

Bacterial endophytes from wild perennial Cicer confer drought tolerance and induced growth promotion in chickpea (*Cicer arietinum* L.)

Zanyar Omer Esmael

Bahman Bahramnejad (✉ b.bahramnejad@uok.ac.ir)

University of Kurdistan <https://orcid.org/0000-0002-8586-2464>

Behrouz Harighi

Nariman S. Ahmad

Hemn Salehi

Hossein Maroofi

Paul H. Goodwin

Research Article

Keywords: *Cicer anatolicum*, *C. oxyodon*, drought tolerance, ACC deaminase, growth promotion

Posted Date: March 15th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1380513/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background and aims

Cicer anatolicum and *C. oxyodon* are the perennial chickpea species found in the Kurdistan region of Iran. They have greater drought tolerance than the most drought tolerant cultivars of chickpea (*Cicer arietinum*). They have not been examined thus far for endophytic bacteria, which could have potential for promoting plant growth and increasing drought stress tolerance. The goal of this study was to isolate and characterize endophytic bacteria from *C. anatolicum* and *C. oxyodon*, and then inoculate selected strains on *C. arietinum* and assess their growth under normal and drought conditions.

Methods

Endophytic bacteria were isolated from stems, roots and leaves of two perennial chickpea species and characterized for growth under temperature and drought stress, production of indole acetic acid, 1-aminocyclopropane-1-carboxylic acid deaminase activity, nitrogen fixation, phosphate solubilization, ammonia production, and enzymatic activities of protease, amylase and lipase. Selected isolates were identified using 16S rDNA sequencing, and used to inoculate soil of cultivated chickpea followed by observations of seed germination, growth and development, and photosynthetic pigment contents.

Results

Among 34 bacterial endophyte isolates from wild perennial chickpeas, *in vitro* activity was observed for 100% of the isolates for indole acetic acid and ammonia production, 50-60% for phosphate solubilization, protease and amylase activities, 30-40% for nitrogen fixation, lipase, drought tolerance and 1-aminocyclopropane-1-carboxylic acid deaminase, and 23% for heat tolerance. For the 14 isolates with the highest levels of most of those traits, there were five *Bacillus* species, *Micrococcus flavus* and *Kocuria rosea*, all of which have not been reported previously as *Cicer* endophytes. Cultivated chickpea, *Cicer arietinum*, was soil inoculated with isolates *Bacillus pumilus* COBR7, *B. pumilus* CAAR3, *Bacillus simplex* CAAR1 or *Bacillus muralis* CASR16. With watering, *B. pumilus* COBR7 most increased seed germination, root/shoot growth and photosynthetic pigments, but with drought, different traits were increased by either *B. simplex* CAAR1, *B. muralis* CASR16 or *B. pumilus* COBR7.

Conclusions

Cicer anatolicum and *C. oxyodon* contains bacterial endophytes not yet reported from *C. arietinum*, some of which promoted plant growth without drought as well as improved drought tolerance of cultivated chickpea in the greenhouse.

Introduction

Drought stress is one of the major important abiotic factors limiting plant growth and crop production under field conditions (Fathi and Tari, 2016). It limits photosynthesis, water and nutrient uptake, and

harms plasma membranes, resulting in decreased growth and yield (Yordanov et al. 2000). One approach to reduce drought stress is the use of endophytes, which are microorganisms that spend at least a part of their life cycle inside plant tissues without causing disease (Hardoim et al. 2015). Endophytes can benefit their host plants through production of phytohormones to promote growth, increased uptake of nutrients like phosphorus or nitrogen, and increased resistance to abiotic and biotic stresses (Santos et al., 2018).

A number of studies have shown that bacterial endophytes can increase plant tolerance to drought stress. For example, application of endophytic *Bacillus subtilis* and *Paenibacillus illinoensis* increased fresh and dry weights of pepper plants under drought (Vigani et al. 2019), and inoculation of endophytic *Burkholderia phytofirmans* and *Enterobacter* sp. increased maize shoot and root dry weights, leaf area, chlorophyll content, photosynthesis, and photochemical efficiency of PSII under drought (Naveed et al. 2014). However, not all endophytes can improve plant growth under drought stress. Only three out of 20 non-identified bacterial endophytes isolated from soybean root tissues significantly increased total chlorophyll content in drought stressed soybean seedlings compared to non-inoculated plants (Dubey et al. 2021). Therefore, many, but not all endophytic bacteria, have the potential to increase plant performance under drought stress.

Cultivated chickpea (*Cicer arietinum* L.) is a member of the Fabaceae plant family that is one of the most widely grown legumes in dryland conditions, and drought is one of the most significant abiotic stresses that limits its yield (Mehta et al. 2015). Chickpea roots contain many endophytic bacterial species. For example, Brigido et al. (2019) cultured 59 endophytic bacteria strains from cultivated chickpea roots belonging to the Proteobacteria, Firmicutes and Actinobacteria with *Enterobacter* and *Pseudomonas* being the most common. Inoculation with some of them can improve plant performance. Egamberdieva et al. (2017a) showed that inoculation of cultivated chickpea with *Mesorhizobium ciceri* and *Bacillus subtilis* isolated from chickpea roots increased yield under saline soil compared to the non-inoculated plants. The only example reported thus far for cultivated chickpea under drought stress is the filamentous bacterium *Streptomyces* isolated from chickpea seeds that improved seed germination and enhanced root and shoot length weight under drought (Kumari et al. 2018).

There are also many wild *Cicer* species. Two examples are the perennial chickpea species, *Cicer anatolicum* and *C. oxyodon*. In western Iran, *C. anatolicum* is normally found in sandy or rocky slopes in scrub or pine forests, and *C. oxyodon* is also normally found in rocky slopes but in oak forests (Van der Maesen, 1987). *Cicer anatolicum* and *C. oxyodon* as well as many other wild perennial species have greater drought tolerance than even the best drought tolerant cultivated chickpea (Toker et al., 2007). While they have been studied for their potential for introducing genes in breeding programs for improved abiotic/biotic stress tolerance and yield of cultivated chickpea (Singh et al. 2008), they also have potential as sources of endophytic bacteria.

The goal of this study was to isolate and characterize endophytic bacteria from wild perennial *C. anatolicum* and *C. oxyodon* from the Kurdistan province in western Iran. They were screened for their tolerance to environmental stress *in vitro* as well as traits related to plant growth promotion and stress

tolerance previously found in bacterial endophytes, such as providing or regulating plant hormones, such as auxin and cytokinin (Asaf et al. 2017), decreasing ethylene, such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase that degrades the ethylene precursor (Glick, 2005), facilitating nutrient acquisition, including nitrogen, phosphorus and iron (White et al. 2018), and producing enzymes, such as protease, amylase, lipase, that can promote plant growth (Carro and Menéndez, 2020). Isolates that showed promising activities were identified to species and then four isolates were used to inoculate cultivated chickpea to evaluate their effect on plant growth promotion and drought tolerance.

Materials And Methods

Plant sampling

Bacterial endophytes were isolated from *C. anatolicum* and *C. oxyodon* collected at the flowering stage from the areas of Todarmella (35°41' N, 46°84' E), Qurichai (36°04' N, 45°95' E), Saral-Divandareh (35°57' N, 48°81' E) and Abidar (35°30' N, 46°95' E) in the Kurdistan province of Iran.

Isolation of endophytic bacteria

Roots, stems and leaves were removed and small pieces of plant samples were washed with tap water, then 1% sodium hypochlorite for 5 min and finally washed 4 times in sterile distilled water (Jasim et al. 2014). 50 µl of the final wash water was plated onto LB agar as a control. The sterilized tissue samples were macerated in 5 ml of sterile-distilled water for 45 min and 100 µl was plated onto Luria-Bertani (LB) agar and incubated at 28°C for 7 days and observed daily for colony formation.

Pigment production and gram stain

Fluorescent pigment production was detected on King's B agar medium with colonies examined at 24 - 48 h after incubation under UV light at 360nm (King et al. 1954). Gram identification was performed with the potassium hydroxide (KOH) test (Buck, 1982).

Growth under temperature and drought stress

Bacteria were grown on LB agar at 30, 35, 40, and 45 °C for 3 days, and colony diameters were measured with the growth expressed relative to growth at 30 °C (Mangla et al. 2014). Bacteria were grown in 20 ml LB broth supplemented with 0, 10.93 or 13.7% polyethylene glycol (PEG) 6000 (Merck, Darmstadt, Germany) to create water potentials of 0, -1.6, and -2.03 MPa, respectively, shaking at 120 rpm at 30°C (Forchetti et al. 2007). Bacterial growth was measured using a spectrophotometer at optical density (OD) 540 nm at two hour intervals for 12 h.

Indole acetic acid (IAA) production

Bacteria were grown in LB broth containing (2% v/v) L-tryptophan and incubated at 28 °C for 10 days shaking at 120 rpm. The amount of IAA in the culture supernatant was determined by centrifuging the

culture at 6000 rpm for 10 min, and adding 1 mL supernatant to 2 ml Salkowski reagent, incubating in the dark for 30 minutes at room temperature, and measuring the OD 530 nm (Gordon and Weber, 1951). A standard curve of IAA (0-250 µg/ml) was also done for quantification.

1-aminocyclopropane-1-carboxylic (ACC) deaminase activity

Bacteria were grown in LB broth with filter sterilized 3 mM ACC instead of 3 mM (NH₄)₂SO₄ as the sole nitrogen source at 30°C with shaking at 120 rpm for 3 days (Honma and Shimomura 1978). α-ketobutyrate production was measured at OD 540 nm, and a standard curve of 0.1 to 1.0 mmol α-ketobutyrate (Merck, Darmstadt, Germany) was done for quantification.

Nitrogen fixation

Bacteria were cultured on Jensen's agar media for 7 days at 28°C (Reddy et al. 2015). Growth of bacterial colonies was considered positive for nitrogen fixation ability.

Phosphate solubilization

Bacteria were cultured on Pikovskaya's agar at 28°C for 7 days and examined for clear zones around the colonies (Pikovskaya, 1948). The solubility index (P-SI) was calculated by:

$$(P-SI) = \frac{\text{Colony diameter (mm)} + \text{Zone diameter (mm)}}{\text{Colony diameter (mm)}}$$

Ammonia production

Bacteria were grown in peptone broth (Malleswari and Bagyanarayana, 2013) at 28°C for 48 h shaking at 120 rpm. The amount of ammonia in the culture supernatant was determined by centrifuging the culture at 3000 rpm for 10 min, and adding 1 mL supernatant to 50 µl Nessler's reagent and incubating for 30 min at room temperature. The OD 450 nm was measured, and a standard curve using 0.5-1 mol/ml ammonium sulfate (Merck, Darmstadt, Germany) was done for quantification (Malleswari and Bagyanarayana, 2013).

Protease, amylase and lipase activities

Bacteria were cultured at 28 °C for 2-3 days on 1% skim-milk agar with a clear zone around a bacterial colony indicating protease production (Sunitha et al. 2013). Bacteria were grown on 1% starch agar at 30°C for 3 days, and the plates flooded with Lugol's iodine to detect a clear zone indicating amylase activity (Sahu et al. 2005). Bacteria were grown on LB agar with 1% CaCl₂·H₂O and 1% Tween 80 at 28 °C for 2-3 days with the presence of white residue around the grown colony indicating lipase production (Kasana et al. 2000).

