PHYLOGENETIC ASSOCIATION OF ENDOPHYTIC HALOPHILES WITH PLANT GROWTH PROMOTING AND BIOCONTROL POTENTIAL FROM *Avicenna marina*

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Plant microbe interaction could occur with advantageous microbes, that help the plants to dwell through terrestrial and support their growth and health, or it may take place with harmful organisms, which challenge plant strength (Vandenkoornhuyse *et. al*., 2015). The former group inhabits the plant tissues without being harmful to their host. Wide range of these endophytes offer many benefits to their host plant (Lata *et. al*., 2018). The associations between the plant and the endophytes have many benefits, the later can promote host plant growth due to production of phytohormones, enhance plant tolerance to adapt/survive in both biotic-infection by plant pathogens- and abiotic stress conditions such as salinity, and in the long run increase biomass production of the plants (Tan & Zou, 2001; Pandey *et. al*., 2017). The endophytic bacteria colonize the plants at the same cells with the plant pathogens, hence adapting various recognized mechanisms of biocontrol activities such as competition for ecological niche or substrate, induced systemic resistance in the host plants against different pathogens and production of inhibitory metabolites (Compant *et. al*., 2005). These metabolites can be valuable in different biotechnological applications. The variation in the endophyte structure and diversity are greatly affected by the plant and are vastly reliant on the geographic location, their interactions with the host and the different environmental factors (*Liu et. al*., 2017).

Mangroves are woody plants adapted to grow in saline environments; they cover approximately 60 to 75% of the Earths' tropical and subtropical regions. They are wetland ecosystems, situated between land and sea (Andreote *et. al*., 2012; De Souza *et. al*., 2013). The major components of this ecosystem are bacteria and fungi which constitute 91% of its total biomass (Alongi, 1988). Large numbers of mangrove plant species have different extract with biological activities such as antiviral, antibacterial, antifungal and insecticidal (Eldeen and Effendy, 2013). A great amount of different active metabolites was previously reported from mangroves and their accompanying organisms (Bibi *et. al*., 2017). Consequently, studies on the endophytes of mangrove plants offer an opportunity to discover new re-

Egypt. J. Genet. Cytol., 49:45-58, January, 2020
Web Site (*www.esg.net.eg*)

sources and compounds with biotechnological potential. This study compared the endophytes from stems, leaves and roots of *Avicennia marina* collected from the Red Sea coastal area and examine their antimicrobial activity against plant pathogenic bacteria as well as their ability to promote plant growth.

MATERIALS AND METHODS

Sampling

Samples were collected from the Red Sea mangrove shore at the Sharm area in Yanbu, Saudi Arabia. The sampling location coordinates are 24°02'26.6"N and 38°06'54.9"E. Plant samples were collected in clean zip-bags and stored at 4°C then transported to the laboratory until proceeding within 24 h.

Endophytic bacterial isolation

Healthy-looking plant parts were surface sterilized for pretreatment of endophytic bacterial isolation, as described by (Deivanai *et. al*., 2014) with some modifications. Soil particles were removed from the different plant parts by washing under running tap water. All the samples were initially rinsed for 30 sec in 70% ethanol, then for 3 min in sodium hypochlorite (4%) and finally washed carefully in sterile water 3 times. The efficiency of the surface sterilization technique was confirmed by plating 150 µl of the last wash water was on nutrient agar and incubated in 37°C. Successful surface sterilization was concluded when no bacterial growth was indicated after 48 h of incubation. Afterwards, two grams from each plant tissue (leaf, stem and root) were grinded separately in 12 mL of phosphate buffer saline (PBS). Then, the solutions were vortexed for 1h at 4°C. Finally centrifuged at maximum 14,000 rpm for 10 min. The pellets were suspended in PBS buffer, plated on nutrient agar, incubated at 37°C and observed up to 72 h for bacterial growth. Bacterial colonies were purified and stored for further use.

