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ORIGINAL ARTICLE

Efficacy of two different microbial consortia on salinity tolerance in chickpea: an in-planta evaluation on biochemical, histochemical, and genomic aspects

Arumugam Sathya[1] · Vahida Rehman[1] · Vadlamudi Srinivas[1] · Himabindu Kudapa[1] · Subramaniam Gopalakrishnan[1,2]

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Abstract
This study aimed to identify and characterize actinobacteria and rhizobia with plant growth-promoting (PGP) traits from chickpea plants. Out of 275 isolated bacteria, 25 actinobacteria and 5 chickpea rhizobia showed 1-aminocyclopropane-1-carboxylate deaminase (ACCd) activity. Selected chickpea rhizobia were tested for their nodulating capacity under sterile and non-sterile soil conditions. Further screening on salinity and PGP traits identified three promising isolates: *Nocardiopsis alba* KG13, *Sinorhizobium meliloti* KGCR17, and *Bacillus safensis* KGCR11. These three isolates were analyzed for their compatibility and made into a consortium (Consortium 1). This along with another consortium made from our salinity-tolerant lab strains *Chryseobacterium indologenes* ICKM4 and *Stenotrophomonas maltophilia* ICKM15 (Consortium 2) was compared *in planta* studies. Trials revealed that Consortium 2 showed significant ($p < 0.05$) tolerance and on above-ground, below-ground traits and yield components than Consortium 1. Moreover, both consortia induced nodulation in saline-stressed plants, alleviated electrolyte leakage (2.3 vs. 0.4 in ICCV 2; 1.8 vs. 0.6 in JG 11), and increased chlorophyll content. Histochemical staining indicated reduced oxidative stress and lipid peroxidation in consortium-treated plants under salinity stress. Further, gene expression studies revealed mixed patterns, with up-regulation of antioxidant and transporter genes observed in consortium-treated plants, particularly in Consortium 2. Overall, Consortium 2 showed better gene expression levels for antioxidant and transporter genes, indicating its superior efficacy in mitigating salinity stress in chickpea plants. This study provides valuable insights into the potential use of these microbial isolates in improving chickpea productivity by enhancing salinity tolerance.

Keywords Plant growth-promotion · Actinobacteria · Rhizobia · Salinity tolerance · ACCd

Introduction

Across the world, the agricultural sector is facing many challenges to meet food and feed demands for increasing human population and one such is salinity (FAO 2021). As per the Global Map of Salt-affected Soils (GSASmap) from FAO, more than 424 M ha of topsoil (0–30 cm) and 833 M ha of subsoil (30–100 cm) are salt-affected. This mapping data includes information from 118 countries covering 73% of global land area. In addition, continuing climate change and persistent droughts are expected to increase the density of salinity challenge. Salt stress affects ionic, oxidative and osmotic balance and hence affects numerous physiological functions of any region of the plants. This leads to lower rate of seed germination, root and shoot development and yields (Chauhan et al. 2022).

On the other hand, global focus on eco-friendly environment by exploring beneficial microorganisms is increasing with innovative techniques including rhizospheric engineering (Pathak et al. 2024). 'Plant growth-promoting bacteria (PGPB)' are one such microbial community resides either in soil, rhizosphere region or inside of the plants as endophytes. They employ many direct and indirect mechanisms, through metabolism, chemotaxis, secretion, and

✉ Subramaniam Gopalakrishnan
s.gopalakrishnan@cgiar.org

[1] International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India

[2] International Institute of Tropical Agriculture (IITA), Dar es Salaam, Tanzania

antibiotic production, etc., on plants and brings out sustainable crop yield and soil health (Gopalakrishnan et al. 2012a, b, 2013a, b, 2014, 2015a, b, c, 2016a, b, c; Srinivas et al. 2020, 2022; Upadhyay et al. 2022). It is observed that multi-microbe application called consortium to plants exhibits better plant and soil health than single strain inoculations (Gopalakrishnan et al. 2022).

PGPB plays an effective role in inducing the abiotic stress tolerance including salinity and many reports are available on various crops (Yang et al. 2009; Etesami and Beattie 2017; Bakka and Challabathula 2020; Prittesh et al. 2020; Singh et al. 2022; Chauhan et al. 2024a, b). In the last 20 decades, production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (ACCd) is observed as one of the key tools in stress mechanism as it converts the stress ethylene precursor ACC into α-ketobutyrate and ammonia which alleviates the stress ethylene consequences. Another area is an effective antioxidant system in manipulating the stress consequences. Reports focusing these areas are available on crops like tomato, wheat, maize, canola, French bean, chickpea, capsicum etc. (del Carmen Orozco-Mosqueda et al. 2020).

Chickpea (*Cicer arietinum* L.), a global staple crop with potential nutritional values, has high demand in global market space (Kotula et al. 2015; Khan et al. 2015). The production of chickpea has increased twice in the last thirty years. Still, biotic and abiotic stress factors challenge the production levels, in specific salinity accounts for 8–10% yield loss. Breeding of high yielding salt resistant varieties is underway involving many genotypes and desirable phenotypic traits (Dodd and Pérez-Alfocea 2012; Soren et al. 2020). Many reports are available on the effect of PGPB's in chickpea (Qurashi and Sabri 2012; Gopalakrishnan et al. 2015a, b, c, d, 2017, 2018, 2022; Mir et al. 2021; Sravani et al. 2021; Pratyusha et al. 2023, Vijayabharthi et al. 2018a, b). However, only very few studies are available on salinity tolerance (Abd-Allah et al. 2018).

Based on these, we attempted a study with the following objectives: (1). Isolation of ACCd-producing PGPB and rhizobia from chickpea rhizosphere and root nodules respectively. (2) Identifying the salt tolerance of isolated bacteria, PGP traits and make a possible consortium. (3) Analyzing the effect of selected PGP bacterial consortia on salinity tolerance of chickpea varieties ICCV 2 and JG 11 on physiological, histochemical, and genomic aspects.

Materials and methods

Sample collection and isolation of actinobacteria and chickpea rhizobia

Chickpea plantlets of 15–30 days old and rhizospheric soil samples were collected during Nov 2016 in Telangana, Andhra Pradesh, and Karnataka states of India. The soil samples were subjected to standard laboratory protocols and actinobacteria isolates were obtained with starch casein agar (SCA) and actinomycete isolation agar (AIA) supplemented with cycloheximide (50 µg mL^{-1}) and nystatin (25 µg mL^{-1}), incubated for a week at 28 °C. Rhizobia from chickpea nodules were isolated as per Somasegaran and Hoben (1994) using yeast mannitol agar (YMA), incubated at 27 °C for 2 weeks. All the actinobacteria and rhizobium were stored in AIA and YMA respectively at 4 °C.

Primary screening by 1-aminocyclopropane-1-carboxylate deaminase activity

All the isolates were subjected to 1-aminocyclopropane-1-carboxylate deaminase (ACCd) activity qualitatively using Dworkin and Foster (DF) minimal medium amended with ACC (30 mmol $plate^{-1}$) as per Penrose and Glick (2003). ACCd positive isolates were further tested for quantitative ACCd activity against the standard curve of α-ketobutyrate.

Salinity tolerance

All the isolates of actinobacteria and chickpea rhizobia were tested for their salinity tolerance at 2, 4, 6, 8 and 10% NaCl. AIA and YMA agar medium amended with various salt concentrations were inoculated with test isolates and incubated at 28 °C for 5 days. The presence of growth of actinobacteria and rhizobia in AIA and YMA was considered as tolerance to salinity levels.

In vitro PGP traits

Actinobacteria and chickpea rhizobia with ACCd-producing capacity and saline tolerance were evaluated for other PGP traits. Siderophore formation (Schwyn and Neilands 1987) was detected using Chrome Azurol S (CAS) reagent and calculated for siderophore units (%). IAA (µg mL^{-1}) was estimated using starch casein (SC) broth supplemented with (1 µg mL^{-1}) and without L-tryptophan using Salkowski reagent (1 mL of 0.5 M FeCl3 in 50 mL of 35% HClO4) (Patten and Glick 1996). Phosphate solubilization (P equivalents µg mL^{-1}) was estimated using National Botanical Research

Institute's Phosphate (NBRIP) medium (Fiske and Subbarow 1925). ß-1,3-Glucanase was estimated from tryptic soy broth supplemented with 1% colloidal chitin using 2% laminarin and the activity was defined as the amount of enzyme that liberated 1 µmol of glucose h^{-1} at defined conditions (Singh et al. 1999). The other enzymes, chitinase (Hirano and Nagao 1988), cellulase (Hendricks et al. 1995), lipase and protease (Bhattacharya et al. 2009) were determined qualitatively. HCN (Lorck 1948) and ammonia (Cappuccino and Sherman 1992) were also determined qualitatively.

Nodulation test for chickpea rhizobia

The chickpea rhizobia isolates producing highest ACCd were tested for their nodulating efficiency in sterilized and non-sterilized soils in five chickpea genotypes (ICCV 2, ICC 4958, ICCV 10, Annigeri and JG 11) under glasshouse conditions. Known nodulating chickpea rhizobia, such as IC76, IC59 (lab strains @ICRISAT) and *Mesorhizobium ciceri* UPM-Ca7T (ATCC® 51,585), were used as reference strains. This nodulation experiment was planned with treatments (NC—Normal control, IC59, IC76, Mc—*Mesorhizobium ciceri* UPM-Ca7T, L1—ACCd-producing chickpea rhizobia 1, L2—ACCd-producing chickpea rhizobia 2, L3—ACCd-producing chickpea rhizobia 3) with 6 replications/treatment. Mc was grown in yeast mannitol broth at 200 rpm, 28 °C for 5–7 days with the cell count of ~ 1×10^9 CFU mL^{-1}. Pot mixture was prepared with black soil, sand and farmyard manure (3:2:1), sterilized and filled in 8" plastic pots. The chickpea seeds were surface-sterilized with 2.5% sodium hypochlorite for 5 min, washed several times with sterilized distilled water and subjected to seed bacterization (10^8 CFU mL^{-1} h^{-1}). The seeds were allowed to dry and sown in pots (4 seeds/pot but thinned to 2 after germination, in a week). Booster doses of chickpea rhizobia (5 mL $seedling^{-1}$, 10^8 CFU mL^{-1}) were applied at 15 and 30 days after sowing (DAS) by soil drench method. Growth responses were determined by shoot dry weight, root dry weight and nodule dry weight at 35 DAS. Nitrogenase activity was estimated by acetylene reduction (µmol C_2H_4 $plant^{-1}$ h^{-1}) activity in Hewlett Packard gas chromatograph (HP4890D), with FID detector, HP-PLOT-Q column and N_2 as carrier gas (Zhang et al. 2016).

Compatibility and biofilm formation capacity of selected actinobacteria and chickpea rhizobia

To prepare a consortium, the selected actinobacteria and chickpea rhizobia with nodulating potential were evaluated for their compatibility in AIA and YMA agar medium. Our lab isolates of chickpea root nodule-associated bacteria *Chryseobacterium indologenes* ICKM4 (GenBank Acc. No: KX583496) and *Stenotrophomonas maltophilia* (ICKM15 (GenBank Acc. No: KX611374) with proven PGP traits in chickpea were also tested for salinity tolerance and compatibility (Gopalakrishnan et al. 2017). This serves as another consortium. All the isolates were also tested for biofilm formation capacity in microtiter plates as per ÓToole (2011) using M9 minimal medium.