Genomic DNA extraction

Genomic DNA was extracted from bacteria by using CTAB method with slight modifications. Bacteria were cultured in 10 ml LB broth at 28°C shaking at 120 rpm for 24 h. After centrifugation at 6000 rpm for 5 minutes, 2 ml CTAB extraction buffer was added to the pellet, mixed and then incubated at 100 °C for 10 minutes. The solution was extracted against 25:24:1 phenol: chloroform: isoamyl alcohol, and after centrifugation at 13,000 rpm for 5 minutes, the supernatant was removed. This was repeated, and then 0.6 volume ice-cold isopropanol and 0.1 volume (supernatant plus isopropanol) 3 M sodium acetate was added. After incubation overnight at -20 °C, the solution was centrifuged at 13,000 rpm for 15 minutes, and the pellet washed with 70% ethanol, air-dried and then dissolved in 20-50 µl Tris-EDTA buffer (TE).

16S rRNA sequencing

PCR was performed in 25µl containing 1µl DNA template, 13µl Master Mix (Yekta Tajhiz, Tehran, Iran), 1µM of each primer [27F (5'AGAGTTTGATCATGGCTCAG 3') and 1492R (5'ACGGTTACCTTGTTACGACTT3')] (Weisburg et al. 1991) and 9 µl H₂O. Amplification was performed in MJ Research thermocycler (BioRad, Hercules, California, USA) with an initial denaturation of 94 °C for 4 min, followed by 35 cycles of 94 °C for 45 s, 57 °C for 45 s, 72 °C for 90 s and final extension of 72 °C for 7 min. The PCR products were electrophoresed in 1.2% TAE agarose and stained with 0.01% Safe Stain (Yekta Tajhiz) with a 10 kb ladder (Yekta Tajhiz) used as a size marker. Samples were sent to BioMagic (Tehran, Iran) for sequencing.

The sequences were used as queries in a BLASTn search of the NCBI nr database (<https://www.ncbi.nlm.nih.gov/>). Sequence alignments were performed with the Clustal Omega online tool (Madeira et al. 2019), and trees were prepared using MEGA X software (version 10.2.2) (Kumar et al. 2018) using the minimum evolution method (Rzhetsky and Nei, 1993). Kimura's two-parameter model was used for calculating the distance matrix, and the tree was evaluated by bootstrap analysis of 1,000 resamplings. The Genbank accession numbers for the sequences are provided in Table 6.

Surface sterilization of chickpea seeds and inoculation of bacteria

Four selected isolates, *B. pumilus* COBR7, *B. pumilus* CAAR3, *B. simplex* CAAR1 and *B. muralis* CASR16, were grown in 50 ml LB broth shaking at 120 rpm at 28 C until OD₆₀₀= 0.7 . The cultures were centrifuged at 5800 rpm for 10 minutes, and resuspended in sterile distilled water at 10⁹ CFUs /ml. Seeds of *C. arietinum* cv. Hazarmard was procured from the Agriculture Research Center, Sulaimani, Kurdistan Region-Iraq. After surface sterilization of seeds (Gupta and Pandey 2019) and rinsed with distilled water, they were incubated with the bacterial suspension for 4 hours at room temperature and then air-dried (Zhu et al. 2017). All endophyte inoculation experiments were conducted with three repetitions, and treated seeds with distilled water were used as control.

Effect of endophytic bacteria on seed germination under drought stress

Twenty inoculated seeds per treatment were placed in Petri dishes with sterile filter paper soaked with 5 mL 0, 10.93 or 13.7% PEG 6000 (Lastochkina et al. 2020). The rate of germination (number of seeds

germinated per day), and percentage of germination (number of seeds germinated at 10 days) was determined.

Effect of endophytic bacteria on plant growth under drought stress

A soil mixture containing clay, manure, compost and sand were prepared at the ratio of 2:1:1:1, respectively. The soil mixture was sterilized by autoclaving and then completely drying. Each pot contained 6 kg soil, and the weight of pots was measured after irrigating and allowing the pots to completely drain to determine field capacity. Drought stress was induced after three weeks of full irrigation by maintaining 30% field capacity as the control, 15% field capacity (-0.5 MPa) and 7.5% field capacity (-0.7 MPa) (Mardeh et al. 2014). After 30 days, fresh and dry weight of roots and shoots, shoot height and root length were measured.

Chlorophyll a, Chl b, total Chl and carotenoid contents were measured following maceration of 0.3 g fresh leaf tissue in 2 ml 0.1% (w/v) trichloroacetic acid with a mortar and pestle, centrifuging at 12000 rpm for 15 min, and then mixing 500 µl supernatant with 500 µl 10 mM potassium phosphate buffer pH=7 followed by 1 ml potassium iodide and then centrifugation at 12000 rpm for 15 min. The absorbance of the supernatant was measured at OD 663, 645 and 470 nm, and pigment concentration calculated as Chl a (ppm) = $12.25A_{663} - 2.79A_{646}$, Chl b (ppm) = $21.21A_{646} - 5.1A_{663}$, Chl total (ppm) = Chl a + Chl b, Carotenoids (ppm) = $(1000 A_{470} - 1.82[Chla] - 85.02[Chlb])/198$ (Lichtenthaler and Buschmann 2001).

Statistical analysis

ANOVA was performed with a Tukey test ($p < 0.05$) using SAS software version 1.0.136. Charts were drawn with Excel 2019, and Venn diagrams were drawn with DrawVenn (<http://bioinformatics.psb.ugent.be/webtools/Venn/>). Principal component analysis (PCA) was performed with XLSTAT Version 2016.02.28451.

Results

Source species, tissue, gram stain and fluorescence of culturable endophytic bacteria from wild perennial chickpea

Among the 34 endophyte isolates, 8, 4 and 15 were from *C. anatolicum* roots, stems and leaves, respectively, while 1, 2 and 4 were from *C. oxyodon* roots, stems and leaves, respectively (Table 1). Most were from Saral (18 isolates) followed by Baneh (7 isolates) and Abidar (7 isolates) with only 2 isolate from Marivan. The 20 Gram positive isolates were mostly from *C. anatolicum* leaves (10 isolates), followed by roots (5 isolates) and stems (1 isolates). The 14 Gram negative isolates were also mostly from *C. anatolicum* leaves (5 isolates), followed by roots (3 isolates) and stems (3 isolates). Among the isolates, 17 produced fluorescent pigment which mostly came from *C. anatolicum* leaves (7 isolates),

roots (5 isolates) and then stems (3 isolates), whereas *C. oxyodon* only had 1 isolate with fluorescent pigment from roots and 1 from stems.

Table 1

Source species, collection location, source tissue, Gram stain and fluorescence of endophytic bacteria isolated from *C. anatolicum* and *C. oxyodon*.

Isolate	Source species	Collection location	Source tissue	Gram	Flr
CAAL4	<i>C. anatolicum</i>	Abidar	Leaf	-	-
CAAL5	<i>C. anatolicum</i>	Abidar	Leaf	+	-
CAAL6	<i>C. anatolicum</i>	Abidar	Leaf	+	+
CAAL7	<i>C. anatolicum</i>	Abidar	Leaf	+	+
CAAR1	<i>C. anatolicum</i>	Abidar	Root	+	+
CAAR2	<i>C. anatolicum</i>	Abidar	Root	+	+
CAAR3	<i>C. anatolicum</i>	Abidar	Root	+	-
CAMS1	<i>C. anatolicum</i>	Mariwan	Stem	+	+
CAMS2	<i>C. anatolicum</i>	Mariwan	Stem	-	+
CASL1	<i>C. anatolicum</i>	Saral	Leaf	+	-
CASL10	<i>C. anatolicum</i>	Saral	Leaf	-	+
CASL11	<i>C. anatolicum</i>	Saral	Leaf	+	+
CASL2	<i>C. anatolicum</i>	Saral	Leaf	+	-
CASL3	<i>C. anatolicum</i>	Saral	Leaf	+	-
CASL4	<i>C. anatolicum</i>	Saral	Leaf	+	+
CASL5	<i>C. anatolicum</i>	Saral	Leaf	+	-
CASL6	<i>C. anatolicum</i>	Saral	Leaf	-	+
CASL7	<i>C. anatolicum</i>	Saral	Leaf	-	-
CASL8	<i>C. anatolicum</i>	Saral	Leaf	+	+
CASL9	<i>C. anatolicum</i>	Saral	Leaf	-	-
CASR12	<i>C. anatolicum</i>	Saral	Root	+	-
CASR13	<i>C. anatolicum</i>	Saral	Root	-	+

Isolate code was based on the source species (*C. anatolicum*; CA, and *C. oxyodon*; CO), collection location (Abidar: A, Baneh: B, Marivan: M, and Saral: S) and source tissue (root: R, stem: S, and leaf: L).

Gram: KOH test (+: yes, -: no)

Flr: fluorescence on King's B medium (+: yes, -: no)

Isolate	Source species	Collection location	Source tissue	Gram	Flr
CASR14	<i>C. anatolicum</i>	Saral	Root	-	+
CASR15	<i>C. anatolicum</i>	Saral	Root	-	-
CASR16	<i>C. anatolicum</i>	Saral	Root	+	+
CASS17	<i>C. anatolicum</i>	Saral	Stem	-	+
CASS18	<i>C. anatolicum</i>	Saral	Stem	-	-
COBL3	<i>C. oxyodon</i>	Baneh	Leaf	-	-
COBL4	<i>C. oxyodon</i>	Baneh	Leaf	+	-
COBL5	<i>C. oxyodon</i>	Baneh	Leaf	+	-
COBL6	<i>C. oxyodon</i>	Baneh	Leaf	+	+
COBR7	<i>C. oxyodon</i>	Baneh	Root	+	+
COBS1	<i>C. oxyodon</i>	Baneh	Stem	-	-
COBS2	<i>C. oxyodon</i>	Baneh	Stem	-	-
Isolate code was based on the source species (<i>C. anatolicum</i> ; CA, and <i>C. oxyodon</i> ; CO), collection location (Abidar: A, Baneh: B, Marivan: M, and Saral: S) and source tissue (root: R, stem: S, and leaf: L).					
Gram: KOH test (+: yes, -: no)					
Flr: fluorescence on King's B medium (+: yes, -: no)					

In vitro bacterial growth under temperature and drought stress

All 34 isolates grew at 28°C (control) and 35°C, but 33 isolates grew at 40°C and only 26 isolates grew at 45°C (Table 2). Growth at 28°C was on average 5.2 mm/day ranging from 2.3 to 10.7 mm/day. Relative to growth at 28°C, growth was on average 101% at 35°C, 72% at 40°C and 43% at 45°C. Overall, the least heat tolerant isolates were CASR13, CASR15 and CASS18 with growth less than 25% at 40°C compared to 28°C and no growth at 45°C, whereas the most heat tolerant isolates were CAAR3, COBR7, CAMS2 and CASL10 growing at 100% or greater at the elevated temperatures compared to 28°C. While the least heat tolerant isolates were from roots and stems of *C. anatolicum*, the most heat tolerant isolates were from all tissue types of *C. anatolicum* and *C. oxyodon*.

Table 2

In vitro hormone production of endophytes isolated from *C. anatolicum* and *C. oxyodon*. Activities measured for indole acetic acid (IAA) and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase.