Antibacterial activity

Two bacterial plant pathogens, the Gram-positive *Clavibacter sp.* isolated form diseased tomato and the Gram-negative *Pseudomonas sp.* isolated form infected pepper (unpublished data) were chosen as test organisms. The antibacterial activity of the endophytic isolates was conducted according to the protocol described by (Moran *et. al.,* 2016) with minor modifications. Briefly, twenty microliters of fresh overnight culture from each bacterial isolate (concentration of about 108 cfu/ml) were spotted into a Müller-Hinton agar plates and allowed to grow at °C for at least 16 h. About 300 µl suspension of the selected plant pathogens (approximately 2×108 cfu/ml) were sprayed into the plates and kept at room temperature for 5 min. The plates were incubated for 24 h at 37°C then the width of the inhibition zones was measured. The experiment was performed in triplicates and the mean inhibition zones were calculated.

Amplification and sequencing of the 16S rRNA gene.

Phenol/chloroform/isoamyl protocol as previously described by (Kheiran-

dish and Harighi, 2015) was used to extract the genomic DNA from the bacterial isolates after they were grown in nutrient broth medium for 24 h at 37°C. The 16S rRNA gene was amplified with the universal primer pairs of 27F (5'-AGA GTT TGA TCA TGG CTC AG-3') and 1492R (5'-ACG GTT ACC TTG TTA CGA CTT-3'). The PCR products were sequenced using Sanger method (Sanger and Coulson, 1975) by MACROGEN, Korea using the same primers.

Sequence similarity and phylogenetic analysis

The 15 partial 16S DNA sequences obtained were first assembled, then scanned for similarity using BlastN in the National Central Bank Database (Altschul *et. al.,* 1990). The taxonomic hierarchies of the tested sequences were obtained by defining the nearest neighbors on the basis of common words between test sequences and query sequences. The 15 rDNA sequences as well as their closely related hits in the GenBank database were aligned using "Clustal W" algorithm (Higgins and Sharp, 1988) with the default parameters. Phylogenetic analyses of partial gene sequences were executed by MEGA X (Kumar, 2018). Tamura-Nei model (Tamura and Nei, 1993) and Maximum Likelihood test were used to reconstruct the Evolutionary background between the 31 selected nucleotide sequences. The tree with the highest log probability (-10587.98) is shown. Bootstrap evolutionary distances (Hillis and Bull, 1993) were calculated using 500 repeats, the values are shown next to the branches with 50% cutoff. The branch lengths was measured by the number of substitutions per site, the tree was drawn to scale. In total, there were 1585 positions in the final dataset.

Hydrogen cyanide (HCN) production

Hydrogen cyanide production for all isolates was investigated according to Alstrom and Burns (1989). In brief, fifty microliters of each bacterial suspension were spread onto nutrient agar plate and a Whatman paper was soaked in picric acid solution (2% Na2CO3, 0.5% picric acid) then placed inside the lids. Culture plates were incubated in an inverted position at 37°C for 7 days while sealed with Parafilm. The change in paper color from yellow to orange or reddish brown indicated HCN production. The production efficiency was measured from zero to five according to the color intensity, with zero as no change in color and five as dark brown.

Plant growth induction

Optical density (OD) of the isolates growth was determined by spectrophotometer at 660nm until it reached (0.6). Seeds of wheat (Qassimi) were sterilized with 20% Clorox commercial bleach for 15 minutes and rinsed three times in sterilized distilled water for 5 minutes/rinse. Then sixty seeds were soaked in fresh bacterial cell suspension of each sample for five min. subsequently, the seeds were dispersed on sterilized tissue and left to

dry for few minutes then allowed to germination in a dark dry environment for six days. Growth parameters means (radical length; longest seminal root; number of seminal roots; coleoptile length; first leaf length; fresh weight) were recorded.

RESULTS AND DISCUSSION

Endophytic bacterial identification and phylogenetic analysis

The culturable endophytic bacterial population of stems, leaves and roots of *A. marina* were assessed. Twelve bacterial isolates were collected in this study, five from the leaves (SAL3, 6, 8 10 and 18); five from the stem (SAS 5, 7, 13, 14 and 16) and finally, two from the root (SAR 1 and 6). The relatively small number of the detected bacteria may be due to the fact that the majority of the endophytes are obligate and the supplementation of the media with plant extract might be necessary for their isolation and propagation. The BLAST search analysis of the 16S rDNA nearly full length sequences indicated that seven samples belonged to different species of genus *Bacillus* SAL6, 8, 10, SAS7, 14, 16 and SAR1. Whereas SAL3 belonged to *Sporosarcina,* SAL18 was *Micrococcus,* SAS5 was *Virgibacillus,* and SAS13 was *Staphylococcus* while ASR6 was *Paenibacillus.* The exact species and the accession number for each match and the identity percentage are shown in Table (1), the e-value for all the matches was zero which indicate true similarity.