Molecular identification of actinobacteria and chickpea rhizobia

Actinobacteria and chickpea rhizobia having the highest ACCd-producing capacity and/or salinity tolerance were selected and identified as per our previous protocols (Vijayabharathi et al. 2014) and the partial nucleic acid sequences were submitted to GenBank, NCBI.

Scanning electron microscopy

Spore morphology of the selected actinobacteria was characterized by scanning electron microscopy (JEOL JSM-5600, Japan) as per standard protocols with reference to Aouar et al. (2012) at RUSKA Laboratories, Hyderabad, Telangana, India.

In planta effects of selected isolates on salinity tolerance of chickpea

This study was conducted in pots in outdoor conditions of ICRISAT, Patancheru, India (17°30′N; 78°16′E; altitude 549 m) during Nov 2017 to Feb 2018. The experiment includes 4 treatments (C—Control, SS—Saline-stressed; C1—Saline-stressed treated with Consortium 1, C2—Saline-stressed treated with Consortium 2) with 12 replications/treatment. Consortium 1 consists of saline-tolerant ACCd-producing actinobacteria, saline-tolerant ACCd-producing chickpea rhizobia and the highest saline-tolerant nodule-associated bacteria. Consortium 2 consists of previously characterized our lab isolates with saline tolerance (Gopalakrishnan et al. 2017). Chickpea genotypes with saline sensitivity (ICCV 2) and tolerance (JG 11) obtained from GenBank, ICRISAT, Patancheru, India were used. Pot mixture was prepared with black soil, sand, and farmyard manure (3:2:1), pasteurized and filled in 8" plastic pots. The pots were saturated with either tap water or saline water as per the treatments. The pots were artificially salinized at 80 mM concentration as two split doses at the time of sowing and 12 days after sowing to mimic field situations. The base of the pots of the saline treatment was sealed to avoid salt leakage, whereas the pots of the non-saline treatment had holes to allow drainage. After salt application and for the remaining crop cycle, pots were watered with tap water and maintained close to a range of 60–90% field capacity to avoid an increase in the salt concentration in the soil

solution. The chickpea seeds were surface-sterilized with 2.5% sodium hypochlorite for 5 min washed several times with sterilized distilled water and subjected to seed bacterization (10^8 CFU mL^{-1} h^{-1}). The seeds were allowed to dry and sowed in pots (6 seeds/pot but thinned to 3 after a week). For control pots, surface-sterilized, non-bacterized seeds were used. Booster doses of isolates (5 mL/seedling, 10^8 CFU/mL) were applied at 15 and 30 days after sowing (DAS) by soil drench method. Shoot length and dry weight, number of branches, number of flowers, number of pods, leaf area, leaf dry weight, root length, root surface area, root volume and root dry weight were determined at 45 DAS. Samples of shoot and root were harvested for gene expression analysis. Chlorophyll, carotenoid content (Wellburn 1994) and electrolyte leakage as relative leakage ratio (Lutts et al. 1996) were also estimated in young leaves. At harvest, seed number and weight, pod number and weight and total biomass were determined.

Histochemical studies

To do histochemical analysis, the *in-planta* study is mimicked as follows: the chickpea seeds were grown in sterile germination sheets in the growth chamber at 28 ± 2 ℃ with a photoperiod of 12 light hours for 15 days to get less damaged roots rather than the sand-cultured roots. Seed sterilization procedure and treatments were the same as in the salinity pot trial. At 15 days after placing the seeds, leaves and roots were harvested for detection of H_2O_2 and $O_2-^{\cdot}$ radicals on chickpea leaves followed by lipid peroxidation products on roots. To detect $O_2\text{-}^{\cdot}$, the chickpea leaves were stained for 30 min with 0.05% nitro-blue tetrazolium (NBT) (w/v) in 50 mM potassium phosphate, pH 7.0. To detect H_2O_2, the chickpea leaves were stained for 5 h with 0.1% 3,3′-diaminobenzidine (DAB) in 10 mM potassium phosphate, pH 7.0. Samples were stained under light at room temperature, after which they were cleared with an ethanol:acetic acid (96:4) solution until photographed. Schiff staining of roots was done as per Pompella et al. (1987). The roots were stained with Schiff's reagent (Jensen 1962) for 20 min and rinsed with a 0.5% $K_2S_2O_5$ in 0.05 M HCl. Later, leaves and roots were imaged using Lecia S8AP0 Stereo microscope at Plant Quarantine Unit, ICRISAT.

Gene expression

RNA extraction

For expression profiling, leaf and root tissues of chickpea plants of 45 days old were collected and washed thoroughly with 0.1% DEPC water, frozen in liquid nitrogen and stored at − 80 °C until RNA extraction. Total RNA was extracted from the harvested tissues using TRIzol (Invitrogen, USA) according to the manufacturer's protocol. RNA quality was assessed on 1.2% formaldehyde agarose gels, while purity of RNA was assessed using a Nanovue spectrophotometer (A260/A280 ratio). First-strand cDNA was synthesized from total RNA (2.5 µg) using a cDNA synthesis kit (Superscript® III, Invitrogen, CA, USA) following manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR was performed using Applied Biosystems 7500 Real-Time PCR System with the SYBR green chemistry (Applied Biosystems, USA) according to the manufacturer's instructions. The expression patterns of eight well-characterized genes relating to super oxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), phenylalanine ammonia lyase (PAL), lipoxygenase (LOX), sodium/hydrogen antiporter (NHX), chloride channel transporter (CLC-b) and high-affinity potassium transporter (HKT) were studied using qRT-PCR. Gene-specific primers were designed using primer 3 software (Rosen and Skaletsky 2000) (Table 1). qRT-PCR was carried out in three biological and two technical replicates. In brief, 10 µL reaction containing 30 ng of first-strand cDNA, 1X PCR buffer, 125 mM dNTPs, 1.5 mM MgCl2, 0.2 mM primers and 1U Taq polymerase. PCR program is as follows: 50 °C for 2 min and denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has been used as endogenous control gene to normalize cDNA samples. The expression

Table 1 Primers used for qRT-PCR in this study

S. no	Primer name	Sequences (5′–3′)	
1	SOD	F	TCCCTCTCACTGGACCAAAC
		R	CGGAGTTGAGAGTGGTGGTT
2	CAT	F	TCAGGCTGATCGTTCTCTT
		R	TTGGCGAGGACCTTAACT
3	APX	F	GGTAGTAAGGTGTTTAGAGAGG
		R	CTTCACATTCATCGTGTCTG
4	PAL	F	ACGCATGGTGGAAGAGTACC
		R	GCACCACCCTGTTTTGTTCT
5	LOX	F	CACGGCCTTCGCCTCGTGATACAGG
		R	GGCCACCATGGCTTGTCTTTCAAG TCACC
6	NHX1	F	CATGCGTGGAGCTGTTTCTA
		R	ACATCCTCTCCATTGCCAAG
7	CLC-b	F	TGTTGGGGGAGTTCTCTTTG
		R	CTGTACCGAAAAGGCCACAT
8	HKT1	F	TGCAAAGATTCATGGATTGG
		R	CATGCATGCTTTTGAGCACT

data from different cDNA samples was compared using the mean of the CT values of the three biological replicates that were normalized to the mean CT values of the endogenous gene. The expression ratios were calculated using the $2^{-\Delta\Delta Ct}$ method and Student's *t* test was used to calculate significance (Livak and Schmittgen 2001). Relative transcription levels are presented graphically.

Statistical analysis

The data of in vitro PGP traits and chickpea nodulation responses were subjected to one-way analysis of variance (ANOVA) and the significant difference between mean values was determined by post hoc Tukey's test. Data of salinity pot experiments were subjected to AVOVA and post hoc Dunnett's test. Statistical analysis was done using Statistical Package for the Social Sciences (SPSS) 13.0 (SPSS Inc., Chicago, Illinois, USA). Gene expression analysis was done by Student's *t* test using SAS GLM (General Linear Model) procedure (SAS Institute 2002-08, SAS version 9.3).

Results

Isolation and strain selection

The rhizospheric soil and nodule samples from chickpea genotypes JG 11 and Annigeri were collected across the 3 southern states of India. A total of 275, including 192 actinobacteria and 83 chickpea rhizobia were isolated (Table 2). Preliminary qualitative screening on ACCd for these 275 isolates identified 25 actinobacteria and 5 chickpea rhizobia (data not shown). Further quantitative estimation showed ACCd activity of 0.5–48.2 and 0.9–38.9 nmoles α-ketobutyrate mg protein^{-1} h^{-1} for actinobacteria and chickpea rhizobia, respectively. The secondary screening on salt tolerance of the 275 isolates identified 2 actinobacteria and 6 chickpea rhizobia with the salinity tolerance of 4–8% (data not shown).

Plant growth-promoting traits of these ACCd-producing and saline-tolerant isolates were characterized and 3 isolates were selected for further study (Table 3; Fig. 1). This includes an actinomycete isolate KG13 with moderate ACCd production (27.4 ± 2.7 nmoles α-ketobutyrate mg protein^{-1} h^{-1}) and high saline tolerance (8% NaCl); two chickpea rhizobia, in which KGCR17 has higher ACCd production (38.9 ± 2.37 nmoles α-ketobutyrate mg protein^{-1} h^{-1}) and lower saline tolerance (4% NaCl); and KGCR11 has lower ACCd production (0.9 ± 0.1 nmoles α-ketobutyrate mg protein^{-1} h^{-1}) and higher saline tolerance (8% NaCl).

Other PGP traits were also evaluated for the selected actinobacteria and rhizobia. IAA production was observed to be high in KGCR17 (12.9 µg mL^{-1}) followed by KG13 (6.1 µg mL^{-1}) and KGCR11 (1.2 µg mL^{-1}). Isolates KG13 and KGCR17 were able to solubilize P with 16 P equivalents µg mL^{-1} and produce ammonia, while KGCR11 was devoid of both the traits. β-1,3-Glucanase was observed in the order of KG13 (10.7 Units) > KGCR17 (6.9 Units) > KGCR11 (1.3 Units). In addition, siderophores, HCN and chitinase production was also noticed in which KGCR17 is devoid of chitinase activity.