Isolate	IAA ($\mu\text{g/ml/hr}$)	ACC deaminase (nmol/mg/hr)
CAAL4	29.3	0
CAAL5	35.69	0
CAAL6	31.09	0.23
CAAL7	28.53	0
CAAR1	36.2	0.18
CAAR2	34.66	0.328
CAAR3	47.7	0.313
CAMS1	92.17	0
CAMS2	0.92	0
CASL1	63.8	0
CASL10	25.46	0
CASL11	38.5	0
CASL2	64.31	0
CASL3	171.92	0
CASL4	27	0
CASL5	21.63	0.151
CASL6	64.57	0.341
CASL7	8.34	0
CASL8	30.83	0
CASL9	35.94	0
CASR12	36.2	0
CASR13	50.77	0
CASR14	29.55	0
IAA was measured at optical density 530 nm quantified with a standard curve of IAA (0-250 $\mu\text{g/ml}$).		
ACC deaminase activity was measured at optical density 540 nm quantified with a standard curve of α -ketobutyrate (0.1 to 1.0 mmol).		

Isolate	IAA ($\mu\text{g/ml/hr}$)	ACC deaminase (nmol/mg/hr)
CASR15	73.01	0
CASR16	34.41	0.71
CASS17	36.96	0.183
CASS18	3.22	0.024
COBL3	73.77	0.037
COBL4	59.2	0.069
COBL5	149.69	0.071
COBL6	30.06	0
COBR7	72.24	0.394
COBS1	2.97	0
COBS2	62.27	0
IAA was measured at optical density 530 nm quantified with a standard curve of IAA (0-250 $\mu\text{g/ml}$).		
ACC deaminase activity was measured at optical density 540 nm quantified with a standard curve of α -ketobutyrate (0.1 to 1.0 mmol).		

All of the isolates could grow at 0 (control), 1.6 and 2.03 MPa indicating some drought tolerance (Table 2). Growth at 0 mPa was on average 0.82 mm/day ranging from 0.09 to 1.42 mm/day. Relative to 0 MPa, growth was on average 69% at 1.6 MPa and 51% at 2.03 MPa. Overall, the least drought tolerant isolates were CASS17 and CASL10 with growth less than 15% at 2.03 MPa compared to 0 MPa, and the most drought tolerant isolate were CAAR3, COBR7, CASL2 and CAAL5 with growth above 70% at 2.03 MPa compared to 0 MPa. While the least drought tolerant isolates were from stems and leaves of *C. anatolicum*, the most drought tolerant isolates were from roots and leaves of both *C. anatolicum* and *C. oxyodon*.

In vitro production of indole acetic acid (IAA) and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity

IAA production was found in all 34 isolates with an average of 45 $\mu\text{g/ml/hr}$ ranging from 0.92 to 171.92 $\mu\text{g/m/hr}$ (Table 3). ACC deaminase activity was observed in 13 isolates with an average of 0.23 nmol/mg/hr ranging from 0.024 to 0.71 nmol/mg/hr . Most of the isolates with ACC deaminase activity were from leaves (3 from *C. anatolicum* and 3 *C. oxyodon*) followed by roots (4 from *C. anatolicum* and 1 *C. oxyodon*) and stems (2 from *C. anatolicum*).

Table 3

In vitro effect on nutrients by endophytes isolated from *C. anatolicum* and *C. oxyodon*. Measurements of nitrogen fixation (NF), phosphate solubilization (PS) and ammonia production.

Isolate	NF	PS (mm/day)	NH ₃ (mol/ml/hr)
CAAL4	+	-	0.65
CAAL5	-	5	0.72
CAAL6	-	1	0.89
CAAL7	-	3	1.85
CAAR1	+	-	1.35
CAAR2	-	1	0.75
CAAR3	+	3	0.21
CAMS1	+	1	0.2
CAMS2	-	4	1.84
CASL1	-	-	1.35
CASL10	-	3	0.96
CASL11	-	-	0.23
CASL2	-	1	0.88
CASL3	-	-	0.21
CASL4	-	2	1.15
CASL5	-	1	0.73
CASL6	+	-	0.06
CASL7	-	-	2.82
CASL8	+	4	0.88
CASL9	+	-	1.84
CASR12	+	-	1.67

NF: nitrogen fixation was measured based on growth on Jensen's media (+: yes, -: no).

PF: phosphate fixation was measured based on the diameter of clear zone around colonies on Pikovskaya's agar medium (mm).

NH₃: ammonia productions was measured at optical density 450 nm quantified with a standard curve of ammonium sulfate (0.5-1 mol/ml).

Isolate	NF	PS (mm/day)	NH ₃ (mol/ml/hr)
CASR13	+	-	1.1
CASR14	+	3	1.9
CASR15	-	4	0.31
CASR16	+	-	2.56
CASS17	+	1	1.85
CASS18	-	1	0.7
COBL3	-	-	0.35
COBL4	-	1.5	2.02
COBL5	+	-	0.52
COBL6	-	4	0.79
COBR7	+	5	0.91
COBS1	-	1	1.52
COBS2	-	3	0.93
NF: nitrogen fixation was measured based on growth on Jensen's media (+: yes, -: no).			
PF: phosphate fixation was measured based on the diameter of clear zone around colonies on Pikovskaya's agar medium (mm).			
NH ₃ : ammonia productions was measured at optical density 450 nm quantified with a standard curve of ammonium sulfate (0.5-1 mol/ml).			

In vitro **nitrogen fixation, phosphate solubilization and ammonia production**

Nitrogen fixation was observed for 14 isolates with most from roots (6 from *C. anatolicum* and 1 from *C. oxyodon*) followed by leaves (4 from *C. anatolicum* and 1 from *C. oxyodon*) and stems (2 from *C. anatolicum*) (Table 4). Phosphate solubilization was detected in 21 isolates with an average of 0.36 mm/day ranging from 0.14 to 0.71 mm/day (Table 4). Most were from leaves (8 from *C. anatolicum* and 2 from *C. oxyodon*), followed by stems (4 from *C. anatolicum* and 2 from *C. oxyodon*) and roots (4 from *C. anatolicum* and 1 from *C. oxyodon*). Ammonia production was detected in all 34 isolates with an average of 1.07 mol/ml/hr, ranging from 0.06 to 2.82 mol/ml/hr (Table 4).

Table 4

In vitro heat and drought tolerance of endophytes isolated from *C. anatolicum* and *C. oxyodon*. The percent of the growth rate for each isolate is shown for each temperature relative to growth at 28°C, and the percent growth rate for each isolate at each water potential relative to growth at 0 MPa .

Isolate	Heat			Drought	
	35°C	40°C	45°C	1.6 MPa	2.03 MPa
CAAL4	69%	52%	19%	76%	68%
CAAL5	53%	53%	56%	86%	51%
CAAL6	80%	78%	84%	77%	68%
CAAL7	107%	64%	54%	73%	65%
CAAR1	130%	104%	0%	76%	68%
CAAR2	88%	81%	67%	66%	53%
CAAR3	131%	192%	192%	82%	76%
CAMS1	107%	104%	54%	59%	46%
CAMS2	173%	159%	123%	50%	33%
CASL1	107%	107%	93%	80%	70%
CASL10	100%	121%	118%	33%	11%
CASL11	87%	63%	0%	39%	32%
CASL2	22%	22%	15%	100%	73%
CASL3	71%	76%	10%	68%	51%
CASL4	157%	29%	0%	40%	20%
CASL5	58%	32%	11%	82%	79%
CASL6	175%	63%	25%	73%	63%
CASL7	100%	76%	0%	100%	50%
CASL8	171%	14%	14%	68%	47%
CASL9	149%	114%	57%	75%	58%
CASR12	79%	21%	14%	79%	68%

Bacterial growth was measured at optical density 540 nm for bacteria in LB broth at 12 h at 28 C (control) or 35, 40, and 45 °C. Flasks were incubated with shaking (120 rpm).

Bacterial growth was measured at optical density 540 nm for bacteria in LB broth at 12 h with 0 (control), 10.93, 13.7% polyethylene glycol 6000 (PEG) to create water potentials of (0, - 1.6, and - 2.03 MPa), respectively. Flasks were incubated at 28°C with shaking (120 rpm).

Isolate	Heat			Drought	
	35°C	40°C	45°C	1.6 MPa	2.03 MPa
CASR13	107%	21%	0%	80%	68%
CASR14	49%	74%	0%	68%	62%
CASR15	36%	0%	0%	60%	54%
CASR16	117%	81%	11%	23%	18%
CASS17	104%	93%	22%	38%	7%
CASS18	42%	8%	0%	81%	68%
COBL3	116%	58%	11%	71%	53%
COBL4	65%	10%	10%	70%	58%
COBL5	133%	83%	61%	74%	22%
COBL6	62%	95%	110%	100%	17%
COBR7	135%	135%	135%	84%	74%
COBS1	86%	100%	36%	66%	52%
COBS2	163%	67%	78%	60%	40%
Bacterial growth was measured at optical density 540 nm for bacteria in LB broth at 12 h at 28 °C (control) or 35, 40, and 45 °C. Flasks were incubated with shaking (120 rpm).					
Bacterial growth was measured at optical density 540 nm for bacteria in LB broth at 12 h with 0 (control), 10.93, 13.7% polyethylene glycol 6000 (PEG) to create water potentials of (0, – 1.6, and – 2.03 MPa), respectively. Flasks were incubated at 28°C with shaking (120 rpm).					

In vitro protease, amylase and lipase activities

Protease activity was observed for 20 isolates, mostly from leaves (8 from *C. anatolicum* and 1 from *C. oxyodon*) followed by roots (7 from *C. anatolicum* and 1 from *C. oxyodon*) and stems (2 from *C. anatolicum* and 1 from *C. oxyodon*) (Table 5). Amylase activity was observed for 19 isolates, mostly from leaves (7 from *C. anatolicum* and 2 from *C. oxyodon*) followed by roots (5 from *C. anatolicum* and 1 from *C. oxyodon*) and stems (3 from *C. anatolicum* and 1 from *C. oxyodon*) (Table 5). Lipase activity was observed for 12 isolates, mostly from leaves (5 from *C. anatolicum* and 2 from *C. oxyodon*) followed by stems (3 from *C. anatolicum*) and roots (1 from *C. anatolicum* and 1 from *C. oxyodon*) (Table 5). The only isolates lacking all three of these activities were COBS2 and CASL8, whereas the only isolates that had all three activities were COBR7, CAAL7, CAAR1 and CASS17.

Table 5

In vitro extracellular enzyme activities of endophytes isolated from *C. anatolicum* and *C. oxyodon*.

Isolate	Protease	Amylase	Lipase
CAAL4	+	+	-
CAAL5	+	+	-
CAAL6	+	+	-
CAAL7	+	+	+
CAAR1	+	+	+
CAAR2	+	+	-
CAAR3	+	+	-
CAMS1	+	+	-
CAMS2	-	-	+
CASL1	+	-	-
CASL10	+	+	-
CASL11	-	-	+
CASL2	-	-	-
CASL3	-	-	+
CASL4	-	+	-
CASL5	-	-	+
CASL6	-	-	+
CASL7	+	-	-
CASL8	-	-	-
CASL9	+	+	-
CASR12	-	+	-
CASR13	+	-	-

Protease activity was detected as clear zone surrounding the colony on skim-milk agar (+: yes, -: no).

Amylase activity was detected as a clear zone surrounding the colony on starch agar medium flooded with Lugal's iodine solution (+: yes, -: no).

Lipase activity was detected as clear zone surrounding the colony on LB agar with $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ and Tween 80 (+: yes, -: no).

Isolate	Protease	Amylase	Lipase
CASR14	+	+	-
CASR15	+	-	-
CASR16	+	-	-
CASS17	+	+	+
CASS18	-	+	+
COBL3	-	-	+
COBL4	-	+	+
COBL5	+	-	-
COBL6	-	+	-
COBR7	+	+	+
COBS1	+	+	-
COBS2	-	-	-
Protease activity was detected as clear zone surrounding the colony on skim-milk agar (+: yes, -: no).			
Amylase activity was detected as a clear zone surrounding the colony on starch agar medium flooded with Lugal's iodine solution (+: yes, -: no).			
Lipase activity was detected as clear zone surrounding the colony on LB agar with CaCl ₂ .H ₂ O and Tween 80 (+: yes, -: no).			

