296.6 mg/g EPS. Cluster analysis based on phenotypical and physiological traits divided the isolates into two divergent groups, the first one included one isolate Ses10, which was salt moderate tolerant, and the second main group included the rest of Sesbania isolates which splits into two subgroups with 6% similarity, the first subgroup comprised all sensitive isolates plus one salt moderate (Ses9), and the second subgroup included all salt tolerant and moderate isolates.

**Molecular characterization**

Molecular methods used in this study was applied on eight representative isolates, salt tolerant (Ses2, Ses13, and Ses20), salt sensitive (Ses17 and Ses28) and salt moderate tolerant (Ses7, Ses18, and Ses32). DNA purity ranged from 1.3 _1.7 O.D. The RAPD-PCR amplification products comprised different bands (fig2,3, and4), table-6 cleared that most efficient and highest discriminatory power was 35.4% and 37%respectively for the primer OPA-10, also the cluster analysis based on RAPD-

PCR products showed two divergent groups with 15% similarity, the first group contained all salt sensitive isolates, while the second group included all salt moderate and tolerant isolates, this group subdivided into two subgroups with 44% similarity, the first subgroup included all salt tolerant isolates which show 100% similarity between them, and the second subgroup comprised the salt moderate tolerant.

The study showed a wide variability for salt tolerance between Iraqi isolates, these results was consistent with Elboutahiriand her coworkers as well as Sharma and his coworkers[6, 17]. Probable cause for genetic diversity could be the short soil moisture perhaps have resulted in genetic adaptations of the strains. Thus, variations between different strains of diverse origins suggested that there is genetic potential to improve tolerance to environmental stress such as low soil moisture.

## CONCLUSIONS

This study demonstrated that we could isolate and purify salt tolerant *Sesbania* isolates from Iraqi soils, the cluster based on physiological and phenotypical traits shows that these isolates represents divers populations and this could offer selection advantage in survival and adaptation to harsh environment conditions. RAPD technique was effectively utilized to discriminate between *Sesbania* isolates. And the genetic potential for increased tolerance to salinity could improve production of high tolerant inoculum strains for legume plants.

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