In addition, our lab strains *Chryseobacterium indologenes* ICKM4—GenBank Acc. No: KX583496 and

Table 2 Diversity of isolated actinobacteria and chickpea rhizobia

Sampling sites				Chickpea variety	Number of samples	Actinobacteria		Chickpea rhizobia	
State	Location	Latitude (N)	Longitude (E)			Isolate code	N° isolates obtained	Isolate code	N° isolates obtained
Telangana	Alampur	15° 53′ 3.6564″ N	78° 7′ 9.7752″ E	JG-11	7	TA	48	TACR	18
	Ramapuram	16° 0′ 55.4256″ N	77° 49′ 51.636″ E	JG-11	5	TR	28	TCR	13
Andhra Pradesh	Banganapalle	15° 19′ 5.1708″ N	78° 13′ 31.962″ E	JG-11	7	APB	39	APCR	8
Karnataka	Sedam rural	17° 10′ 57.8064″ N	77° 19′ 15.438″ E	Annigeri, JG-11	7	KG	34	KGCR	17
	Kurikota	17° 29′ 34.4076″ N	76° 55′ 44.454″ E	Annigeri					
	Mahagaon	17° 31′ 15.3516″ N	76° 54′ 52.5888″ E	Annigeri					
	Byalhalli	17° 53′ 36.924″ N	77° 18′ 44.424″ E	Annigeri, JG-11	9	KB	43	KBCR	27

Table 3 In vitro PGP traits, salinity tolerance and identification details of selected actinobacteria and chickpea rhizobia

Isolates	ACCd*	IAA¶	P solubilization"	Siderophore	β-1,3-Glucanase#	Ammonia	HCN	Chitinase	NaCl tolerance				Identification by 16 s rDNA	% Similarity in Entrez	GenBank Accession number
									2%	4%	6%	8%			
KG13	27.4 ± 2.7	6.1 ± 0.7	16.0 ± 1.9	1	10.7 ± 0.5	+	2	1	+	+	+	+	*Nocardiopsis alba*	100%	MH333283
KGCR11	0.9 ± 0.1	1.2 ± 0.2	–	1	1.3 ± 0.2	–	2	1	+	+	+	+	*Bacillus safensis*	100%	MH333097
KGCR17	38.9 ± 2.3	12.9 ± 0.9	16.3 ± 1.9	2	6.9 ± 0.3	+	2	–	+	+	–	–	*Sinorhizobium meliloti*	99.93%	MF374790
Isolates of our previous study (Gopalakrishnan et al. 2017)															
ICKM4	–	9 ± 1.4	–	2	2.03	–	1	3	+	+	+	–	*Chryseobacterium indologenes*	–	KX583496
ICKM15	–	10 ± 1.5	10 mm	2	1.73	–	3	–	+	+	–	–	*Stenotrophomonas maltophilia*	–	KX611374

Values are Mean ± SE ($n = 3$). IAA—Indole acetic acid; HCN—Hydrocyanic acid; * nmoles α-ketobutyrate mg protein^{-1} h^{-1}; ¶ µg mL^{-1}; " P equivalents µg mL^{-1}; # Units, One unit of β-1,3-glucanase activity was defined as the amount of enzyme that liberated 1 µmol of glucose h^{-1} at defined conditions. + and—indicates positive and negative for ammonia production; Ratings scale for HCN production are 0 = no color change; 1 = light reddish brown; 2 = medium reddish brown and 3 = dark reddish brown. The rating scale for Siderophore and Chitinase are 0 = no change; 1 = 1–6 mm; 2 = halo zone of 7–12 mm; 3 = halo zone of 19–24 mm and 4 = halo zone of 25–30 mm and above

Stenotrophomonas maltophilia ICKM15—GenBank Acc. No: KX611374 isolated from chickpea root nodules with identified PGP traits in chickpea (Gopalakrishnan et al. 2017) were also tested for salinity tolerance. ICKM4 and ICKM15 showed salinity tolerance of 6 and 4%, respectively (Fig. 1). They both were devoid of ACCd activity. Other PGP traits are shown in Table 3.

Nodulation efficiency of chickpea rhizobia

Among the isolated chickpea *Rhizobi*um (CR) strains with ACCd activity, 3 (KGCR17: L1, KBCR12: L2, APCR20: L3) were tested for nodulating capacity in both sterile soil (SS) and non-sterile soil (NS) conditions. All were found to nodulate irrespective of the chickpea genotypes ICCV2, ICC4958, ICCV10, Annigiri and JG11 (Fig. 2). Under SS conditions, *CR* was found to induce shoot and/or root growth in all the chickpea genotypes except JG11 where none of the strains showed significant growth responses. In case of NS conditions, this phenomenon was shifted to ICC4958 in which none of the *CR* showed significant shoot or root growth responses.

During the initial selection process in SS conditions, all the isolated *CR* (L1, L2, L3) were found to nodulate and fix nitrogen significantly similar to known *CR* strains IC59, IC76 and ATCC. Minor degree of nodulation was noticed on NC plants except in ICCV 10. In contrast, under NS conditions, NC plants also showed nodulation, which indicates the presence of native rhizobia with chickpea nodulating capacity. The NC plants also show nitrogenase activity to some extent. Significance of test and reference *CR* strains in competing or mutualizing the native rhizobia is documented by the nodulating and nitrogen fixing capacity in NS conditions in all the tested chickpea genotypes.

Results obtained in SS and NS system are clearly indicated by the ANOVA in Table 4, where higher significance was noticed between the genotypes, treatment, and genotype × treatment interaction in SS conditions, than NS conditions. Principal component analysis (Fig. 3) further confirms this by the closer grouping of genotypes in SS conditions and distant grouping of genotypes under NS conditions. Grouping of shoot and root growth, and nodule weight and ARA was confirmed by significant positive correlations in Table 5.

To select one *CR* for further studies, ranking was done in which L1 scored the highest ranking in ICC 4958 and JG 11 followed by ATCC, IC59 and IC76 in other chickpea genotypes under NS conditions. The ranking among L1, L2 and L3 also shows the highest ranking by L1 in ICC 4958 and JG 11, by L2 in ICCV 2 and ICCV 10, by L3 in Annigeri.

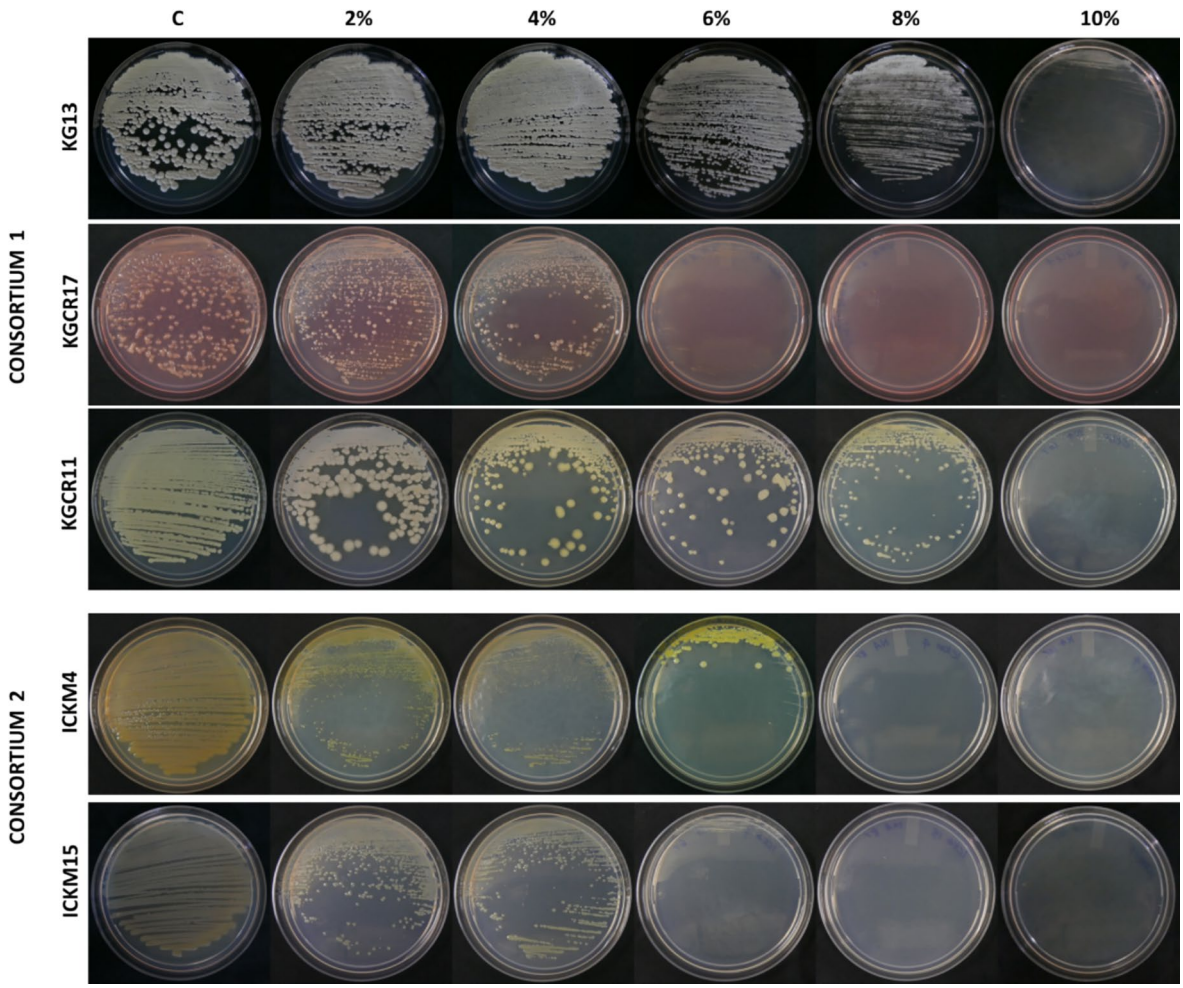

Fig. 1 Salinity tolerance of the selected isolates in consortium 1 (KG13 + KGCR11 + KGCR17) and consortium 2 (ICKM4 + ICKM15). 2–10%–NaCl concentration

Compatibility analysis and consortium preparation

The selected isolates were subjected to compatibility analysis, which showed absence of inhibition under co-culturing conditions (Fig. 4). This indicates the compatibility between the isolates and hence two sets of consortia were prepared for further *in-planta* studies. Consortium 1 consists of KG13, KGCR17 and KGCR11; and consortium 2 consists of *C. indologenes* ICKM4 and *S. maltophilia* ICKM15.

Biofilm forming capacity

Invariably, all the five isolates were found to form biofilms (Fig. 5). KG13, *C. indologenes* ICKM4 and *S. maltophilia* ICKM15 were found to be strongly adherent; and KGCR11 and KGCR17 were found to be moderately adherent.

Strain identification

The selected isolates KG13, KGCR17, and KGCR11 were identified by 16 s rDNA sequencing and the partial nucleic acid sequences were deposited in GenBank, NCBI and the accession numbers were obtained (Table 3). They are (i) A non-streptomycete actinomycete *Nocardiopsis alba* KG13—GenBank Acc. No: MH333283; (ii) a chickpea rhizobia *Sinorhizobium meliloti* KGCR17—GenBank Acc. No: MF374790, (iii) a root nodule-associated bacteria *Bacillus safensis* KGCR11—GenBank Acc. No: MH333097.

Scanning electron microscope

The isolate KG13 identified as *Nocardiopsis alba* is further visualized under scanning electron microscope (Fig. 6). It showed smooth-surfaced and rod-shaped spore morphology.