Comparison of *in vitro* activities

A comparison of the commonality of nitrogen fixation, protease, amylase, lipase and ACC deaminase activity showed that only 3 isolates had all five traits (Fig. 1). The most commonly shared traits were protease with amylase (14 isolates), protease with nitrogen fixation (11 isolates), and amylase with nitrogen fixation (9 isolates). The least commonly shared traits were protease with lipase (4 isolates), nitrogen fixation with lipase (4 isolates) and amylase with lipase (6 isolates).

A bi-plot ordination of PCA for the *in vitro* traits of the 34 isolates showed that the traits were not related to the host species and host tissue (Fig. 2). There was also no clustering of isolates based on any of the other traits.

16S rDNA -based species determination

The 14 isolates with the highest levels of production of IAA and ammonia, activities of protease, amylase, lipase and ACC deaminase, and tolerance to drought and heat were selected for 16S rDNA sequencing. Ten were related to *Bacillus* species (*B. pumilis*, *B. muralis*, *B. safensis*, *B. simplex* and *B.*

zhangzhouensis), three were related to *Micrococcus flavus*, and one was related to *Kocuria rosea* (Table 6). Thus, all were Gram positive with most isolates being in the phylum Firmicutes in the family Bacillaceae, and the remainder being in the phylum Actinobacteria in the family Micrococcaceae. Table 6. Identification of selected endophytic bacteria based on matches of 16S ribosomal DNA sequences.

Isolate	Chickpea species	GenBank accession number	Fragment length (bp)	Closest match in GenBank
CAAR1	<i>C. anatolicum</i>	MW474753.1	1397	<i>Bacillus simplex</i> MT124545.1
CAAR2	<i>C. anatolicum</i>	MW474743.1	1398	<i>Bacillus zhangzhouensis</i> MN826587.1
CAAR3	<i>C. anatolicum</i>	MW474750.1	1419	<i>Bacillus pumilus</i> MT072159.1
CASR16	<i>C. anatolicum</i>	MW474747.1	1422	<i>Bacillus muralis</i> MT081085.1
COBR7	<i>C. oxyodon</i>	MT279500.1	1404	<i>Bacillus pumilus</i> MT279500.1
CASS17	<i>C. anatolicum</i>	MW474748.1	1376	<i>Micrococcus flavus</i> MN511780.1
CASS18	<i>C. anatolicum</i>	MZ713408	1386	<i>Bacillus pumilus</i> NR_043242.1
CAAL4	<i>C. anatolicum</i>	MW474744.1	1421	<i>Bacillus simplex</i> MT065712.1
CAAL6	<i>C. anatolicum</i>	MW474745.1	1447	<i>Bacillus safensis</i> MK123505.1
CASL1	<i>C. anatolicum</i>	MW474746.1	1382	<i>Micrococcus flavus</i> JX094953.1
CASL3	<i>C. anatolicum</i>	MW474752.1	1352	<i>Micrococcus flavus</i> MH282422.1
CASL5	<i>C. anatolicum</i>	OK035711	1459	<i>Bacillus subtilis</i> NR_113265.1
COBL3	<i>C. oxyodon</i>	MW474749.1	1370	<i>Kocuria rosea</i> MT081097.1
COBL4	<i>C. oxyodon</i>	OK035712	1454	<i>Bacillus subtilis</i> NR_113265.1

Discussion

In this study, 34 bacterial endophytes were isolated from healthy tissues of *C. oxyodon*, and *C. anatolicum*. In culture, most of the endophyte strains showed drought and heat tolerance, but this was less common in other studies. Only three of twenty bacterial endophyte strains from soybean root showed drought tolerance at -0.3 MPa (Dubey et al. 2021), and only one of 59 bacterial endophyte strains from roots of *Artemisia princeps*, *Chenopodium ficifolium*, *Oenothera biennis* and *Echinochloa crus-galli* showed heat tolerance at 45°C (Khan et al. 2020). The relatively widespread drought and heat tolerance of the endophytes in this study may be an adaptation to their perennial chickpea hosts that grow in warm arid areas.

IAA production was found in all the endophyte strains, but ACC deaminase activity was observed only in about a third of the strains. This is higher than bacterial endophytes from roots and leaves of wild tea species where 23 of 46 strains showed IAA production and 13 showed ACC deaminase activity (Borah and Thakor, 2020). Selecting for strains with these hormone-related traits have been related to plant growth promotion. For example, pepper growth was promoted by inoculation with IAA producing endophytes through stimulation of root lengthening and development of adventitious roots (Borah and Thakur, 2020), and maize plants by inoculation with ACC deaminase producing endophytes through reduced stress-induced ethylene via degradation of the ethylene precursor ACC (Naveed et al. 2014).

Nitrogen fixation and phosphate solubilization was observed for approximately half of the strains, whereas ammonia production was observed in all strains but only 40% of the strains showed relatively high production (above 1.0 mol/ml/hr). By comparison, Saini et al. (2015) and Brigido et al (2019) found 42% and 18% of bacterial root endophytes from cultivated chickpea had phosphate solubilization, respectively, whereas 75% and 70% of the strains showed ammonia production, respectively. However, among those ammonia producing strains, Saini et al. (2015) found only 33% of strains with production above 1.0 mol/ml/hr, whereas Brigido et al (2019) did not report the level of production. Phosphate solubilization inside plants is important as free phosphate reacts with calcium and other metals inside plant tissues, making them insoluble (Varga et al. 2020). Inoculation with phosphate solubilizing endophytic bacteria increased phosphate content and growth of pepper and tomato (Mei et al. 2021). Ammonia production inside plant tissues can make the nitrogen more available for plant growth (Banik et al. 2019). Inoculation with ammonia-producing endophytic *Stenotrophomonas* and *Pseudomonas* species increased growth and chlorophyll content of tea bushes (Hazarika et al. 2021). Inoculation with nitrogen fixing endophytes can also be beneficial, such as nitrogen-fixing endophytic bacteria that alleviated nitrogen deficiency and promoted growth of maize, tomato, pepper, squash, sunflower and grasses (Khan et al. 2012).

Protease and amylase activities were observed for approximately half the strains, whereas lipase activity was only observed for about a third of the strains. This is similar to other studies, such as endophytic bacterial strains from *Jacaranda decurrens* with 60% having protease, 60% having amylase and 40% having lipase activities (Carrim et al. 2006), and endophytic bacteria from two mangrove species with

75% of the strains having protease, 45% having amylase and 52% having lipase activities (Castro et al. 2014). Endophytic bacterial amylases can help hydrolyze starch providing additional energy for plant growth (Islam et al. 2016), as well as induction of phytohormones, such as gibberellins, to stimulate plant growth (Kaneko et al. 2002). Endophytic bacterial lipases can hydrolyze storage lipids also providing additional energy for plant growth as well as acting as stress signals (Matos and Pham-Thi, 2009). Endophytic microbial proteases can help recycle proteins, releasing nutrients and acting as nutritional signals (Sims, 2006). Thus, endophyte strains with protease, amylase and lipase activities could provide multiple benefits to plants.

Identification of several of the endophyte strains in this study showed that they were five species of *Bacillus* as well as one species each of *Micrococcus* and *Kocuria*. All have been described previously as endophytes, such as *B. simplex* in canola roots (Martínez-Hidalgo et al. 2021), *B. zhangzhouensis* in *Arctotheca calendula* roots (Thompson, 2020), *B. pumilus* in poplar stems and *Ocimum sanctum* stems and leaves (Murugappan et al. 2013), *B. muralis* in bean leaves, cucumber shoots and sorghum roots (Gamalero et al. 2020), *B. safensis* in *Chloris virgata* roots (Wu et al. 2019), *M. flavus* in *Vernonia anthelmintica* roots (Rustamova et al. 2020) and *K. rosea* in *Lolium perenne* stems (Kukla et al. 2014).

There have been at least seven previous studies identifying bacterial endophytes from *Cicer*, but all have examined endophytes of only *C. arietinum* (Rangeshwaran et al, 2008; Egamberdieva et al, 2017b; Brigido et al., 2019; Maheshwari et al, 2019; Chhabra and Sharma 2019; Alok et al. 2020; Gorai et al. 2021). Those studies have shown that many endophytic *Bacillus* species occur in *C. arietinum*, such as *B. subtilis*, *B. thuringiensis*, *B. cereus* (Egamberdieva et al, 2017b), *B. megaterium*, *B. circulans* (Rangeshwaran et al, 2008) and *B. siamensis* (Gorai et al. 2021), but none of the five *Bacillus* species described in this work as well as *M. flavus* and *K. rosea* have been previously described as endophytes in *Cicer*. Therefore, it appears that many of the endophytes of *C. anatolicum* and *C. oxyodon* may be different from those from *C. arietinum*.

The percent and rate of seed germination of *C. arietinum* was most improved with *B. pumilus* COBR7 without drought and *B. simplex* CAAR1 with the highest level of drought. Other reports of seed inoculation with bacterial endophytes enhancing seed germination include increased *Lepidium perfoliatum* seeds with 28 out of 62 endophytic bacterial strains (Li et al. 2017), and maize seeds with 6 endophytic bacterial strains (Gholami et al. 2009). Seed germination under drought can be inhibited by a variety of factors, such as reduced water availability for enzymatic reactions and hydrolytic cleavage of storage compounds, as well as reduced movement of metabolites (Bialecka and Kepczynski, 2009). For fungal endophytes that increased wheat seed germination, Hubbard et al. (2012) proposed that they could have increased stress-response gene and hormone levels, osmolyte and proline levels, and antioxidant enzyme activities. Thus, the better seed performance with *B. simplex* CAAR1 and *B. pumilus* COBR7 could have been for similar reasons.

Root growth of *C. arietinum* was most improved with *B. pumilus* COBR7 (dry and fresh weight) and *B. muralis* CASR16 (length) without drought and *B. pumilus* COBR7 for all three parameters with the highest

level of drought. Similarly, seed inoculation enhanced root growth of maize seeds with *B. cereus* and *B. subtilis* endophytes increasing root fresh and dry weights (Fouda et al. 2021) and rice seeds with seven bacterial endophytes increasing root length, fresh weight and dry weight (Etesami et al. 2014). Drought can reduce root growth by mechanisms, such as changing hormone balance (Khan et al. 2018) and reducing mineral uptake (Bista et al. 2018). Under drought stress, root growth was increased following inoculation of soybean with endophytic *B. cereus* by reducing ethylene via ACC deaminase activity (Dubey et al. 2021), alfalfa with endophytic *B. megaterium* by phosphate solubilization (Kang et al. 2021), and *Arabidopsis* with endophytic *B. subtilis* by induction of auxin synthesis (Zhang et al. 2007). The improved root growth of *B. pumilus* COBR7 may have involved one or more of those mechanisms and appears to have been effective both under drought and non-drought conditions.