The phylogenetic analysis using Maximum Likelihood method was able to affiliate most of the isolates to their best match (Fig. 1). However, SAR1 was found to be closer to *Micrococcus luteus* than *Bacillus galactosidilyticus*, which require additional examination to confirm its identity. The BLASTN search was not conclusive about the species of isolate SAS5, as it provided the same identity score and coverage value for different *Virgibacillus* species. Similarly, the phylogenetic analysis was unable to affiliate the isolate to a specific species which might indicate that it could be novel specie. Further identification analysis is required to confirm this assumption. The phylogenetic analysis divided the isolates into two main groups, the first incorporated eight isolates branching from two nodes. The first node included (SAL6, SAL10, SAS5 and SAS13); the second node covered (SAL8, SAS7, SAS14 and SAS16). Meanwhile the second group comprised four isolates (SAL3, SAL18, SAR1 and SAR6). Accordingly, the data obtained in this study indicated that endophytic bacterial populations constituted mostly by spore forming Gram positive bacilli bacteria except for SAL8 and SAS16. Bacterial endophytes have been reported in various plant tissues, unlike our results, both Gram positive and negative bacteria were reported (Tashi-Oshnoei *et. al*., 2017).

Evaluation of plant growth promotion ability

Endophytic bacteria can positively affect plant growth either directly by con-

trolling the concentrations of its hormones or assisting the acquirement of nutrients for instance phosphorus, nitrogen, and iron, or indirectly by acting as biological control agents. Strains were tested *in vitro* for their ability to induce plant growth by measuring, the growth parameters before and after inoculation. There antibacterial effect on plant pathogenic bacteria as well as their ability to produce hydrogen cyanide.

Effect on growth parameters

The effect of the inoculated bacteria was variable, some isolates had significant positive effect on plant growth compared to the control while others showed inhibitory influence. This might be due to the cultivation of the host plants in sea water meaning that the endophytes are supposedly helping the plant to tolerate the stress condition hence some of them may produce abscisic acid and/or ethylene which are known as suppressing factors for plant growth. Abscisic acid and ethylene are plant hormones that help in different physiological processes among which closing the stomata to increase stress tolerance (Srivastava, 2002). The ability of these isolates to enhance plant stress tolerance should be evaluated in a distinct study. Figure (2) showed representatives of the germinated seeds demonstrating the various effects of the bacteria on the plant growth. Out of the 12 isolates screened, inoculation with 4 isolates (SAL6, SAL8, SAS13 and SAS14) were observed to significantly improve shoot elongation (Fig. 3) which might indicate that these isolates could produce cytokinins and /or gibberellic acid that are responsible for shoot initiation and stem elongation. Isolate SAL6 showed the highest shoot length, followed by SAL8, while the other two isolates (SAS13 and SAS14) improved shooting equally.

The three isolates; SAL3, SAL6 and SAL8 significantly improved seminal root number while root length was significantly enhanced by isolates SAL6, SAS5, and SAS7. Root initiation and elongation might be related to the ability of the inoculated isolates to produce different auxins including indole acetic acid (Ludwig-Müller, 2011) while showing high number of seminal roots might be related to production of excess amount of cytokinins which are known to induce adventitious root formation. Highest seminal root length was observed after inoculation with isolates SAL6, SAS13, and SAS14. Only a few number (8) of the studied isolates were able to affect the first leaf germination and length ***????***, cytokinins also affects leaf expansion. Figure (3) shows that isolate SAS13 and SAS7 had the greatest positive effect on the first leaf length. The infection with the endophytic bacteria showed limited effect on the fresh weight. Isolate SAL8 was the only bacteria to significantly increase the fresh weight when related to the control. Over all, isolates SAL6, SAL8, SAS13 and SAS14 showed the greatest effect on plant growth compared to the control as presented in Fig. (3). Previous studies proved that inoculation with plant growth promoting endo-