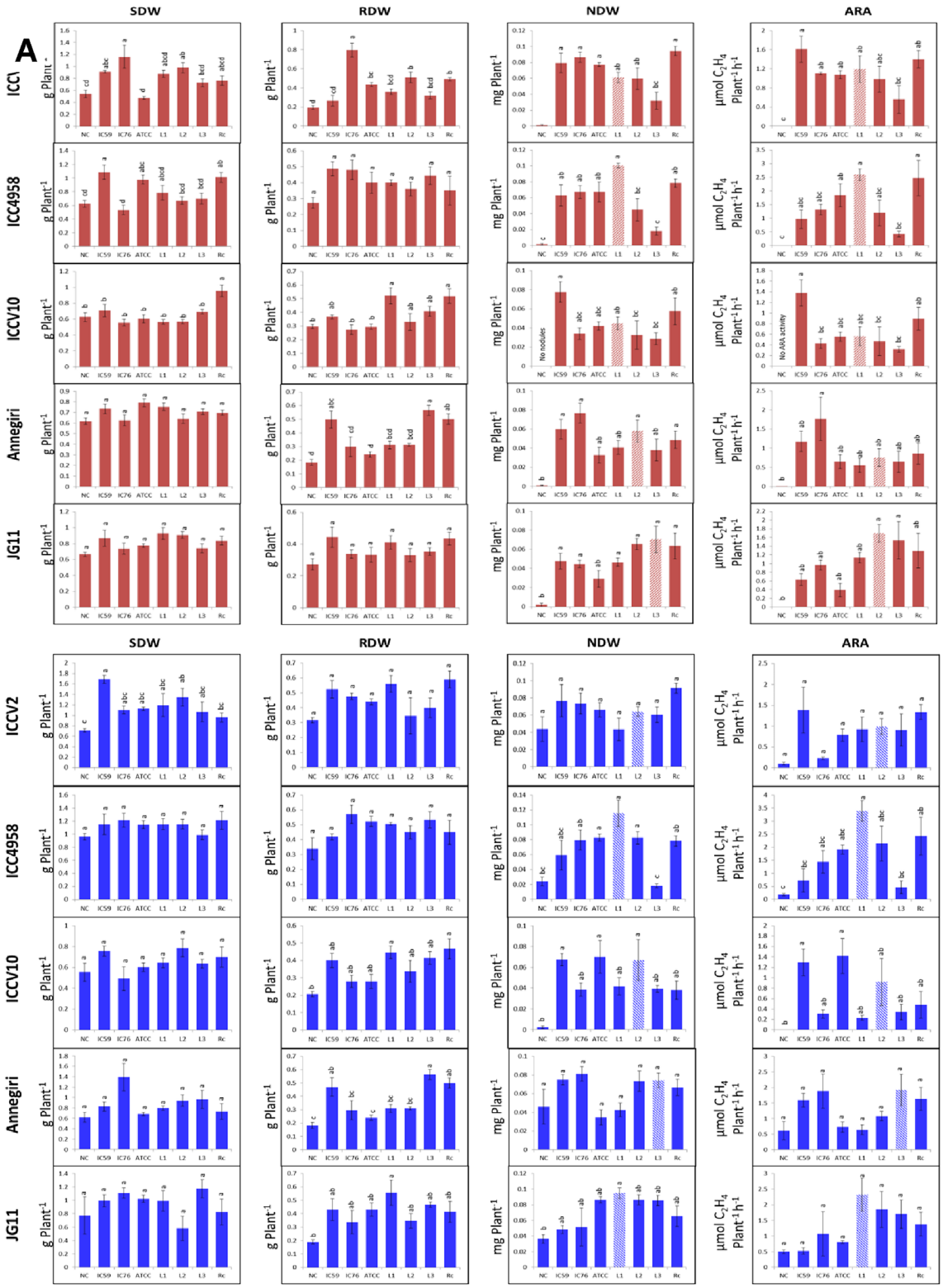

Fig. 2 Chickpea growth responses toward CR treatment at SS (**A**) and NS (**B**) conditions. Values are Mean ± SE ($n = 3$). Error bar indicates SE. Bars within a graph not sharing the same letter are significantly different as per DMRT ($p < 0.05$). Bars with pattern are the highest ranking CR strains on nodulation and nitrogenase activity

Table 4 Analysis of variance (ANOVA) for growth parameters of chickpea genotypes under SS and NS conditions

Source of variation	*df*	Mean square			
		SDW	RDW	NDW	ARA
SS					
Genotype	4	0.11964**	0.01487^{NS}	0.0018571^{NS}	1.9465**
Treatment	7	0.09609**	0.06889***	0.0071572***	2.7709***
Genotype × treatment	28	0.06810***	0.036946***	0.0008585***	0.7059***
NS					
Genotype	4	1.01634***	0.07327**	0.0022876*	3.5015***
Treatment	7	0.1859^{NS}	0.0984***	0.0028894***	2.2681**
Genotype × treatment	28	0.10101**	0.0157*	0.0013097***	1.2506***

NS non-significant, *SDW* shoot dry weight, *RDW* root dry weight, *NDW* nodulation dry weight, *ARA* Nitrogenase activity

*Significant at $p < 0.05$; **significant at $p < 0.01$; ***significant at $p < 0.001$

Salinity experiments

In planta salinity trial

Effect of salinity and its treatment by microbial consortium on chickpea phenotypic traits is depicted in Tables 6, 7 and 8. Traits like shoot length, shoot dry weight, number of branches, leaf area, number of flowers, number of pods and reproductive parts weight were significantly ($p < 0.05$, over the SS groups) influenced either both or any one of the microbial treatments irrespective of the genotype (Table 6). There were no nodules in SS group. However, both the consortia induced nodulation in both the genotypes. The below-ground traits like root length, root surface area, root volume and root dry weight were also significantly ($p < 0.05$) influenced by both the microbial treatments irrespective of the genotype (except root dry weight by C1 in JG 11; still, it is higher than SS group). On the 38th day sampling, C2 treatment significantly ($p < 0.05$) increased the total chlorophyll in both ICCV 2 (2.56 ± 0.28 mg. g FW^{-1}) and JG 11 (2.64 ± 0.09 mg. g FW^{-1}), rather carotenoids (0.53 ± 0.05 mg. g FW^{-1}) were found to be increased only in ICCV 2 (Table 7). C1 has no significant effect on chlorophyll and carotenoids in both ICCV 2 and JG 11. The highest electrolyte leakage index was noticed on saline-stressed groups rather than control (2.3 vs. 0.4 in ICCV 2; 1.8 vs. 0.6 in JG 11); however, both the consortium treatments have reduced the leakage index to 0.6–1.2 (Fig. 7).

Table 8 showed significant ($p < 0.05$) decrease of harvest traits on SS group than control groups. Consortium treatment has overcome the saline stress and surprisingly C2 showed significance on both the genotypes ICCV 2 and JG 11 on all the estimated harvest traits like total biomass (C2 vs. SS; 11.8 vs. 7.2; 10.2 vs. 6.4 g. $Plant^{-1}$), number of pods (C2 vs. SS; 24.4 vs. 15.6; 19.9 vs.8.7 $Plant^{-1}$), pod weight (C2 vs. SS; 6.8 vs. 4.3; 5.1 vs. 2.0 g. $Plant^{-1}$), number of seeds (C2 vs. SS; 21.4 vs. 13; 17.3 vs. 7.6 $Plant^{-1}$) and seed weight (C2 vs. SS; 5.4 vs. 3; 4.2 vs. 1.6 g. $Plant^{-1}$). On the other hand, C1 has showed significance on all the harvest traits of ICCV 2 and only total biomass and no. of seeds of JG 11. Still the values indicate that it was able to overcome the salinity effects to some extent. The data of salinity trial depicts that both the microbial treatment provided salinity tolerance to both the genotypes besides their in-built salinity tolerance levels. Specifically, C2 provided better tolerance and yield traits.

Histochemical studies

Histochemical staining of chickpea leaves for H_2O_2 and $O_2-^{\cdot}$ and roots for lipid peroxidation was depicted in Fig. 8. This is to analyze whether microbial treatments have protective mechanism against salinity stress is the result of reduced oxidative stress and consequent membrane lipid peroxidation. The pattern of DAB and NBT staining reveals that C1 and C2 provide oxidative stress relief against the salinity tolerance, which was indicated by the presence of higher radical formation in SS followed by consortium-treated groups and control plants. Similarly, Schiff staining identifies aldehydes that originate from lipid peroxides, higher lipid peroxidation on saline stress roots rather than saline stress treated by consortium groups.

Gene expression studies

In the present study, antioxidant genes (SOD, APX, CAT, PAL, and LOX) and transporter genes (NXH, CLC-b and HKT) expression patterns were studied in leaves and roots at 45 DAS.

In the case of antioxidant defense genes, mixed pattern of gene expression was observed and there was no standard response among the treatments or the cultivars. In leaf, C1 has scored higher expression levels for APX-gene with a fold change of 4.95 in JG 11 genotype and vice versa in C2.