Shoot growth of endophyte inoculated *C. arietinum* was most improved with *B. pumilus* COBR7 for dry weight, fresh weight and length without drought, but with the highest level of drought, only *B. pumilus* COBR7 increased both dry and fresh shoot weight and *B. muralis* CASR16 increased shoot length. Examples of seed inoculation with bacterial endophytes enhancing shoot growth include *C. arietinum* with endophytic *Mesorhizobium ciceri* and *Bacillus subtilis* for shoot length and dry weight (Egamberdieva et al. 2017b), and tomato with endophytic *B. megaterium* for shoot length (Yavarian et al. 2021). Shoot growth can be inhibited by drought due to factors, such as decreased photosynthesis from stomatal closure, membrane damage and reduced ATP synthesis (Kumawat and Sharma, 2018). Increased shoot growth of mung bean under drought stress following inoculation with an endophytic *Pseudomonas aeruginosa* was related to increased expression of drought stress-responsive gene, like dehydrin (Sarma and Saikia, 2014) and maize with an endophytic *Burkholderia phytofirmans* due to reduced ethylene related to ACC deaminase activity (Naveed et al. 2014). Improved shoot growth of *B. pumilus* COBR7 appears to have been effective both under drought and non-drought conditions, and may have involved one or more of those mechanisms.

Levels of photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll and carotenoid) in this study were most increased with *B. pumilus* COBR7 for all the measured parameters without drought, but with the highest level of drought, only *B. pumilus* COBR7 was effective for chlorophyll a, total chlorophyll and carotenoid, whereas only *B. muralis* CASR16 was effective for chlorophyll b. Except for chlorophyll b, drought stress reduced levels of the photosynthetic pigments, which could be due to oxidative stress resulting in pigment photo-oxidation as well as chlorophyll degradation related to stomatal closure (Reddy et al. 2004). Endophyte-induced increases in photosynthetic pigments under drought stress have been reported for wheat with endophytic *Azotobacter chroococcum* and *Pseudomonas* possibly due to increased cytokinin (El-Afry et al. 2012), sugar beet with endophytic *Bacillus pumilus* and *Acinetobacter johnsonii* producing an unknown compound promoting electron transport (Shi et al. 2010), and rice with endophytic *Citrobacter* with nitrogenase activity increasing available nitrogen (Hongrattipun et al. 2014). Thus, *B. pumilus* COBR7 and *B. muralis* CASR16 may have increased photosynthetic pigments by one or more of those mechanisms.

Conclusions

Among the endophytes tested in this study, one can conclude that *B. pumilus* COBR7 was the most promising without drought as it most often increased the percent and rate of seed germination, root and shoot growth, and levels of photosynthetic pigments. This may be related to one or more of the multiple traits detected in pure culture of *B. pumilus* COBR7, which compared to the other endophytes, had the second highest growth under temperature stress and second highest growth under drought stress, as well as having the fourth highest IAA production, second highest ACC deaminase activity and highest phosphate solubilization. However, it showed relatively low ammonia production (16th highest). In addition, *B. pumilus* COBR7 showed protease, lipase, amylase and nitrogen fixation activities. However, under drought conditions *B. simplex* CAAR1 and/or *B. muralis* CASR16 were also the best endophytes for certain traits. Thus, some endophytes may differ in their growth promoting activities under drought stress, which may be critical as most chickpea fields are grown in dry lands. Future studies can examine combinations of bacterial endophytes, different chickpea genotypes and other dryland crops and other traits, such as yield. However, this study has shown that at least three bacterial endophytes strains from wild chickpea plants are potentially useful bio-inoculants of cultivated chickpea.

Declarations

Acknowledgements

This work was financially supported by the University of Kurdistan.

Authorship contribution statement

Conceptualization, BB and PHG; methodology, ZOE, HS, HM and BH; writing-original draft preparation, ZOE, HS; writing—review and editing, PHG, BB, BH and NAS; supervision, BB. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

We confirm that all the data generated and material used in this current study are available within this manuscript.

References

1. Asaf, S., Khan, M.A., Khan, A.L., Waqas, M., Shahzad, R., Kim, A.Y., Kang, S.M. and Lee, I.J. (2017) Bacterial endophytes from arid land plants regulate endogenous hormone content and promote

- growth in crop plants: an example of *Sphingomonas* sp. and *Serratia marcescens*. *Journal of Plant Interactions* **12**: 31-38.
2. Alok, D., Annapragada, H., Singh, S., Murugesan, S. and Singh, N.P. (2020) Symbiotic nitrogen fixation and endophytic bacterial community structure in Bt-transgenic chickpea (*Cicer arietinum* L). *Scientific reports* **10**:1-12.
 3. Banik, A., Dash, G. K., Swain, P., Kumar, U., Mukhopadhyay, S. K., and Dangar, T. K. (2019) Application of rice (*Oryza sativa* L.) root endophytic diazotrophic *Azotobacter* sp. strain Avi2 (MCC 3432) can increase rice yield under green house and field condition. *Microbiological Research* **219**: 56-65.
 4. Bialecka, B. and Kepczynski, J. (2009) Effect of ethephon and gibberellin A3 on *Amaranthus caudatus* seed germination and alpha-and beta-amylase activity under salinity stress. *Acta Biologica Cracoviensia. Series Botanica* **2**:119-125.
 5. Bista, D. R., Heckathorn, S. A., Jayawardena, D. M., Mishra, S., and Boldt, J. K. (2018) Effects of drought on nutrient uptake and the levels of nutrient-uptake proteins in roots of drought-sensitive and-tolerant grasses. *Plants* **7**: 28.
 6. Borah, A., and Thakur, D. (2020) Phylogenetic and functional characterization of culturable endophytic actinobacteria associated with *Camellia* spp. for growth promotion in commercial tea cultivars. *Frontiers in Microbiology* **11**: 318.
 7. Brigido, C., Singh, S., Menéndez, E., Tavares, M. J., Glick, B. R., Félix, M. D. R Oliveira, S. and Carvalho, M. (2019) Diversity and functionality of culturable endophytic bacterial communities in chickpea plants. *Plants* **8**: 42.
 8. Buck, J.D. (1982) Nonstaining (KOH) method for determination of gram reactions of marine bacteria. *Applied and Environmental Microbiology* **44**: 992-993.
 9. Carrim, A.J.I., Barbosa, E.C. and Vieira, J.D.G. (2006) Enzymatic activity of endophytic bacterial isolates of *Jacaranda decurrens* Cham. (Carobinha-do-campo). *Brazilian Archives of Biology and Technology* **49**: 353-359.
 10. Carro, L., and Menéndez, E. (2020) Knock, knock-let the bacteria in: Enzymatic potential of plant associated bacteria. In V. Sharma, R. Salwan, and L. Tawfeeq (Eds.), *Molecular Aspects of Plant Beneficial Microbes in Agriculture* (pp. 169-178). Academic Press.
 11. Castro, R.A., Quecine, M.C., Lacava, P.T., Batista, B.D., Luvizotto, D.M., Marcon, J., Ferreira, A., Melo, I.S. and Azevedo, J.L. (2014) Isolation and enzyme bioprospection of endophytic bacteria associated with plants of Brazilian mangrove ecosystem. *Springer Plus*, **3**: 1-9.
 12. Chhabra, D. and Sharma, P. (2019) Non rhizobial endophytic bacteria from Chickpea (*Cicer arietinum* L.) tissues and their antagonistic traits. *Journal of Applied and Natural Science* **11**:346-351.
 13. Dubey, A. Saiyam, D. Kumar, A. Hashem, A. Abd_Allah, E.F. Khan, M.L. (2021) Bacterial root endophytes: Characterization of their competence and plant growth promotion in soybean (*Glycine max* (L.) Merr.) under drought stress. *International Journal of Environmental Research and Public Health* **18**: 931.

14. Egamberdieva, D., Wirth, S. J., Alqarawi, A. A., Abd_Allah, E. F., and Hashem, A. (2017a) Phytohormones and beneficial microbes: essential components for plants to balance stress and fitness. *Frontiers in Microbiology* **8**: 1-14.
15. Egamberdieva, D., Wirth, S. J., Shurigin, V. V., Hashem, A., and Abd_Allah, E. F. (2017b) Endophytic bacteria improve plant growth, symbiotic performance of chickpea (*Cicer arietinum* L.) and induce suppression of root rot caused by *Fusarium solani* under salt stress. *Frontiers in Microbiology* **8**: 1887.
16. El-Afry, M. M., El-Nady, M. F., Belal, E. B. A., and Metwaly, M. M. S. (2012) Physiological responses of drought stressed wheat plants (*Triticum aestivum* L.) treating with some bacterial endophytes. *Journal of Plant Production* **3**: 2069-2089.
17. Etesami, H., Mirsyed Hosseini, H., and Alikhani, H. A. (2014) *In planta* selection of plant growth promoting endophytic bacteria for rice (*Oryza sativa* L.). *Journal of Soil Science and Plant Nutrition* **14**: 491-503.
18. Fathi, A., and Tari, D.B. (2016) Effect of drought stress and its mechanism in plants. *International Journal of Life Sciences* **10**: 1-6.
19. Forchetti, G., Masciarelli, O., Alemano, S., Alvarez, D., and Abdala, G. (2007) Endophytic bacteria in sunflower (*Helianthus annuus* L.): isolation, characterization, and production of jasmonates and abscisic acid in culture medium. *Applied Microbiology and Biotechnology* **76**: 1145-1152.
20. Fouda, A., Eid, A.M., Elsaied, A., El-Belely, E.F., Barghoth, M.G., Azab, E., Gobouri, A.A. and Hassan, S.E.D. (2021) Plant growth-promoting endophytic bacterial community inhabiting the leaves of *Pulicaria incisa* (Lam.) DC inherent to arid regions. *Plants* **10**: 76-98.
21. Gamalero, E., Favale, N., Bona, E., Novello, G., Cesaro, P., Massa, N., Iick, B.R., Orozco-Mosqueda, M.D.C., Berta, G. and Lingua, G. (2020) Screening of bacterial endophytes able to promote plant growth and increase salinity tolerance. *Applied Sciences* **10**: 5767.
22. Gholami, A., Shahsavani, S., and Nezarat, S. (2009) The effect of plant growth promoting rhizobacteria (PGPR) on germination, seedling growth and yield of maize. *World Academy of Science, Engineering and Technology* **49**: 19-24.
23. Glick, B. R. (2005) Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. *FEMS Microbiology Letters* **251**: 1-7.
24. Gorai, P.S., Ghosh, R., Mandal, S., Ghosh, S., Chatterjee, S., Gond, S.K. and Mandal, N.C. (2021) *Bacillus siamensis* CNE6-a multifaceted plant growth promoting endophyte of *Cicer arietinum* L. having broad-spectrum antifungal activities and host colonizing potential. *Microbiological Research* **252**: 126859.
25. Gordon, S. A., and Weber, R. P. (1951) Colorimetric estimation of indoleacetic acid. *Plant Physiology* **26**: 192.
26. Gupta, S., and Pandey, S. (2019) ACC deaminase producing bacteria with multifarious plant growth promoting traits alleviates salinity stress in French bean (*Phaseolus vulgaris*) plants. *Frontiers in Microbiology* **10**: 1506.