phytic bacteria significantly reduced the effect of drought stress by enhancing biomass production in maize (Naveed *et. al*., 2014). Additionally, *Burkholderia phytofirmans* Ps JN was observed as an endosphere colonizer that promoted growth and enhanced abiotic and biotic stress tolerance in different crops, e.g. potatoes, tomato and grapevine (Mitter et al., 2013). The most frequently described plant response facilitated by plant growth promoting bacteria (PGPB) in several plant species is the increase in the root system (Lucy *et. al*., 2004). Observations of the current study are in accordance with earlier reports on the prospective of endophytic bacteria in improving plant yield and enhancing their drought tolerance (Vardharajula *et. al*., 2011). Different bacteria including *Bacillus*, *Micrococcus* and *Paenibacillus* have been utilized as Plant growth-promoting rhizobacteria (McSpadden-Gardener, 2004; Bhattacharyya and Jha, (2012); Akinrinlola *et. al*., 2018). Specifically, *Bacillus* strains that are well known to be used as biofertilizers and biocontrol agents in agriculture (Borriss 2011). *Staphylococcus pasteuri* and *Micrococcus luteus* were also stated as endophytic bacteria that have PGP activity (Vendan *et. al*., 2010) while a *Virgibacillus sp.* were reported (Dias *et. al*., 2009) to have IAA producing and phosphate solubilisation potential, while (Kavamura *et. al*., 2013) reported that they can produces exopolysaccharide and have the ability to grow under water stress.

Antagonistic activity

Amongst the tested isolates, *B. subtilis*-SAL6 had the greatest inhibitory effect on the plant pathogenic *Pseudomonas sp*., the inhibition zone diameter mean was 13 mm, while 6 mm against *Clavibacter sp*. *B. subtilis*-SAL10 and *B. foraminis*-SAS16 strains had mean inhibition zone diameter of 9 mm against *Pseudomonas sp*., while their inhibitory effect on *Clavibacter sp*. was slightly higher than SAL6. Moderate or weak inhibition was noticed from the rest of the isolates as shown in fig. (4). The results from this study are in agreement with several previously reported observations; in a study on the bacteria isolated from coastal ecosystems that evaluated their antimicrobial potential, *B. foraminis* was found to have polyketide biosynthesis type II (pks2) gene for macrolactin Also various *Virgibacillus* species as well as *Paenibacillus* spp. detected were found to have antimicrobial activity (Al-Amoudi *et. al*., 2016). Meanwhile, the capacity of *B. subtilis* group strains to produce numerous secondary metabolites facilitating antibiosis was recognized for long time (Stein, 2005), since various antimicrobial byproducts were reported (Caulier *et. al*., 2019).

HCN production

The ability of all the endophytic bacterial isolates were evaluated for their ability to produce hydrogen cyanide (HCN) as one of the indirect mechanisms that supports plant growth The results showed that, four isolates (~18.2%) were found to be positive HCN producers but, the amount of HCN production differed according to the isolate. Maximum production of HCN was obtained from isolates SS6 and SS16, while isolates SL8 and SL10 showed lower amounts. The remaining 18 isolates showed no sign of

production (Fig. 5). Hydrogen cyanide is a toxic chemical which is naturally produced as byproduct by some fungi, plants and certain bacterial species that harm wind range of organisms and act as biocontrol agent. Previous studies reported HCN production by endophytic bacteria as well as many rhizobacterial species. Hydrogen cyanide producers and has been involved in inhibition of vast array soil borne pathogens, hence, playing a major role in biological control of plant disease (Reetha *et. al*., 2014; El-Rahman *et. al*., 2019).

To conclude, this study was able to identify a group of important bacterial isolates that have potentials in promoting the plant growth via different mechanisms. However, further investigation for the levels of the plant growth hormones in each individual bacterial isolates should be evaluated independently.