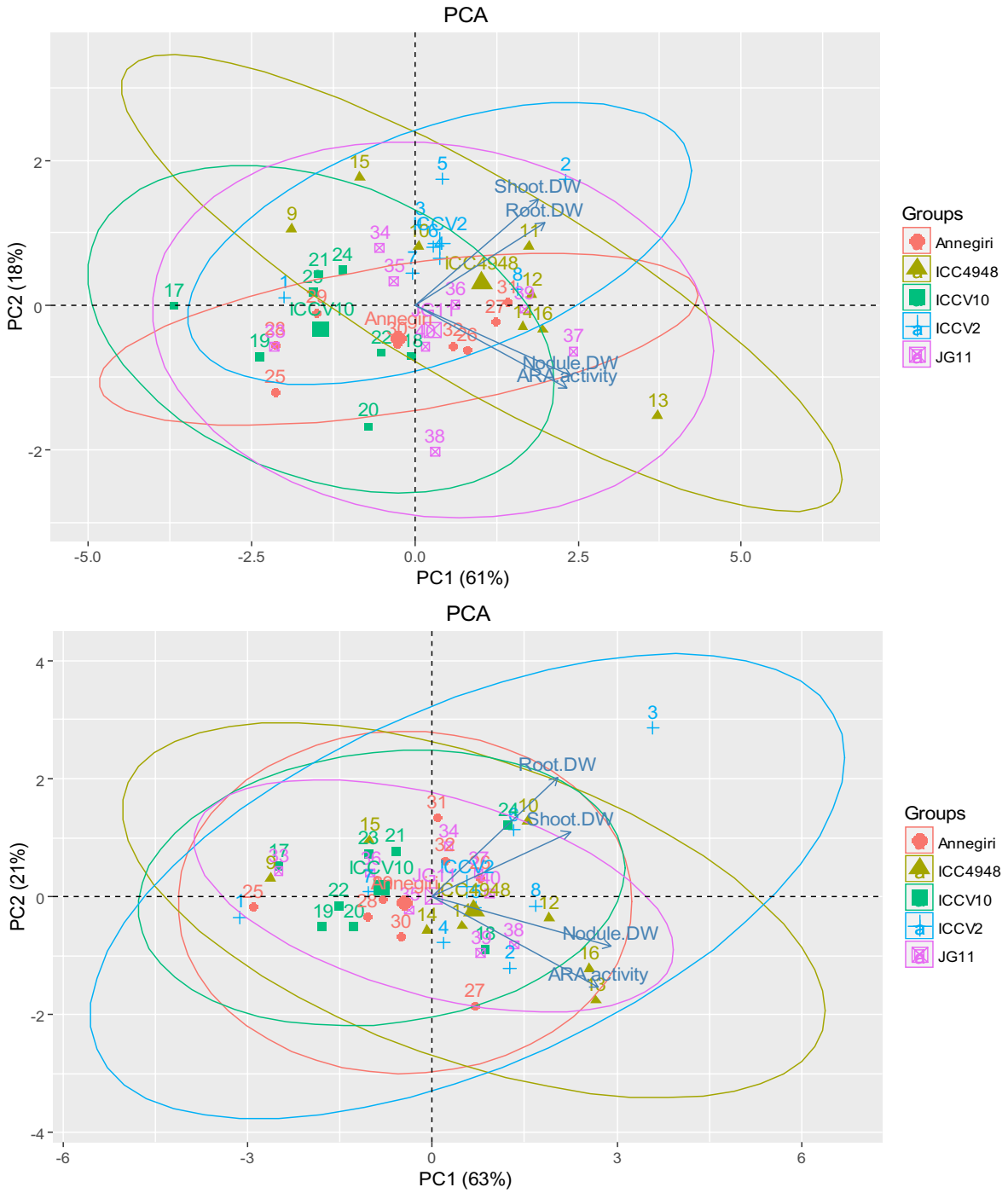

Fig. 3 Principal component analysis on evaluation of growth responses of chickpea genotypes toward CR treatment under SS (**A**) and NS (**B**) conditions. 1, 9, 17, 25, 33—NC; 2, 10, 18, 26, 34—IC59 treated; 3, 11, 19, 27, 35—IC76 treated; 4, 12, 20, 28, 36—ATCC treated; 5, 13, 21, 29, 37—L1 treated; 6, 14, 22, 30, 38—L2 treated; 7, 15, 23, 31, 39—L3 treated; 8, 16, 24, 32, 40—Rc treated

In the case of chickpea roots, no significant gene regulation was observed at many instances than leaves. However, C2 scored a higher gene expression.

Gene expression values were compared against normal control groups. In case of leaf antioxidant gene expression, all the treatment groups SS, C1 and C2 of both the genotypes showed up-regulation ranging from 2.23 to 10.71 (Fig. 9). No significant gene expression was noticed on C1 and SS group of ICCV 2 in CAT and APX, respectively. However, there was no standard response among the treatments or the cultivars. In the context of root gene expression, up-regulation was noticed by 2.37–10.82-fold change. Further,

Table 5 Pearson correlation coefficient analysis on chickpea growth parameters over *CR* treatments under SS (above the diagonal) and NS conditions (below the diagonal)

	Shoot dry weight	Root dry weight	Nodule dry weight	Nitrogenase activity
SDW	1	0.441**	0.446**	0.445**
RDW	0.483**	1	0.493**	0.246^{NS}
NDW	0.435**	0.485**	1	0.888**
ARA	0.403**	0.433**	0.792**	1

**Correlation is significant at $p < 0.01$; NS—Non-significant

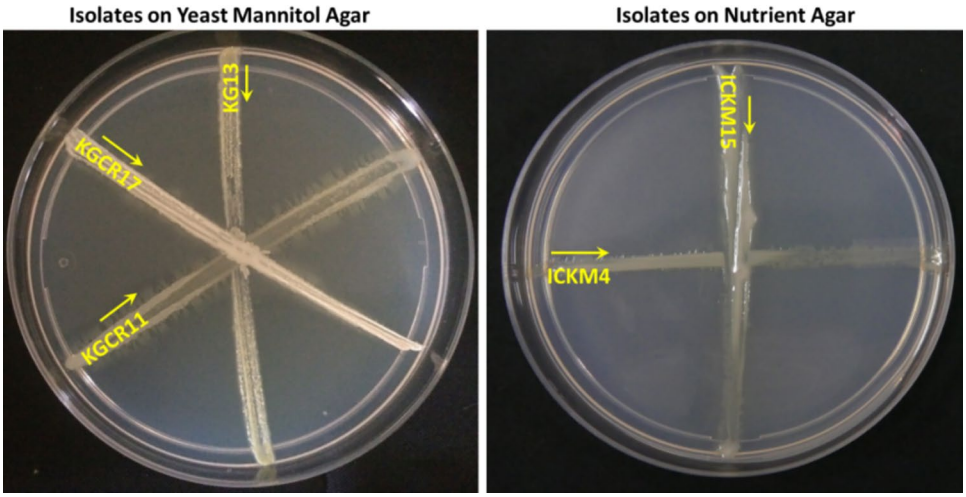

Fig. 4 Compatibility of the selected isolates in consortium 1 (KG13 + KGCR11 + KGCR17) and consortium 2 (ICKM4 + ICKM15)

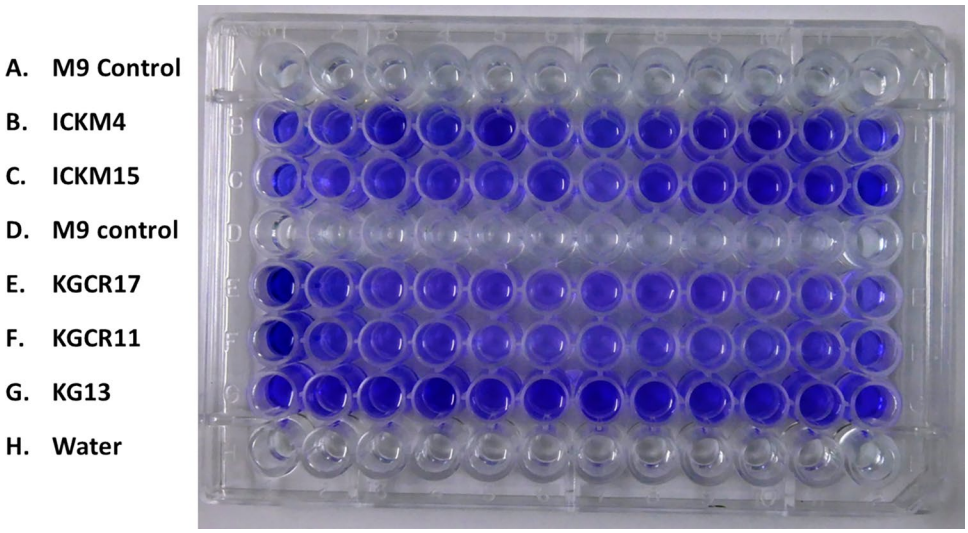

Fig. 5 Biofilm formation of the selected isolates in consortium 1 (KG13 + KGCR11 + KGCR17) and consortium 2 (ICKM4 + ICKM15)

C2 treatment has scored maximum up-regulation in JG 11 on both leaf (8.05/SOD; 8.6/CAT; 10. 71/PAL; 9.86/LOX) and roots (7.44/SOD; 4.96/APX; 6.74/PAL; 6.63/LOX). The root catalase gene has shown no significant expression in all the treated groups (except SS group of JG 11 which showed up-regulation of 3.14).

The mechanism by which the selected microbes help in alleviating the salinity stress is complex. However, an increase in antioxidant machinery is related in increased stress tolerance of plants (Jaspers and Kangasjärvi 2010). While in gene expression studies of transporter genes, there has been an up-regulation of gene expression in the treatment groups in JG 11 genotype. In the case of leaf, all the treatment groups SS, C1, and C2 have shown up-regulation of 2.22–8.93-fold change (Fig. 10). C2 has shown higher gene expression levels in both the genotypes JG 11 and ICCV 2 (2.22/NXH; 2.43/CLC-b; 8.93/HXT and 4.77/NXH) except for the HXT and CLC-b genes in ICCV 2 genotype

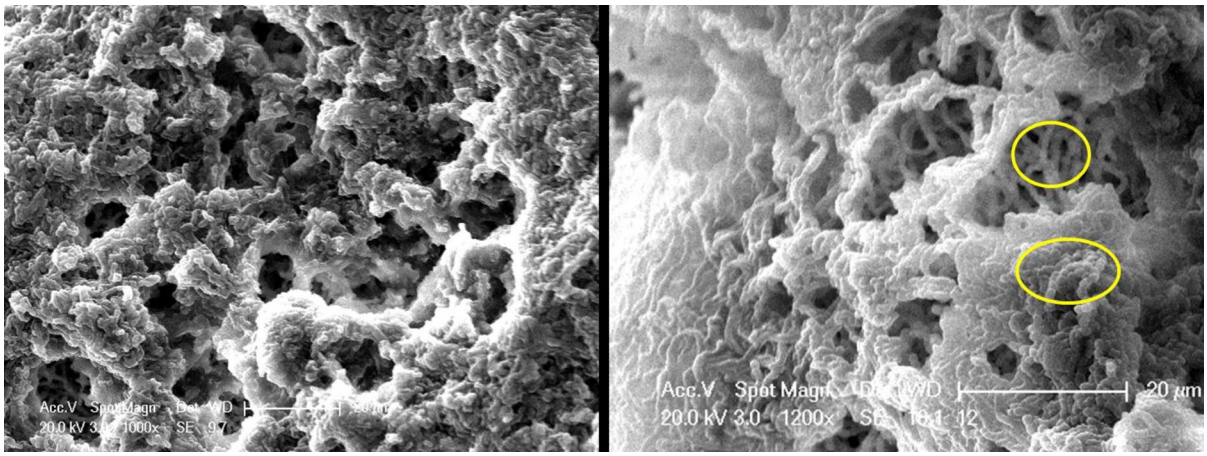

Fig. 6 Spore chain morphology of *Nocardiopsis alba* KG13 under scanning electron microscope

where it shows no significant regulation of when compared to control. No significant gene expression was observed in genotype of ICCV 2 treatment groups SS, C1 and C2 in NXH and CLC-b genes respectively, whereas in the case of roots gene expression studies of transporter genes, there was no standard gene expression patterns, but a significant up-regulation has been observed from fold change of 2.07–6.07. However, no significant regulation has also been observed in SS group of ICCV 2 in NXH and HKT and also in SS, C1, and C2 groups of ICCV 2 in CLC-b gene. C1 has shown up-regulated of NXH and CLC-b gene expression in the genotype JG 11 (3.88/NXH; 3.41/CLC-b) and in NXH gene of ICCV 2 genotype with 2.75-fold change than C2. But in the case of HXT gene, C2 has shown higher levels of up-regulated gene expression in both the genotypes (6.87/ICCV 2; 3.87/JG-11). In overall, C2 showed up-regulated gene expression of both antioxidant and transporter genes in both the genotypes when compared to C1 under saline-stressed conditions.

Discussion

Plants live with the microbial communities in their root system and thus plant–microbe interactions serve as a key determinant for plant fitness. Among these communities, plant growth-promoting microbes including bacteria, fungi and rhizobia either as free cells or as endophytes help plants in nutrient acquisition, growth, biocontrol activity and stress tolerance by direct or indirect mechanisms (Ma 2020; Upadhyay and Chauhan 2022; Upadhyay et al. 2022). Understanding of associated microbes in the context of chickpea under salinity is superficial. Hence, the current study is intended to identify some chickpea rhizosphere and root-associated microbes and their role in chickpea growth under saline conditions.