27. Hardoim, P.R., Van Overbeek, L.S., Berg, G., Pirttilä, A.M., Compant, S., Campisano, A., Döring, M., and Sessitsch, A. (2015) The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiology and Molecular Biology Reviews* **79**: 293-320.
28. Hazarika, S. N., Saikia, K., Borah, A., and Thakur, D. (2021) Prospecting endophytic bacteria endowed with plant growth promoting potential isolated from *Camellia sinensis*. *Frontiers in Microbiology* **12**: 738058
29. Hongrittipun, P., Youpensuk, S., and Rerkasem, B. (2014) Screening of nitrogen fixing endophytic bacteria in *Oryza sativa* L. *Journal of Agricultural Science* **6**: 66.
30. Honma, M., and Shimomura, T. (1978) Metabolism of 1-aminocyclopropane-1-carboxylic acid. *Agricultural and Biological Chemistry* **42**: 1825-1831.
31. Hubbard, M., Germida, J., and Vujanovic, V. (2012) Fungal endophytes improve wheat seed germination under heat and drought stress. *Botany* **90**: 137-149.
32. Islam, S., Akanda, A. M., Prova, A., Islam, M. T., and Hossain, M. M. (2016) Isolation and identification of plant growth promoting rhizobacteria from cucumber rhizosphere and their effect on plant growth promotion and disease suppression. *Frontiers in Microbiology* **6**: 1360.
33. Jasim, B., Joseph, A. A., John, C. J., Mathew, J., and Radhakrishnan, E. K. (2014) Isolation and characterization of plant growth promoting endophytic bacteria from the rhizome of *Zingiber officinale*. *3 Biotech* **4**: 197-204.
34. Kaneko, M., Itoh, H., Ueguchi-Tanaka, M., Ashikari, M., and Matsuoka, M. (2002) The α -amylase induction in endosperm during rice seed germination is caused by gibberellin synthesized in epithelium. *Plant Physiology* **128**: 1264-1270.
35. Kang, S. M., Khan, M. A., Hamayun, M., Kim, L. R., Kwon, E. H., Kang, Y.S., Kim, K.Y., Park, J.J. and Lee, I.J. (2021) Phosphate-solubilizing *Enterobacter ludwigii* AFFR02 and *Bacillus megaterium* Mj1212 rescues alfalfa's growth under post-drought stress. *Agriculture* **11**: 485.
36. Kasana, R. C., Salwan, R., Dhar, H., Dutt, S., and Gulati, A. (2008) A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. *Current Microbiology* **57**: 503-507.
37. Khan, M. A., Asaf, S., Khan, A. L., Jan, R., Kang, S. M., Kim, K. M., and Lee, I. J. (2020) Thermotolerance effect of plant growth-promoting *Bacillus cereus* SA1 on soybean during heat stress. *BMC Microbiology* **20**: 1-14.
38. Khan, Z., Guelich, G., Phan, H., Redman, R., and Doty, S. (2012) Bacterial and yeast endophytes from poplar and willow promote growth in crop plants and grasses. *International Scholarly Research Notices* **2012**: 890280
39. King, E. O., Ward, M. K., and Raney, D. E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *Translational Research* **44**: 301-307.
40. Kukla, M., Płociniczak, T., and Piotrowska-Seget, Z. (2014) Diversity of endophytic bacteria in *Lolium perenne* and their potential to degrade petroleum hydrocarbons and promote plant growth.

41. Kumari, V., Germida, J., and Vujanovic, V. (2018) Legume endosymbionts: Drought stress tolerance in second-generation chickpea (*Cicer arietinum*) seeds. *Journal of Agronomy and Crop Science*, **204**: 529-540.
42. Kumawat, K. R., and Sharma, N. K. (2018) Effect of drought stress on plants growth. *Popular Kheti* **6**: 239-241.
43. Lastochkina, O., Garshina, D., Ivanov, S., Yuldashev, R., Khafizova, R., Allagulova, C., Fedorova, K., Avalbaev, A., Maslennikova, D. and Bosacchi, M. (2020) Seed priming with endophytic *Bacillus subtilis* modulates physiological responses of two different *Triticum aestivum* L. cultivars under drought stress. *Plants* **9**: 1810.
44. Li, Y., Cheng, C., and An, D. (2017) Characterisation of endophytic bacteria from a desert plant *Lepidium perfoliatum* L. *Plant Protection Science* **53**: 32-43.
45. Lichtenthaler, H. K., and Buschmann, C. (2001) Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. *Current Protocols in Food Analytical Chemistry* **1**: 4-3.
46. Madeira, F., Park, Y.M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., Basutkar, P., Tivey, A.R., Potter, S.C., Finn, R.D., and Lopez, R. (2019) The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Research* **47**: 636-641.
47. Maheshwari, R., Bhutani, N., and Suneja, P. (2020) Isolation and characterization of ACC deaminase producing endophytic *Bacillus mojavensis* PRN2 from *Pisum sativum*. *Iranian Journal of Biotechnology*, **18**: e2308.
48. Malleswari, D. and Bagyanarayana, G. (2013) *In vitro* screening of rhizobacteria isolated from the rhizosphere of medicinal and aromatic plants for multiple plant growth promoting activities. *Journal of Microbiology and Biotechnology Research* **3**: 84-91..
49. Mangla, S., Kumar, P., and Barua, M. K. (2014) An evaluation of attribute for improving the green supply chain performance via DEMATEL method. *International Journal of Mechanical Engineering and Robotics Research* **1**: 30-35.
50. Mardeh, A. S. S., Gholami, S., Bahramnejad, B., Kanouni, H., and Sadeghi, F. (2014) Effect of drought stress on compatible osmolytes content, enzyme activity and grain yield in chickpea (*Cicer arietinum* L.) genotypes. *Iranian Journal of Crop Sciences* **16**: 109-124
51. Martínez-Hidalgo, P., Flores-Félix, J. D., Sánchez-Juanes, F., Rivas, R., Mateos, P. F., Santa Regina, I., Peix, Á., Martínez-Molina, E., Igual, J.M. and Velázquez, E. (2021) Identification of canola roots endophytic bacteria and analysis of their potential as biofertilizers for canola crops with special emphasis on sporulating bacteria. *Agronomy* **11**: 1796.
52. Matos, A. R., and Pham-Thi, A. T. (2009) Lipid deacylating enzymes in plants: old activities, new genes. *Plant Physiology and Biochemistry* **47**: 491-503.
53. Mehta, G., Verma, P. K., and Ravi, M. (2015) Correlation studies in chickpea grown under rainfed and irrigated conditions in Northern Plains of India. *Journal of Agroecology and Natural Resource Management* **2**: 388-390.

54. Mei, C., Chretien, R. L., Amaradasa, B. S., He, Y., Turner, A., and Lowman, S. (2021) Characterization of phosphate solubilizing bacterial endophytes and plant growth promotion *in vitro* and in greenhouse. *Microorganisms* **9**: 1935.
55. Murugappan, R. M., Begum, S. B., and Roobia, R. R. (2013) Symbiotic influence of endophytic *Bacillus pumilus* on growth promotion and probiotic potential of the medicinal plant *Ocimum sanctum*. *Symbiosis* **60**: 91-99.
56. Naveed, M., Mitter, B., Reichenauer, T. G., Wieczorek, K., and Sessitsch, A. (2014) Increased drought stress resilience of maize through endophytic colonization by *Burkholderia phytofirmans* PsJN and *Enterobacter* sp. FD17. *Environmental and Experimental Botany* **97**: 30-39.
57. Pikovskaya, R. I. (1948) Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Mikrobiologiya* **17**: 362-370.
58. Rangeswaran, R., Raj, J., and Kumar, P. S. (2008) Identification of endophytic bacteria in chickpea (*Cicer arietinum* L.) and their effect on plant growth. *Journal of Biological Control* **22**: 13-23.
59. Reddy, A. R., Chaitanya, K. V., and Vivekanandan, M. (2004) Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *Journal of Plant Physiology* **161**: 1189-1202.
60. Rustamova, N., Wubulikasimu, A., Mukhamedov, N., Gao, Y., Egamberdieva, D., and Yili, A. (2020) Endophytic bacteria associated with medicinal plant *Vernonia anthelmintica*: diversity and characterization. *Current Microbiology* **77**: 1457-1465.
61. Rzhetsky, A., and Nei, M. (1993) Theoretical foundation of the minimum-evolution method of phylogenetic inference. *Molecular Biology and Evolution* **10**: 1073-1095.
62. Sahu, M. K., Sivakumar, K., and Kannan, L. (2005) Degradation of organic matters by the extra-cellular enzymes of actinomycetes isolated from the sediments and molluscs of the Vellar estuary. *Journal of Aquatic Biology* **20**: 142-144.
63. Saini, R., Dudeja, S. S., Giri, R., and Kumar, V. (2015) Isolation, characterization, and evaluation of bacterial root and nodule endophytes from chickpea cultivated in Northern India. *Journal of Basic Microbiology* **55**: 74-81.
64. Santos, M.L.D., Berlitz, D.L., Wiest, S.L.F., Schunemann, R., Knaak, N. and Fiuza, L.M. (2018) Benefits associated with the interaction of endophytic bacteria and plants. *Brazilian Archives of Biology and Technology* **61**: e18160431
65. Sarma, R. K., and Saikia, R. (2014) Alleviation of drought stress in mung bean by strain *Pseudomonas aeruginosa* GGRJ21. *Plant and Soil* **377**: 111-126.
66. Shi, Y., Lou, K., and Li, C. (2010) Growth and photosynthetic efficiency promotion of sugar beet (*Beta vulgaris* L.) by endophytic bacteria. *Photosynthesis Research* **105**: 5-13.
67. Sims, G. K. (2006) Nitrogen starvation promotes biodegradation of N-heterocyclic compounds in soil. *Soil Biology and Biochemistry* **38**: 2478-2480.
68. Singh, R., Sharma, P., Varshney, R.K., Sharma, S.K., and Singh, N.K. (2008) Chickpea improvement: role of wild species and genetic markers. *Biotechnology and Genetic Engineering Reviews* **25**: 267-

69. Sunitha, V. H., Devi, D. N., and Srinivas, C. (2013) Extracellular enzymatic activity of endophytic fungal strains isolated from medicinal plants. *World Journal of Agricultural Sciences* **9**: 01-09.
70. Thompson, B. (2020) *Isolation and characterization of bacterial endophytes for growth promotion of Phaseolus vulgaris under salinity stress*. University of the Western Cape, Cape Town, South Africa.
71. Toker, C., Canci, H. and Yildirim, T. (2007) Evaluation of perennial wild *Cicer* species for drought resistance. *Genetic Resources and Crop Evolution* **54**: 1781–1786.
72. Vn der Maesen, L. J. G. (1987) Origin, history and taxonomy of Chickpea. In M. C. Saxena, and K. B. Singh (Eds.), *The Chickpea* (pp. 11-34). C.A.B. International.
73. Varga, T., Hixson, K. K., Ahkami, A. H., Sher, A. W., Barnes, M. E., Chu, R. K., Battu, A.K., Nicora, C.D., Winkler, T.E., Reno, L.R. and Fakra, S.C. (2020) Endophyte-promoted phosphorus solubilization in *Populus*. *Frontiers in Plant Science* **11**: 1585.
74. Vigani, G., Rolli, E., Marasco, R., Dell'Orto, M., Michoud, G., Soussi, A., Raddadi, N., Borin, S., Sorlini, C., Zocchi, G. and Daffonchio, D. (2019) Root bacterial endophytes confer drought resistance and enhance expression and activity of a vacuolar H⁺-pumping pyrophosphatase in pepper plants. *Environmental Microbiology* **21**: 3212-3228.
75. Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**: 697-703.
76. White JG, Kingsley KL, Verma SK and Kowalski K. (2018) Rhizophagy cycle: an oxidative process in plants for nutrient extraction from symbiotic microbes. *Microorganisms* **6**:95
10.3390/microorganisms6030095.
77. Wu, T., Xu, J., Liu, J., Guo, W. H., Li, X. B., Xia, J. B., Xie, W.J., Yao, Z.G., Zhang, Y.M. and Wang, R.Q. (2019) Characterization and initial application of endophytic *Bacillus safensis* strain ZY16 for improving phytoremediation of oil-contaminated saline soils. *Frontiers in Microbiology* **10**: 991.
78. Yavarian, S., Jafari, P., Akbari, N., and Feizabadi, M. M. (2021) Selective screening and characterization of plant growth promoting bacteria for growth enhancement of tomato, *Lycopersicon esculentum*. *Iranian Journal of Microbiology* **13**: 121.
79. Yordanov, I., Velikova, V., and Tsonev, T. (2000) Plant responses to drought, acclimation, and stress tolerance. *Photosynthetica* **38**: 171-186.
80. Zhang, H., Kim, M. S., Krishnamachari, V., Payton, P., Sun, Y., Grimson, M., Farag, M.A., Ryu, C.M., Allen, R., Melo, I.S. and Paré, P.W. (2007) Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in Arabidopsis. *Planta* **226**: 839-851.
81. Zhu, Y. L., She, X. P., Wang, J. S., and Lv, H. Y. (2017) Endophytic bacterial effects on seed germination and mobilization of reserves in *Ammodendron biofolium*. *Pakistan Journal of Botany* **49**: 2029-2035.