SUMMARY

Endophytic bacteria inhabit plant tissue and offer different benefits to their host which includes growth promotion as well as biotic and a biotic stress tolerance. Plants under stress such as *Avicenna marina* will selectively harbor halophilic bacteria that can help them adapt to high salt concentration. The present study focuses on the isolation, molecular identification and reconstructing the phylogenetic affiliation of endophytic bacteria associated with different tissues of *A. marina*. Antimicrobial potential of the isolated endophytes have also been investigated. A total of twelve isolates belonging to six different genera were collected from this plant. About 53% of the isolates have plant growth promoting activity, while 20% showed strong antimicrobial activities in agar assay against the tested plant pathogens. Two isolates were found to produce significant amount of hydrogen cyanide. These results open a wide door of benefits to be gained from endophytic isolates showing advantageous potentials in enhancing economically important plant health.

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Table (1): BLASTN result for each bacterial endophyte isolate showing their best match, accession number and percentage of identity.

No.	Sample name	Best match		Identity %
		Name	Accession number	
1	SAL3	*Sporosarcina newyorkensis*	AM910326.1	99.6
2	SAL6	*Bacillus subtilis*	MN417011.1	99.2
3	SAL8	*Bacillus foraminis*	KU983819.1	96.1
4	SAL10	*Bacillus subtilis*	EU071579.1	99.5
5	SAL18	*Micrococcus luteus*	KT003279.1	99.8
6	SAS5	*Virgibacillus sp.*	KR347234.1	99.7
7	SAS7	*Bacillus korlensis*	MG595373.1	99.1
8	SAS13	*Staphylococcus pasteuri*	KT003275.1	100
9	SAS14	*Bacillus_sp*	MH118520.1	97.2
10	SAS16	*Bacillus foraminis*	KU983819.1	98.4
11	SAR1	*Bacillus galactosidilyticus*	KY680233.1	99.9
12	SAR6	*Paenibacillus_taichungensis*	KX959965.1	99.4