The 275 isolates were obtained from rhizospheric soil and root nodules of chickpea, subjected to three stages of screening by qualitative and quantitative estimation of ACCd and salinity tolerance. Three potential isolates were selected and identified by 16 s rDNA sequencing. This includes (i) actinomycete *Nocardiopsis alba* KG13 with moderate ACCd production (27.4 nmoles α-ketobutyrate mg protein^{-1} h^{-1}) and high salt tolerance (8% NaCl); chickpea rhizobia *Sinorhizobium meliloti* KGCR17 with high ACCd production (38.9 nmoles α-ketobutyrate mg protein^{-1} h^{-1}) and low salt tolerance (4% NaCl); (iii) root nodule-associated bacteria *Bacillus safensis* KGCR11 with low ACCd production (0.9 nmoles α-ketobutyrate mg protein^{-1} h^{-1}) and high salt tolerance (8% NaCl). These isolates produced other PGP traits like P solubilization and production of IAA, siderophore, β-1,3-glucanase, ammonia and HCN.

Molecular identification of the selected strains gives us some distinct features. To the best of our knowledge, this is the first report stating the isolation of *Nocardiopsis alba*, a non-streptomycete with PGP properties and saline tolerance from chickpea rhizospheric soil. Many reports identified *Streptomyces* as the predominant genus (Sathya et al. 2017). Even in our lab, we identified many PGP *Streptomyces* strains from chickpea with proven growth-promoting activities (Gopalakrishnan et al. 2015a, b, c, d a, b) and biocontrol activity against *Fusarium* wilt (Gopalakrishnan et al. 2011; Sravani et al. 2021) in chickpea under field conditions. Another interesting point is the identification of *Sinorhizobium meliloti* with PGP traits from chickpea root nodule because very few reports only available as supporting data. Chickpea rhizobia diversity studies from Morocco (Maâtallah et al. 2002) and Portugal (Alexandre et al. 2009) identified *Sinorhizobium* as the least strains and *Mesorhizobium* as the major strains. The other bacterium *B. safensis* KGCR11 associated with chickpea root nodule in the current study correlates with the report of Benjelloun et al. (2019), who

Table 6 Effect of two microbial consortia on physiological traits of chickpea under saline-stressed conditions at 38 DAS

Treatments	Above ground parameters							Below ground parameters				
	Shoot length (cm plant^{-1})	Shoot dry weight (g plant^{-1})	No. of branches (plant^{-1})	Leaf area (cm^2 plant^{-1})	No. of flowers (plant^{-1})	No. of pods (plant^{-1})	Reproductive parts dry weight (g plant^{-1})	No. of nodules (plant^{-1})	Root length (cm)	Root surface area (cm^2)	Root volume (cm^3)	Root dry weight (g plant^{-1})
ICCV 2												
C	24.4 ± 0.7*	1.4 ± 0.1*	4.4 ± 0.4*	88.0 ± 6.6*	14.7 ± 1.3*	3.0 ± 0.5*	0.07 ± 0.01	1.1 ± 0.7	5938.4 ± 867.4*	1715.2 ± 281.4*	27.0 ± 2.9*	1.4 ± 0.2*
SS	19.8 ± 1.0	1.0 ± 0.0	3.0 ± 0.3	61.7 ± 6.1	8.6 ± 1.0	1.4 ± 0.3	0.04 ± 0.01	–	2998.1 ± 115.4	791.4 ± 16.6	17.7 ± 0.5	0.8 ± 0.1
C1	24.5 ± 0.3*	1.3 ± 0.1*	4.1 ± 0.4	82.6 ± 7.7	12.1 ± 1.1	3.1 ± 0.5*	0.1 ± 0.01*	7.6 ± 3.8	5795.1 ± 247.3*	1580.1 ± 51.1*	31.0 ± 2.5*	1.3 ± 0.1*
C2	26.3 ± 0.8*	1.6 ± 0.1*	6.2 ± 0.5*	98.5 ± 7.8*	19.1 ± 1.7*	3.3 ± 0.4*	0.1 ± 0.01*	6.4 ± 5.0	7545.5 ± 853.1*	1795.2 ± 212.8*	35.8 ± 3.1*	1.3 ± 0.1*
JG 11												
C	23.1 ± 0.7*	1.4 ± 0.1*	6.0 ± 0.4*	71.9 ± 3.0*	17.8 ± 1.2*	1.1 ± 0.3*	0.05 ± 0.01	–	10,672.8 ± 1027.3*	2583.9 ± 331.1*	49.4 ± 3.7*	1.9 ± 0.2*
SS	19.9 ± 0.3	1.0 ± 0.0	4.0 ± 0.2	55.0 ± 2.3	11.8 ± 0.5	0.1 ± 0.1	0.03 ± 0.01	–	5984.1 ± 997.9	1576.8 ± 233.8	32.1 ± 4.9	1.1 ± 0.0
C1	22.8 ± 0.5*	1.4 ± 0.1*	6.3 ± 0.4*	75.3 ± 4.8*	13.3 ± 1.2	0.3 ± 0.2	0.05 ± 0.01	4.6 ± 2.6	10,652.1 ± 677.1*	2720.4 ± 99.7*	55.5 ± 0.6*	1.5 ± 0.0
C2	23.8 ± 0.4*	1.5 ± 0.1*	7.0 ± 0.8*	79.6 ± 4.3*	17.0 ± 2.5*	0.1 ± 0.1	0.05 ± 0.01	1.7 ± 1.7	11,530.3 ± 1585.2*	2942.6 ± 332.8*	60.0 ± 5.3*	1.8 ± 0.2*

Values are Mean ± SE ($n = 9$ for above-ground parameters; $n = 3$ for below-ground parameters). C-Control; SS-Saline-stressed; C1-Saline stress treated with Consortium 1; C2-Saline stress treated with Consortium 2. * – Values are statistically significant as per Dunnett's test ($p < 0.05$) over the SS

observed one *Bacillus* sp., among the 46 nodulating and 59 non-nodulating strains from chickpea root nodules. Similarly, Egamberdieva et al. (2017) identified *Bacillus cereus, Bacillus thuringiensis* and *Bacillus subtilis* from root nodules of chickpea grown under saline conditions and found to have PGP traits. Sharma et al. (2019) identified some saline-tolerant (up to 10% NaCl) *Bacillus* strains including *B. safensis* from chickpea rhizospheric soil. These so-called 'guest bacteria' adhere like pathogen or PGP microbes and provide their effects on crops (Shiraishi et al. 2010).

Similarly, Pandey et al. (2019) observed PGP microbes of *Azotobacter* and *Bacillus* sp., from chickpea rhizospheric samples with multifaceted growth-promoting traits like ACCd production, salinity tolerance, IAA production, P solubilization, siderophore production and ammonia production. Additionally, the isolates were found to be tolerant to both salinity and water stress (drought), which are significant environmental stressors for plants. The consortium of these PGP microbes showed potential in improving the growth of chickpea plants, likely through their combined action on various growth-promoting mechanisms and stress tolerance traits.

Many reports are available for the benefits of multi-species/strain mixtures rather than single species for enhanced plant growth, pathogen control and stress tolerance (Woo and Pepe 2018) because of the members of the consortium besides competing for rhizospheric adherence and establishment, and they provide complement functionality with each other (Pandey et al. 2012). The pre-requisite for consortia preparation is compatibility. In the current study, we made two consortia C1 and C2, and there was no growth inhibition between them under in vitro co-culturing conditions. This indicates the absence of suppressive effect, and they may colonize on plant roots, which was further supported by their biofilm-forming trait. As per the calculations of Stepanovic et al. (2000), two categories of biofilms were observed which includes one strong adherent member KG13 in C1 and entire crew of C2, ICKM4 and ICKM15 possesses strong adherents. Others are with moderately adherent property. These biofilm-forming candidates enhance higher root exudate secretions, which in turn serves as an energy source for PGP microbes (Upadhyay et al. 2022). Study of Kasim et al. (2016) observed that *Bacillus amyloliquefaciens* HM6, a biofilm-forming saline-tolerant isolate, influences seedling length, relative water content and dry mass of barley under saline conditions. Ansari and Ahmad (2019) observed both planktonic and biofilm modes of growth of a consortia of PGP microbes *Pseudomonas fluorescens* FAP2 and *Bacillus licheniformis* B642, and the significant enhancement of vegetative growth and photosynthetic parameters of wheat upon consortia application.

In *in planta* salinity trials, the consortia C1 and C2 have increased chickpea growth traits. Similarly, a multispecies

Table 7 Effect of two microbial consortia on chlorophyll and carotenoid content of chickpea under saline-stressed conditions

Treatments	Chlorophyll a (mg g FW^{-1})	Chlorophyll b (mg g FW^{-1})	Total chlorophyll (mg g FW^{-1})	Carotenoids (mg g FW^{-1})
ICCV 2				
C	1.75 ± 0.08	0.35 ± 0.02	2.10 ± 0.10	0.44 ± 0.02
SS	1.66 ± 0.06	0.37 ± 0.02	2.04 ± 0.08	0.43 ± 0.02
C1	1.76 ± 0.09	0.40 ± 0.03	2.16 ± 0.12	0.46 ± 0.02
C2	2.13 ± 0.23*	0.43 ± 0.05	2.56 ± 0.28*	0.53 ± 0.05*
JG 11				
C	2.07 ± 0.06	0.40 ± 0.01	2.47 ± 0.08	0.52 ± 0.02
SS	1.95 ± 0.07	0.38 ± 0.01	2.33 ± 0.08	0.46 ± 0.02
C1	2.08 ± 0.09	0.39 ± 0.01	2.47 ± 0.10	0.51 ± 0.02
C2	2.20 ± 0.08*	0.44 ± 0.02*	2.64 ± 0.09*	0.51 ± 0.02

Values are Mean ± SE ($n = 9$). C-Control; SS-Saline-stressed; C1-Saline stress treated with Consortium 1; C2-Saline stress treated with Consortium 2. * – Values are statistically significant as per Dunnett's test ($p < 0.05$) over the SS

Table 8 Effect of two microbial consortia on harvest traits of chickpea under saline-stressed conditions

Treatments	Total biomass (g $plant^{-1}$)	No. of pods ($plant^{-1}$)	Pod weight (g $plant^{-1}$)	No. of seeds ($plant^{-1}$)	Seed weight (g $plant^{-1}$)
ICCV 2					
C	8.6 ± 0.5*	22.2 ± 2.3*	5.4 ± 0.5	20.1 ± 2.0*	4.2 ± 0.4*
SS	6.4 ± 0.3	15.6 ± 0.8	4.3 ± 0.3	13.0 ± 0.7	3.0 ± 0.2
C1	9.5 ± 0.2*	24.7 ± 1.7*	5.8 ± 0.4*	21.7 ± 1.4*	4.7 ± 0.3*
C2	10.2 ± 0.4*	24.4 ± 2.0*	6.8 ± 0.5*	21.4 ± 1.8*	5.4 ± 0.4*
JG 11					
C	9.2 ± 0.7	15.7 ± 2.8*	3.5 ± 0.6*	13.7 ± 2.2*	3.1 ± 0.5*
SS	7.2 ± 0.4	8.7 ± 1.6	2.0 ± 0.4	7.6 ± 1.2	1.6 ± 0.3
C1	10.8 ± 0.6*	13.7 ± 1.3	3.0 ± 0.4	12.7 ± 1.4*	2.4 ± 0.3
C2	11.8 ± 0.8*	19.9 ± 1.4*	5.1 ± 0.3*	17.3 ± 1.2*	4.2 ± 0.2*