Figures

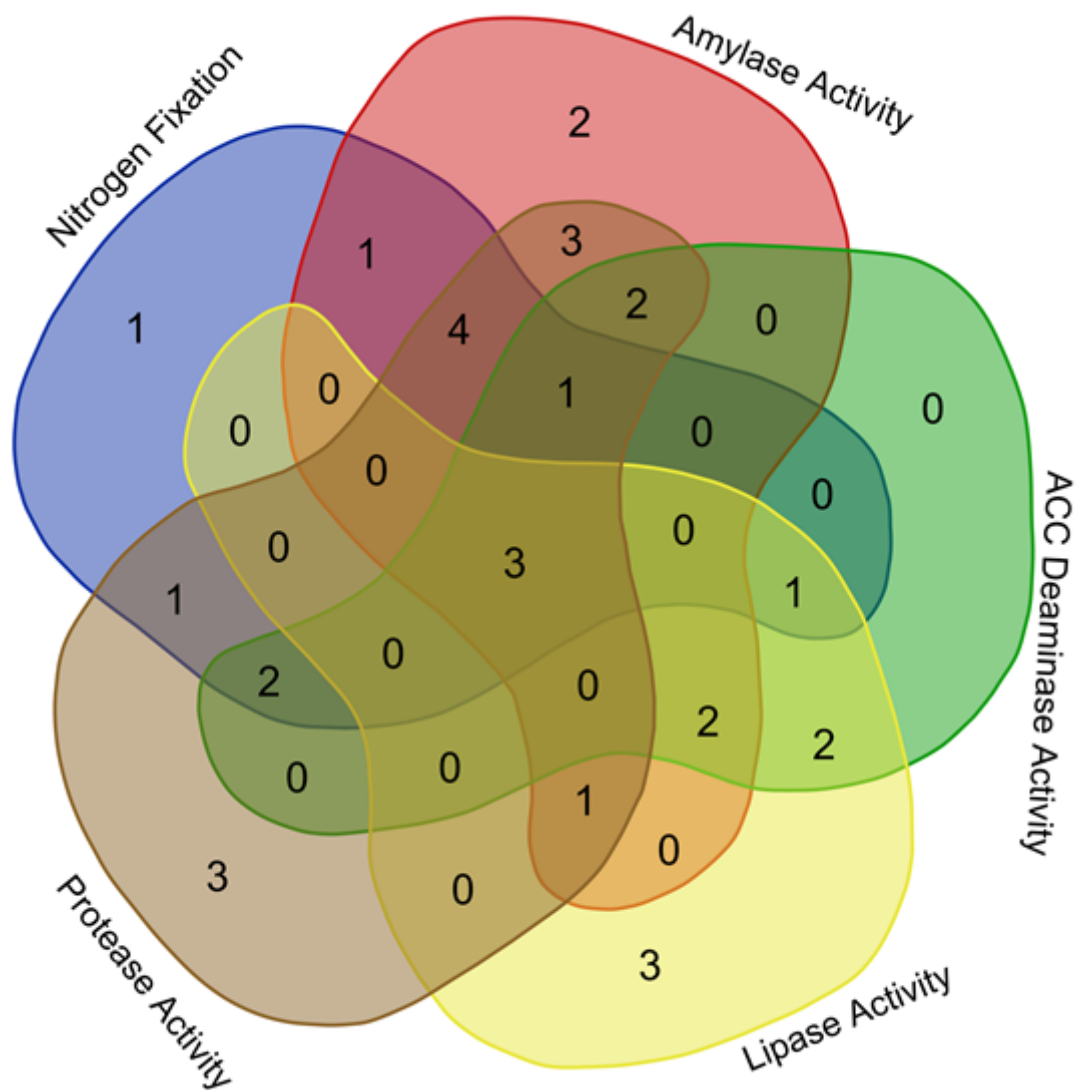


Figure 1

Venn diagram for showing commonality of nitrogen fixation, protease, amylase, lipase and ACC deaminase activity between the 34 endophytic bacteria isolates (Table 1).

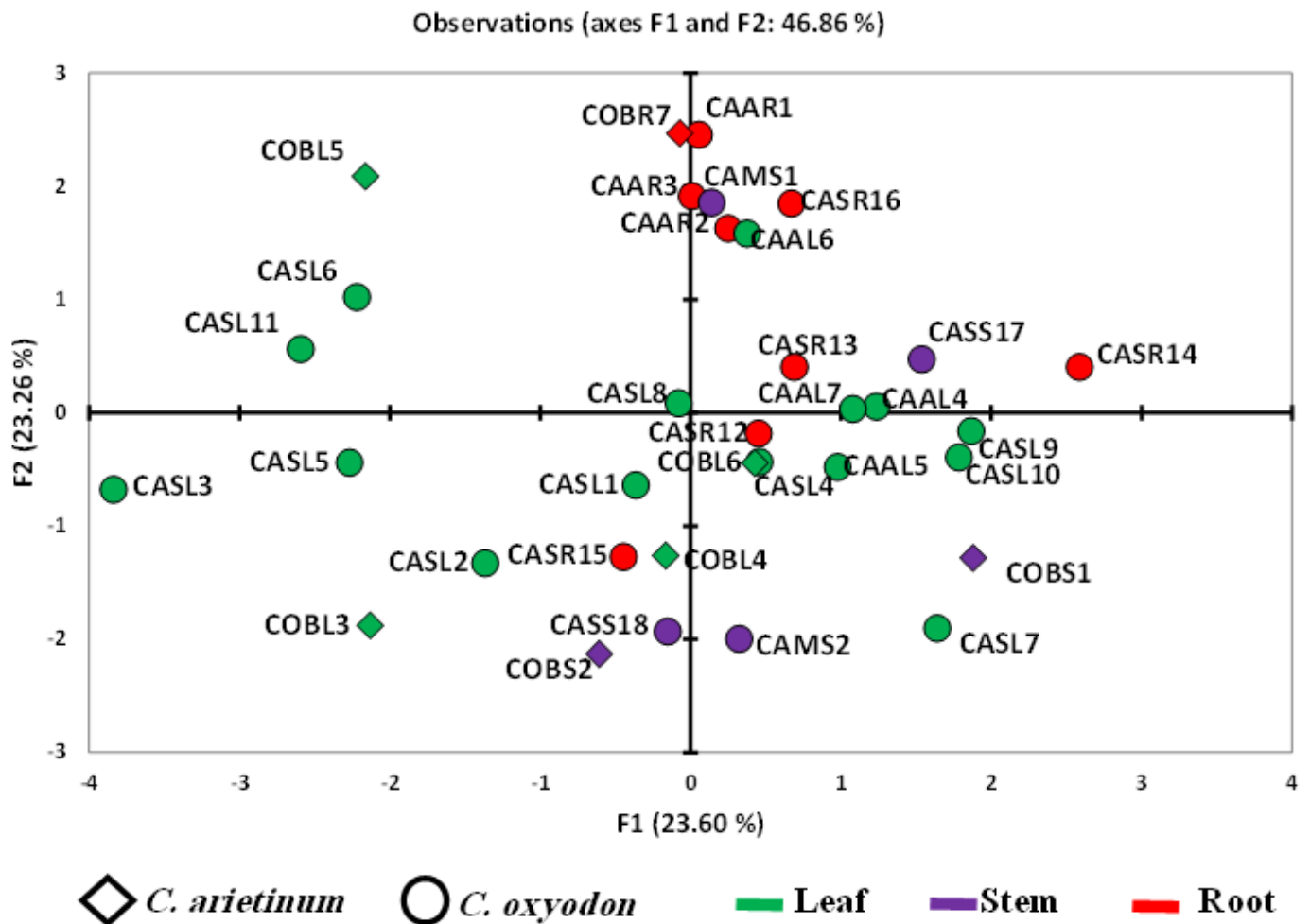


Figure 2

PCA (Principal Component Analysis) of IAA production, ACC deaminase activity, nitrogen fixation, phosphate solubilization, ammonia production, protease, amylase and lipase activities relative to the source species and source tissue of the 34 endophytic bacteria isolates (Table 1).

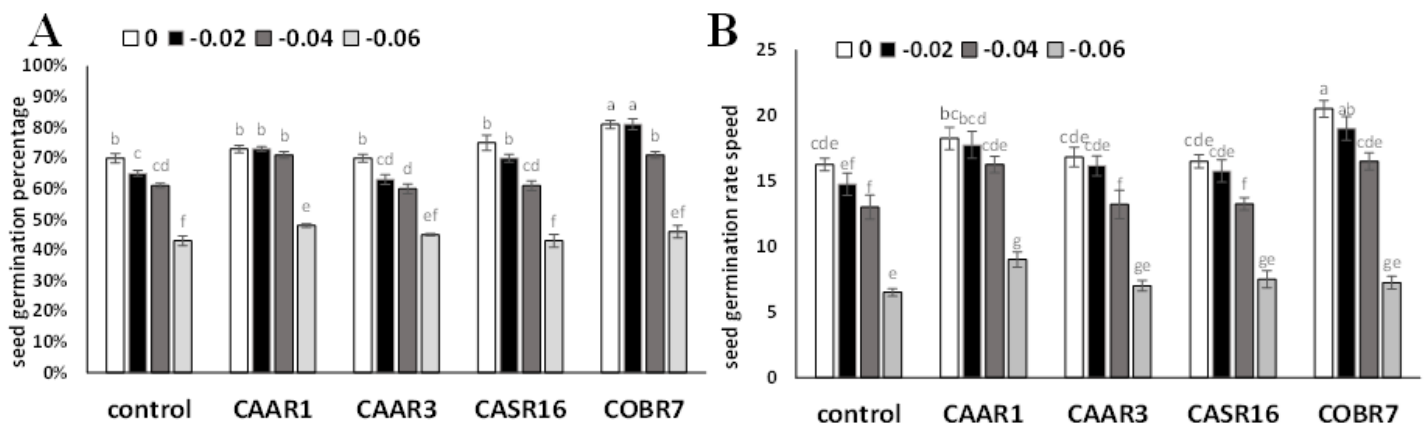


Figure 3

Effect of seed inoculation of *B. simplex* CAAR1, *B. pumilus* CAAR3, *B. muralis* CASR16, and *B. pumilus* COBR7 on chickpea plants (*C. arietinum*) on seed germination under drought. A. percentage of seeds germinated to total seeds at 10 days' post seeding), B. number of seeds germinated per day. Seeds were germinated on culture medium with water potentials of -0.02, -0.04, and -0.06 MPa created with PEG. Control seeds were inoculated with water only.