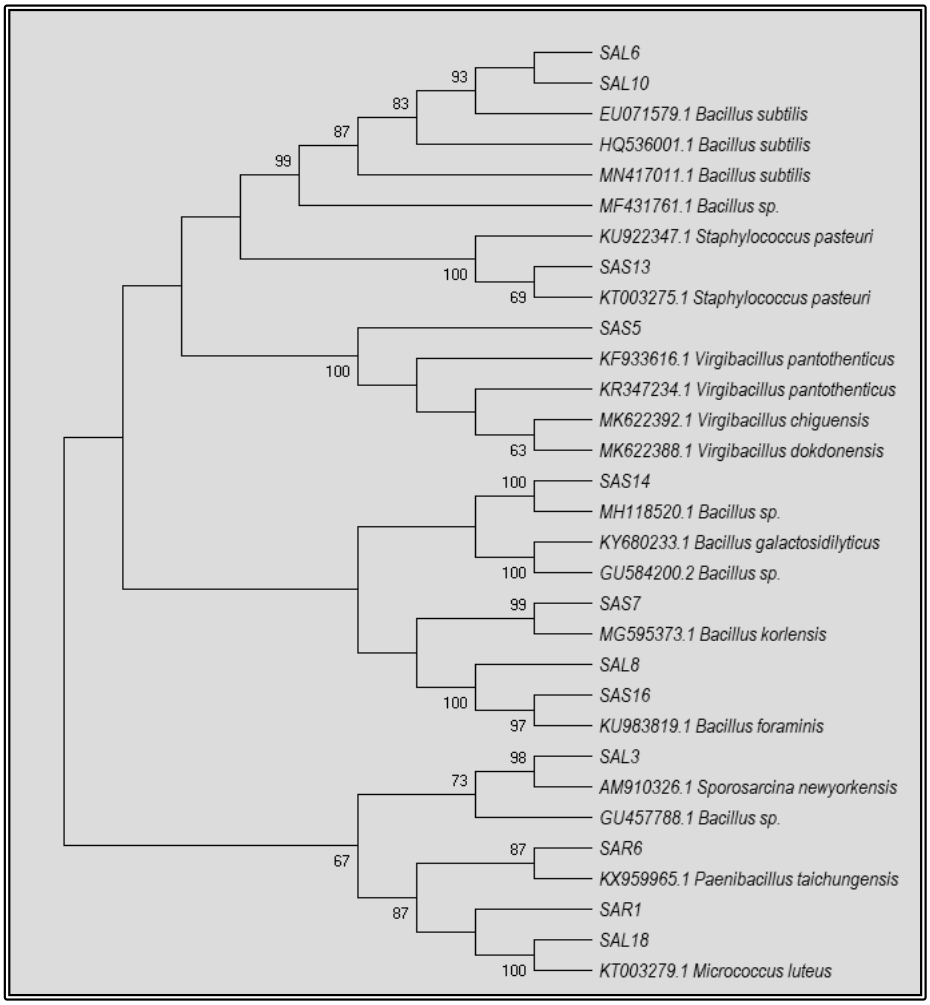


Fig. (1): Phylogenetic analysis of endophytic bacterial isolates with their closest relatives. Maximum Likelihood tree illustrating the evolutionary history, the tree with the highest log likelihood is shown. The bootstrap values higher than 50% are shown on their nodes. The rDNA sequences collected from the GenBank are represented with their accession number followed by the genus and species names.

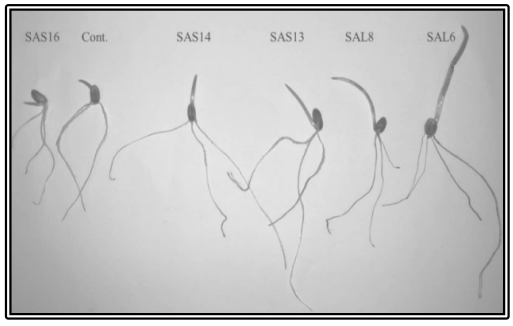


Fig. (2): Seedlings of wheat plant inoculated with endophytic bacterial isolates that induced its growth. Seedlings inoculated with isolates that showed the highest effect are shown relative to a non-inoculated seedling (control).

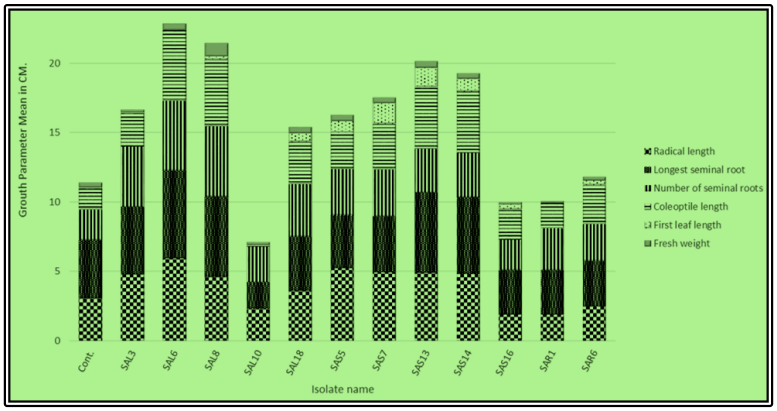
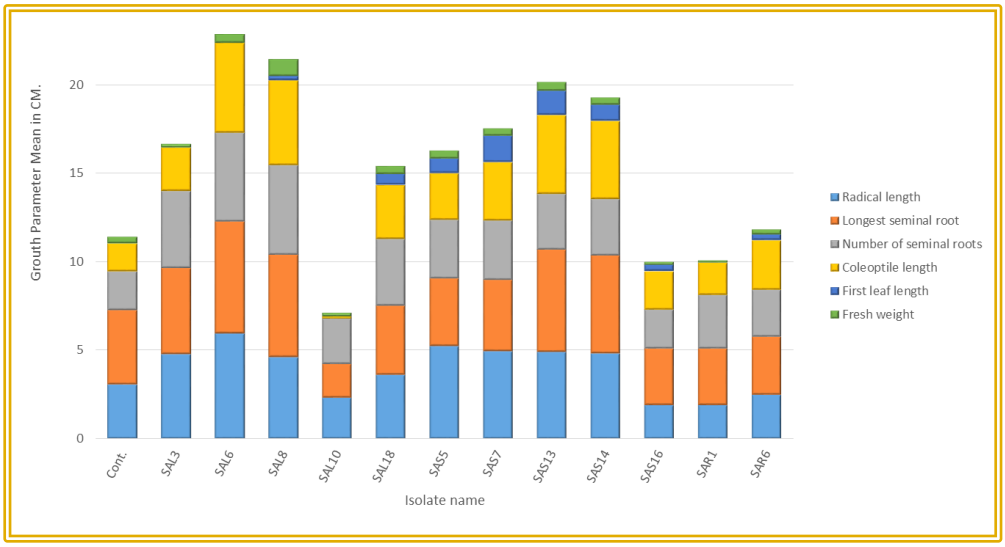