Values are Mean ± SE ($n = 9$). C-Control; SS-Saline-stressed; C1-Saline stress treated with Consortium 1; C2-Saline stress treated with Consortium 2. * – Values are statistically significant as per Dunnett's test ($p < 0.05$) over the SS

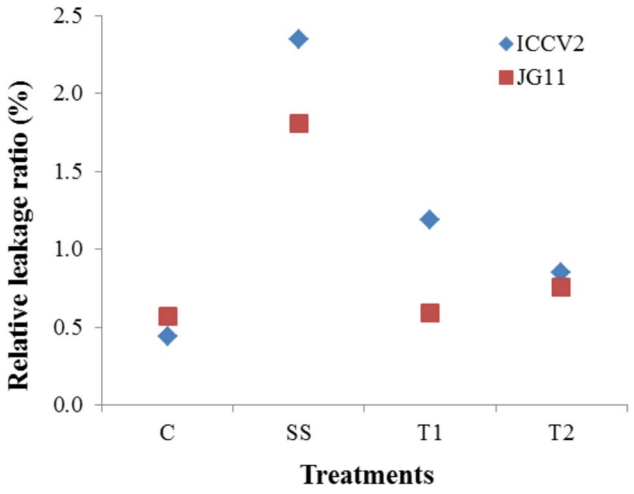

Fig. 7 Electrolyte leakage of chickpea ICCV 2 and JG 11

consortium consisting of halotolerant *Bacillus* sp., *Delftia* sp., *Enterobacter* sp., *Achromobacter* sp., has significantly increased leaf numbers (75%), shoot (92%) and root lengths (146%), leaf (105%), shoot (105%) and root (109%) dry weights of tomatoes under salt stress (Kapadia et al. 2021). Root data of the present study reveal that both the consortia treatments enhanced the entire root system result in higher nutrient and water uptake, which in turn promotes above-ground parameters. This might be due to microbial growth-stimulating hormone IAA, which serves as a boost for plants IAA levels in addition to plant's endogenous IAA. Shutsrirung et al. (2013) observed the highest shoot growth of mandarin seedlings by endophyte *Nocardia*, the higher IAA-producing strain (62–222 µg/mL), whereas the lower shoot growth was by endophyte *Microbispora*, the lower IAA-producing strain (0.3–3 µg/

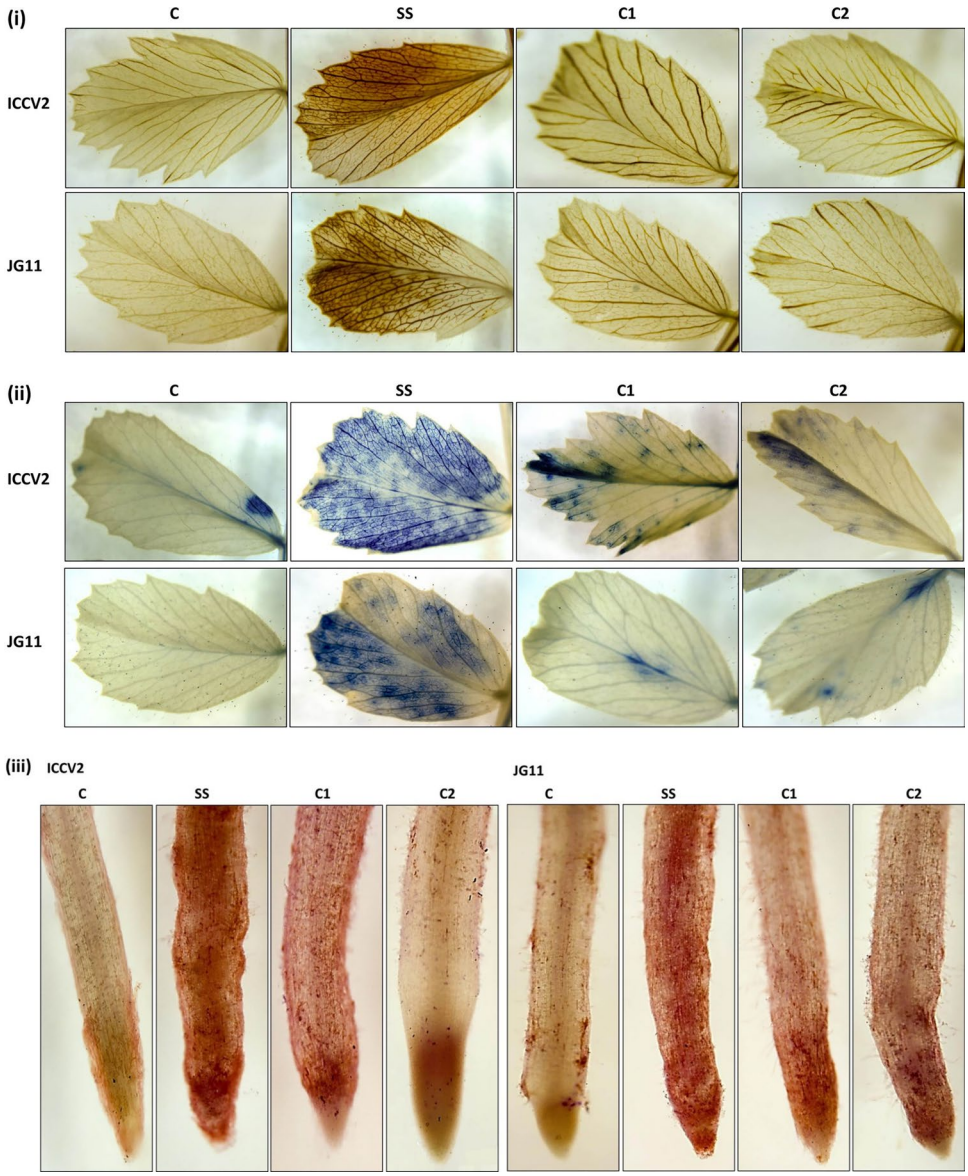

Fig. 8 Staining for the detection of oxidative stress in chickpea ICCV 2 and JG 11. **Panel i**—In vivo detection of H2O2 in chickpea leaves by DAB staining. **Panel ii**—In vivo detection of O2-˙ in chickpea leaves by NBT staining. **Panel iii**—In vivo detection of lipid peroxidation in chickpea roots by Schiff staining. C-Control; SS-Saline stressed; C1-Saline stress treated with Consortium 1; C2-Saline stress treated with Consortium 2

mL). Though total IAA production in vitro of both the consortia was equivalent to ~ 19 µg mL^{-1}, C2 has induced a higher shoot and root growth in both chickpea genotypes besides the statistical significance over the saline controls. Studies of Mir et al. (2021) on chickpea ICCV 2 with the consortia inoculation of red gram and chickpea root nodule-associated rhizobia *Rhizobium tropici* IHRG and *Mesorhizobium* sp. IHGN3, respectively, showed increased nodulation, growth, and yield traits under greenhouse conditions than solo inoculations. Recently, Chauhan and Upadhyay (2024) explored a saline-tolerant (6%) PGP *Klebsiella* and made into a consortium with *Kluyvera* sp. and *Enterobacter* sp. Consortia were able to alleviate 1% saline stress in maize and influence phosphate solubilization, IAA, proline activity, CAT, POD, and exopolysaccharides than single inoculations.

Besides the growth-promoting factors, PGP microbes influence plant growth by providing stress relief by lowering plant ethylene levels through ACCd under stress conditions (Glick et al. 2007). Gupta and Pandey (2019) identified two strains *Aneurinibacillus aneurinilyticus* ACC02 and *Paenibacillus* sp., ACC06 with a higher ACCd production of > 1500 nmoles α-ketobutyrate mg $protein^{-1}$ h^{-1} and salinity tolerance of 6% NaCl with other PGP traits. They observed an increase of shoot and root fresh weight by 255 and 45%; shoot and root length by 60 and 110%; shoot and root biomass by 425 and 220%; and chlorophyll content by 57% by the consortia of ACC02 and ACC06. In our case, C1 was able to produce considerably moderate quantity of ACCd, whereas C2 did not produce ACCd activity; however, they were able to alleviate the stress tolerance till harvest, and interestingly C2 leads the role on both the genotypes.

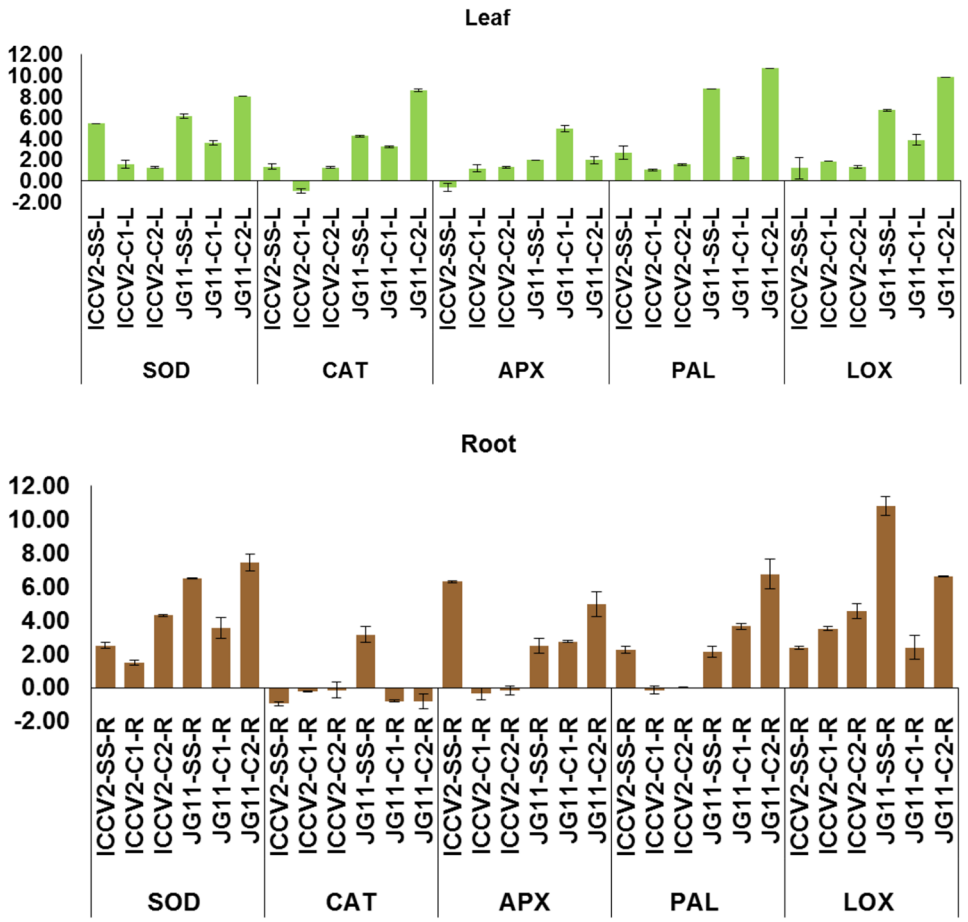

Fig. 9 Effect of two microbial consortia on antioxidant gene expression of chickpea leaf and root under saline-stressed conditions

The two possible reasons for the salinity tolerance without ACCd production were first, it might be the microbial consortium with synergistic interactions between different species may enhance overall saline tolerance. Cross-feeding, resource sharing, or cooperative stress responses can contribute to improved resilience to salt stress. A second possible reason may be up-regulation of genes involved in stress response pathways, including those related to osmotic stress, ion transport, and compatible solute biosynthesis. The present investigation has also studied the gene expression analysis of chickpea genotypes under saline stress conditions, in which C2 has showed the up-regulation of both antioxidant and transporter genes compared to C1 and control suggesting salinity tolerance of the consortium without ACCd production.