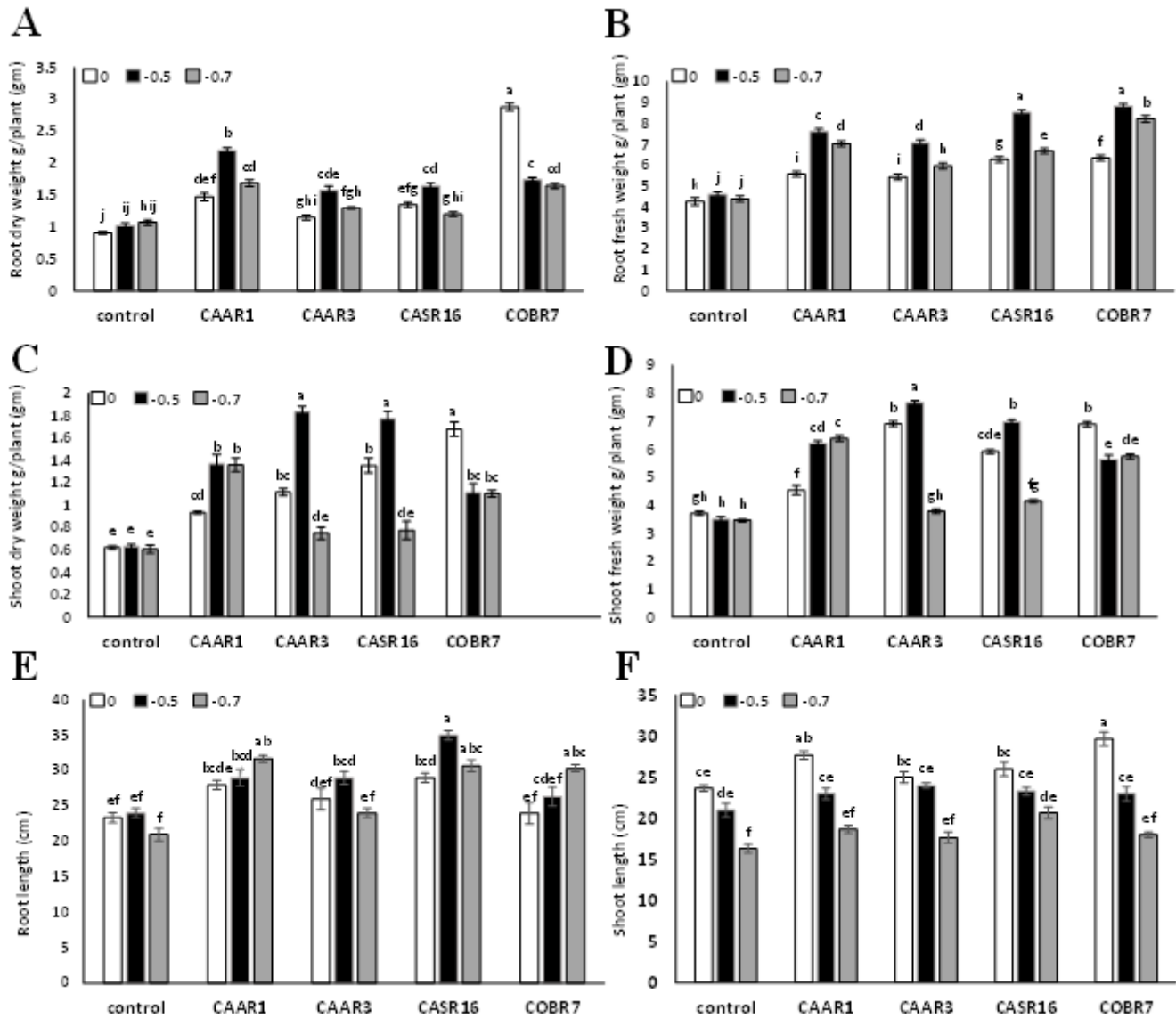


Figure 4

Effect of seed inoculation of *B. simplex* CAAR1, *B. pumilus* CAAR3, *B. muralis* CASR16, and *B. pumilus* COBR7 on chickpea (*C. arietinum*) growth under drought. A. root dry weight, B. root fresh weight, C. shoot dry weight, D. shoot fresh weight, E. root length, F. shoot length. Plants were grown in soil for 20 days with water potentials of 0, -0.5 and -0.7 MPa. Control seeds were inoculated with water only.

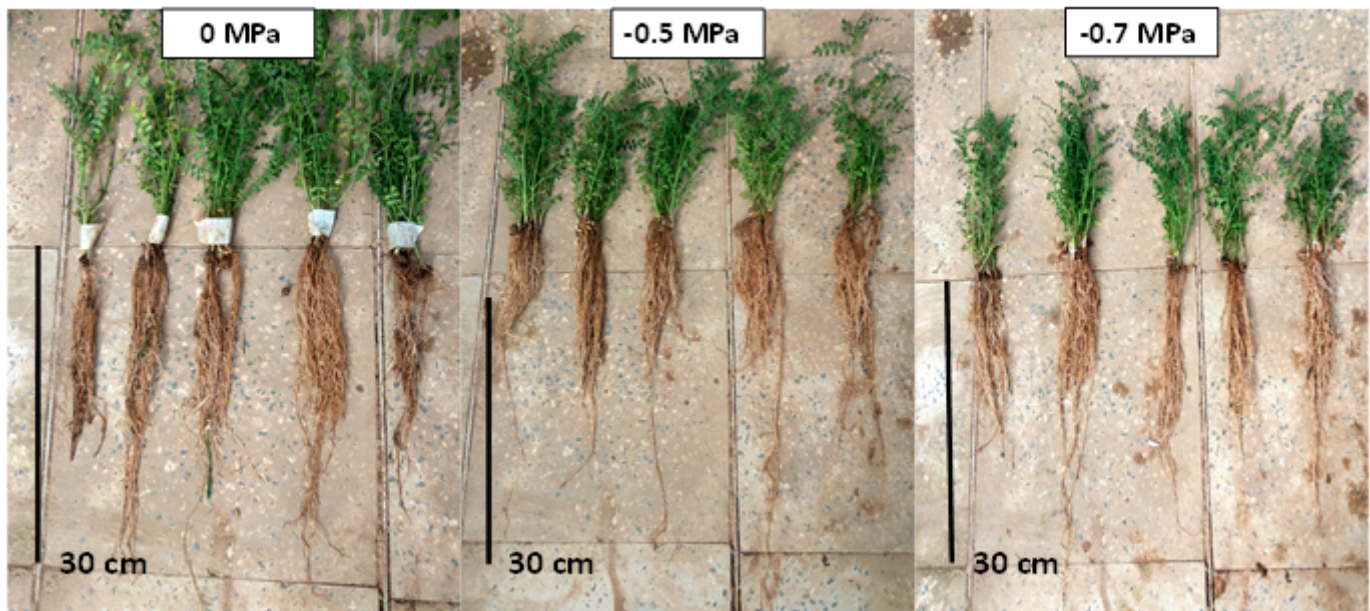


Figure 5

The appearance of chickpea (*C. arietinum*) control plants and plants inoculated with *B. simplex* CAAR1, *B. pumilus* CAAR3, *B. muralis* CASR16, or *B. pumilus* COBR7 (from left to right) at 0, -0.5 and -0.7 MPa.

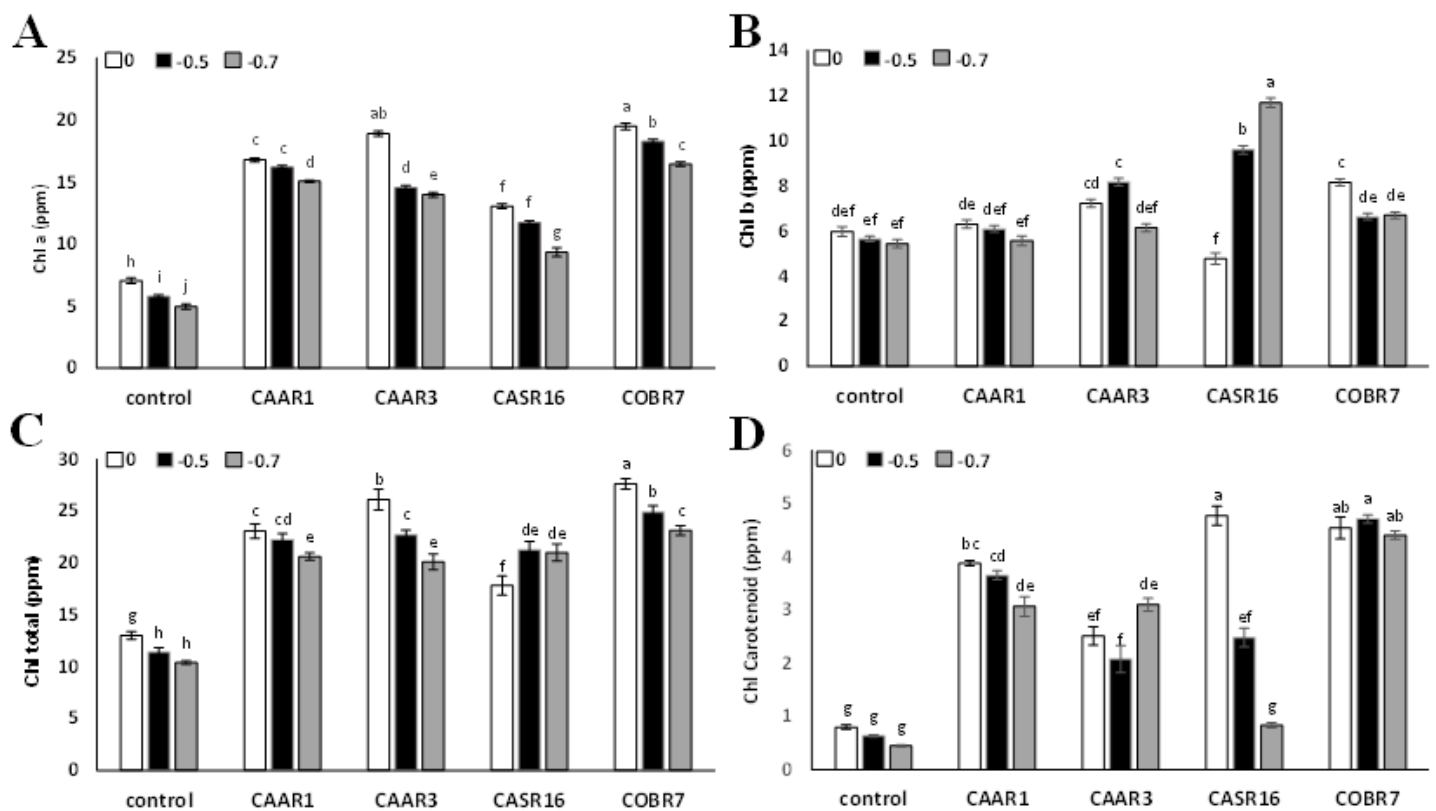


Figure 6

Effect of seed inoculation of *B. simplex* CAAR1, *B. pumilus* CAAR3, *B. muralis* CASR16, and *B. pumilus* COBR7 on chickpea (*C. arietinum*) chlorophyll content and hydrogen peroxide activity at 20 days after seeding under three levels of drought (0, -0.5 and -0.7 MPa). Control plants had non-inoculated seed A. chlorophyll a content, B. chlorophyll b content, C. total chlorophyll content and D. carotenoid content