Fig. (3): The effect of endophytic bacterial inoculation on (Qassimi) wheat seeds plant growth parameters. The Y axis indicate each growth parameter in (mm) except for the fresh weight it is presented in grams, while the X axis indicates the isolate name. Each column represents the different parameters for a single isolate. The mean value of all the replica is presented.



Colored copy of Figure (3)

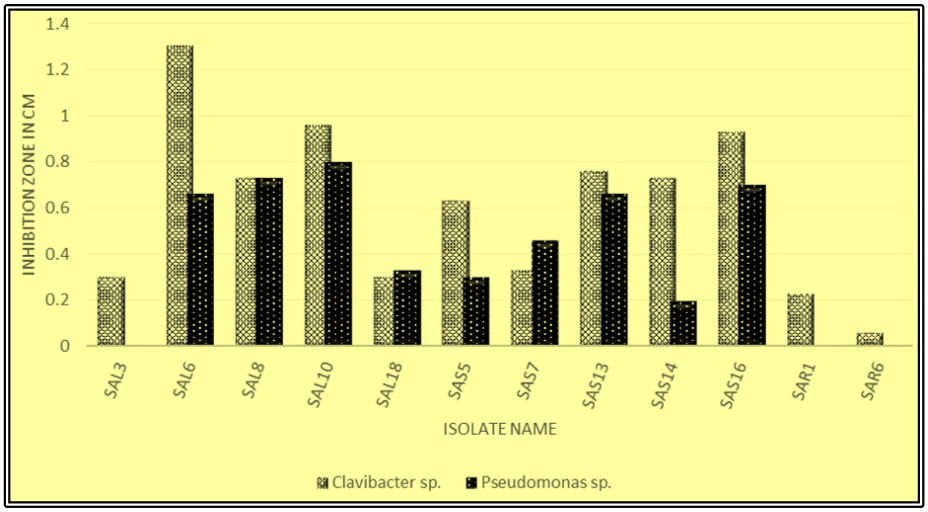


Fig. (4): Histogram illustrating the inhibition zone dimeter mean for all the isolates against two plant pathogenic bacteria *Clavibacter sp* (light color) and *Pseudomonas sp.* (dark color).

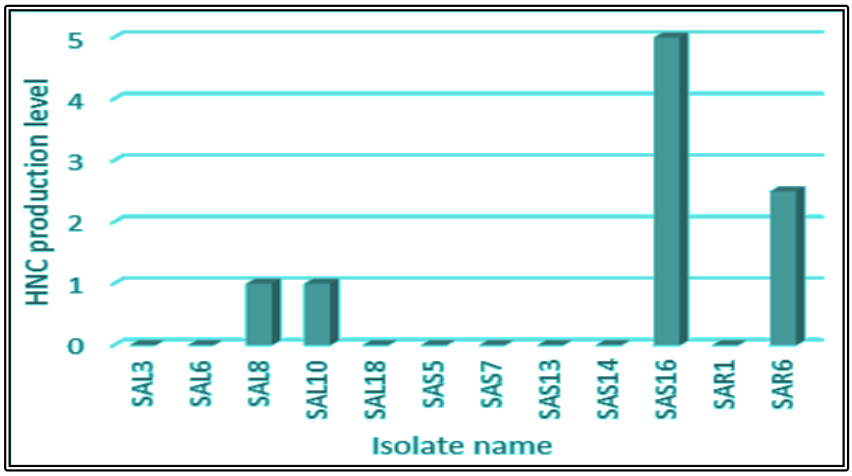


Fig. (5): HCN production level for the endophytic bacterial isolates. The production level was measured from zero to five according to the color intensity, with zero as no change in color and five as dark brown.