Our study was well-supported by Kumawat et al. (2021) who investigated consortium of salt-tolerating *Rhizobium* sp. LSMR-32 and *Enterococcus mundtii* LSMRS-3 with multifarious PGP traits on salinity tolerance of mung bean under field conditions. The consortium was found to yield better seed germination, shoot and root growth traits, symbiotic traits, macro- and micronutrient uptake, and Na^+, K^+ ion homeostasis.

Also, the study by Kukreja et al. (2005) highlights the complex physiological and biochemical responses of chickpea plants to salinity stress, including water relations, oxidative stress, defense mechanisms, and ion homeostasis, as well as their ability to partially recover upon stress relief. Salinity stress has decreased the leaf water potential, osmotic potential, and relative water content of roots and leaves has increased ACCd production, H_2O_2 content, lipid peroxidation, activated antioxidant enzymes, ascorbic acid content, accumulation of proline which increases the potential for plant resilience and adaptive responses to salinity stress.

Akram et al. (2020) showed that plants have developed different mechanisms, such as ionic stress pathways, oxidative stress pathways, and detoxification signaling, to cope with the high soil salinity and toxicity of Na^+ and Cl^- ions. Many cellular processes conferring stress tolerance and regulating plant growth and development are dependent upon pH and ion homeostasis. Ion-specific salinity is caused by the accumulation of toxic concentrations of sodium (Na^+) and/or chloride (Cl^-) ions, especially in the older leaves. In most plant species, Na^+ reaches the toxic concentration earlier than other salts. Two non-selective cation channels (NSCC) are the major source of entry of Na^+ into the cell:

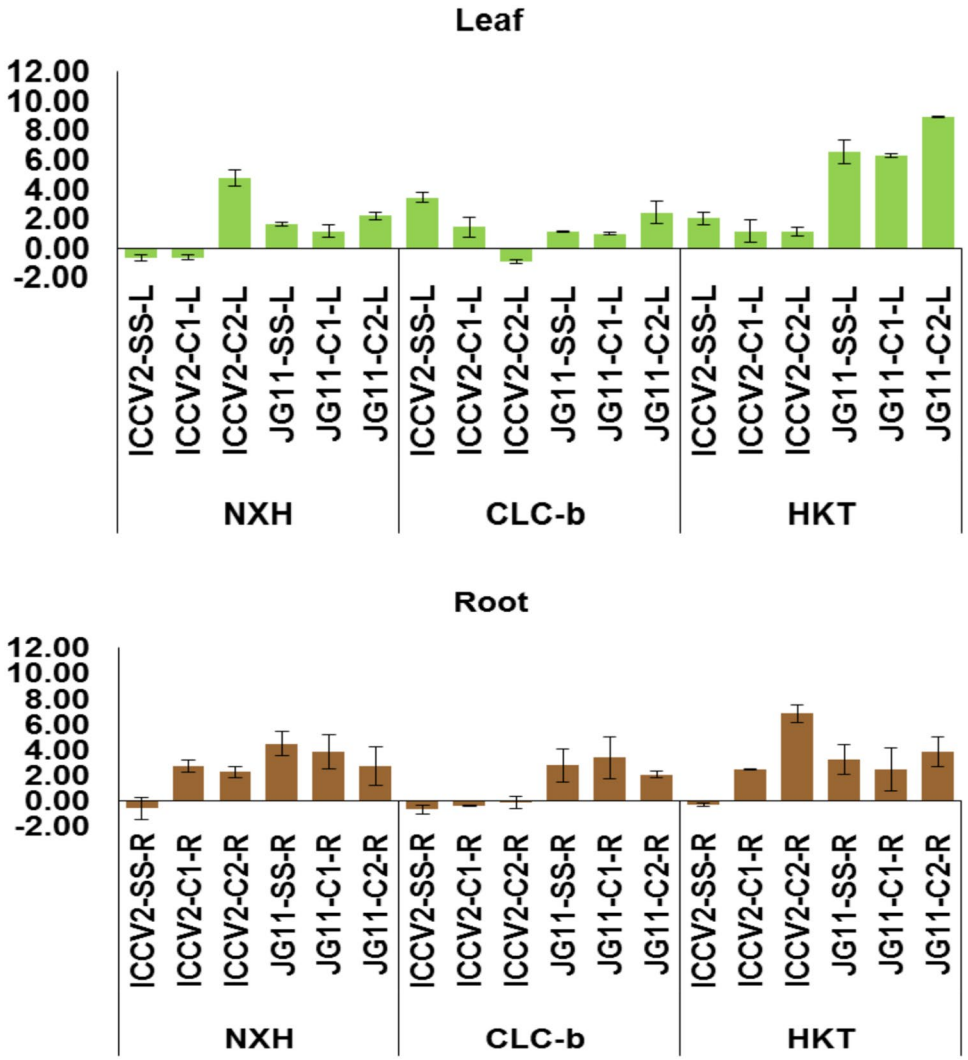

Fig. 10 Effect of two microbial consortia on transporter gene expression of chickpea leaf and root under saline-stressed conditions

voltage-dependent and voltage-independent cation channels. The voltage-independent cation channels are thought to be a significant way of entering for Na^+ ions. Sodium–hydrogen antiporters (NHX) are important antiporter genes which can help plants exclude Na + and Cl^- ions through membranes or deposits in the vacuole to maintain the cell osmotic level. Vacuole-bounded NHX antiporters regulate pH by countering acidity due to H^+ pumps and functions such as H^+ leaks to maintain the pH. Besides the compartmentalization of Na+, NHXs could play a role in increasing the salinity tolerance by adjusting the K^+ homeostasis.

Subba et al. (2021) revealed that even in plants, loss of CLC protein function severely impairs various cellular processes critical for normal growth and development. These proteins sequester Cl^- into the vacuole, thus making them an attractive target for improving salinity tolerance in plants caused by high abundance of salts, primarily NaCl. Besides, some CLCs are involved in NO_3^- transport and storage function in plants, thus influencing their nitrogen use efficiency. However, despite their high significance, not many studies have been carried out in plants. Here, we have attempted to concisely highlight the basic structure of CLC proteins and critical residues essential for their function and classification. We also present the diverse functions of CLCs in plants from their first cloning back in 1996 to the knowledge acquired as of now. We stress the need for carrying out more in-depth studies on CLCs in plants for they may have future applications toward crop improvement.

The current study has also investigated the expression of antioxidant (SOD, APX, CAT, PAL and LOX) and transporter (NXH, CLC-b and HKT) genes in leaves and roots of chickpea genotypes (JG 11 and ICCV 2) at an early reproductive stage under saline stress conditions, along with the effects of selected microbes in alleviating salinity stress. In the leaf, the study observed up-regulation of antioxidant genes in response to saline stress across all treatment groups. However, the significant fold changes were random, indicating that there was no consistent or standard response among

the treatments or cultivars. This suggests that the expression of antioxidant genes in the leaf is influenced by various factors, including the specific genotype, the severity of saline stress, and potentially other environmental factors. Despite the overall trend of up-regulation, down-regulation of antioxidant genes was also noticed in specific instances. This could be due to various reasons, such as the activation of different signaling pathways, feedback regulation mechanisms, or the allocation of resources toward other stress response mechanisms, whereas in roots, up-regulation of antioxidant genes was observed, but non-significant fold changes were noticed in many treatment groups. Catalase gene expression was consistently downregulated in all treated groups, except for one instance. Similarly, transporter gene expression in leaves has shown upward regulation in all treatment groups with varying fold changes. The mechanism by which selected microbes alleviate salinity stress is complex, but an increase in antioxidant machinery is related to increased stress tolerance. C2 treatment showed better and upward-regulated gene expression of both antioxidant and transporter genes in both genotypes compared to C1 under saline stress conditions.

Liu et al. (2020) specified that in a salinized environment, mostly caused by high NaCl, the foliar salt damage of some plants was mainly caused by Na+, while that of other plants, such as tobacco (*Nicotiana tabacum*), grape (*Vitis vinifera*), citrus (*Citrus aurantium*) and soybean (*Glycine max*), was mainly caused by Cl^-. Previous researchers reported that the accumulation patterns of anions, such as Cl^-, NO_3^-, HCO^{3-}, and SO_4^{2-} in plant tissues were associated with the plant salt tolerance. Also, the $NO3^-/Cl^-$ even equals to the K+/Na+, which was confirmed as one of the critical determinants of plant salt resistance.

Conclusion

The present investigation focused on the isolation and selection of microbial strains from chickpea rhizospheric soil and nodules for their potential to alleviate salinity stress in chickpea plants. 3 potential isolates (out of 275 isolates) were further selected for consortium-based *in planta* studies for salinity stress tolerance based on their ACCd production, their salinity tolerance and other plant growth-promoting traits by preliminary and quantitative estimation. Both the consortium treatments have overcome the saline stress over control, and surprisingly C2 showed significance on all of the estimated harvest traits like total biomass, number of pods, pod weight, number of seeds and seed weight on both the genotypes ICCV 2 and JG 11. The microbial treatments improved various phenotypic traits and reduced electrolyte leakage in chickpea plants under salinity stress. Histochemical studies indicated that both microbial consortia provided oxidative stress relief against salinity tolerance, reducing membrane lipid peroxidation. Gene expression analysis revealed up-regulation of antioxidant and transporter genes in response to microbial treatments, particularly in roots. Consortium 2 showed better and more consistent up-regulation of both antioxidant and transporter genes compared to Consortium 1. Overall, this study suggests that the selected microbial consortia have the potential to alleviate salinity stress in chickpea plants through various mechanisms, including PGP traits and modulation of gene expression related to stress tolerance.

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Author contributions SG and AS designed, supervised and finalized the experiments; AS and VS completed the experiments; AS wrote the original draft and VR, VS, HK and SG reviewed and finalized the manuscript

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Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval This manuscript does not contain any experiments involving human or animal participants.

Consent to participate The authors declare consent to participate in this work.

Consent for publication The authors declare consent to publish this work.

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