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REGULAR ARTICLE

# Revealing the underlying mechanisms mediated by endophytic actinobacteria to enhance the rhizobia - chickpea (*Cicer arietinum* L.) symbiosis

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**Abstract**

*Purpose* The effects of endophytic actinobacterial strains, *Microbispora* sp. CP56, *Actinomadura* sp. CP84B, *Streptomyces* spp. CP200B and CP21A, on the chickpea-*Mesorhizobium* symbiosis, were investigated *in planta*, with the aim of revealing the underlying mechanisms of action.

*Methods* The actinobacterial endophytes were co-inoculated with *Mesorhizobium ciceri* onto chickpea seedlings to study the effect on plant growth parameters, nodulation development and grain yield. The role of actinobacterial exudates on rhizobial growth was investigated, as was the role of root exudates of actinobacteria-colonized plants on the expression of rhizobial *nod* factors and biofilm formation. Changes in expression of plant flavonoids and bacterial N-fixation genes resulting from actinobacterial co-inoculation were assessed using qPCR.

*Results* Application of actinobacterial endophytes, together with *M. ciceri*, showed growth promotion of chickpea with an increase in root nodule number and weight. Enhanced nodulation was accompanied by increases in total plant nitrogen, larger total plant weight and a 2–3-fold increase in grain yield. Factors associated with this tripartite symbiosis are promotion of rhizobial growth, earlier nodule formation, increased secondary root formation, up-regulated expression of genes related to flavonoid synthesis and *nif* genes. In addition, exudates of chickpea roots colonised with actinobacteria increased nodulation-related biological processes - rhizobial chemotaxis, biofilm formation and *nod* gene expression.

*Conclusion* These endophytic actinobacteria positively affect many aspects of the chickpea-*Mesorhizobium* symbiosis resulting in increases in grain yield. Similar improvements recorded in chickpea growing in potted field soils, shows the potential to enhance chickpea production in the field.

**Keywords** Endophytic actinobacteria · Chickpea · *Mesorhizobium ciceri* · Nodulation, mechanisms

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## Introduction

Nitrogen (N) is an essential macro-element that is crucial for plant growth and development. Legumes are able to meet most of this requirement through their symbiotic association with rhizobia where atmospheric N is converted to ammonium in root nodules and transported within the plants as glutamine. Chickpea (*Cicer arietinum* L.) is one of the most important food legumes worldwide because of its multiple uses as a source of protein for human consumption and also as an animal feed. Australia is the second largest chickpea producing country accounting for 14% of global chickpea production after India (Merga and Haji 2019). However, fluctuations in soil pH, nutrient availability, temperature and moisture greatly influence chickpea production (Singh and Singh 2018) and nitrogen fixation is reported as much as 30–40% lower than for other legume species such as field pea (Unkovich and Pate 2000). Numerous studies have shown that plant symbionts other than rhizobia can boost growth and confer biotic and abiotic stress tolerance to their host plants. Among these, endophytes are a large microbial resource which can occupy plant tissues without causing symptoms of disease (Wilson 1995). They have received increasing attention because they exert multiple beneficial functions such as growth promotion, nutrient capture, disease resistance and stress tolerance (Wallace and May 2018; Brígido et al. 2019). Actinobacteria have been frequently reported for their capacity to produce an array of bioactive metabolites, such as antibacterial and antifungal compounds, herbicidal and pesticidal substances that have been used as commercial agricultural products (Goudjal et al. 2016). As endophytes they also can develop symbiotic associations with plants, which can protect plants from disease and regulate plant growth by colonizing special ecological niches (Misk and Franco 2011; Shimizu 2011; Gopalakrishnan et al. 2015).

Recently, a series of research papers have shown that a variety of leguminous plants can form tripartite symbiotic associations with nodule-inducing rhizobia and other plant beneficial microorganisms, such as arbuscular mycorrhiza fungi (AMF), fungal endophyte *Phomopsis liquidambari*, mineral phosphate-solubilising bacteria, endophytic *Bacillus*, *Methylobacterium oryzae* and *Micromonospora* strains to enhance plant growth, nodulation and $N_2$-fixation. (Alemneh et al. 2021; Benito et al. 2017; Gull et al. 2004; Subramanian et al. 2015; Wang et al. 2011; Zhang et al. 2016).

However, while the synergistic effects of N-fixing rhizobia with other beneficial microbes on plants has been reported, little information is available on the mechanisms that improve nodulation and legume growth. The infection of legume roots by rhizobia and formation of N fixing nodules involves a complex signalling between plant and rhizobium (Sharma et al. 2020). In this process, flavonoids from roots stimulate production of nod factors in rhizobia which initiate root hair curling, followed by colonisation of roots and formation of nodules containing N fixing bacteroids (Caetano-Anollés and Gresshoff 1991; Mathesius 2009). This process is highly regulated with structural changes in roots and bacteria controlled through complex phytohormone signalling between rhizobia and plants (Buhian and Bensmihen 2018).

Soil actinomycete *Streptomyces lydicus* WYEC108 influenced root nodulation of pea by increasing the level of infection of rhizobia when colonized within the surface cell layers of nodules, which was due to the improvement of bacteroids vigor within the nodules through enhancing nodule assimilation of iron (Tokala et al. 2002). Colonisation by the endophytic fungus *P. liquidambari,* together with its rhizobial partner, was found to significantly increase nodulation, $N_2$ fixation and peanut yield (Xie et al. 2019a). These positive effects correlated with improvement of the physiological status of peanut plants and the rhizosphere soil microenvironment, with enhanced N, P and K assimilation and suppression of disease incidence (Xie et al. 2019a). This endophytic fungus produced specific root exudates to decrease soil nitrate concentration and increase the population and biological activities such as chemotaxis, biofilm formation, expression of nodC genes of *Bradyrhizobium* (Xie et al. 2019b). Furthermore, activation of $H_2O_2$ and NO signaling pathways (Xie et al. 2017) and auxin signaling pathway (Zhang et al. 2018) were also involved in this enhanced nodulation process.

As a large portion of the N requirement of chickpea is provided by symbiotic $N_2$-fixing bacteria (Esfahani et al. 2014) the legume-rhizobia symbiosis is easily affected by environmental factors (Singh and Singh 2018). Endophytic actinobacteria-mediated nodulation improvement seems to be a promising strategy to overcome the suboptimum chickpea N fixation and growth. Although studies on the inoculation with

both $N_2$-fixing rhizobia and endophytic actinobacteria is limited for chickpea, studies with other legumes have shown beneficial effects. In lucerne, endophytic *Streptomyces* boosted plant growth and $N_2$ fixation in combination with *Sinorhizobium meliloti* strain RRI128, at different levels of soil N concentration (Le et al. 2016a, b). Whilst our previous studies have shown that the application of the four actinobacterial strains: *Microbispora* sp. CP56, *Actinomadura* sp. CP84B, *Streptomyces* spp. CP200B and CP21A, increased chickpea growth and nodulation (Vo et al. 2021), the underlying mechanisms responsible for the improvements are examined in this paper. Understanding the modes of action is critical to improve their efficacy, and to assist future endophyte selection and optimization of their application in agriculture. The aim of the present study is to verify that co-inoculation with *M. ciceri* strain CC1192 and strains of endophytic actinobacteria improve nodulation and nodule activity in chickpea and to clarify the physiological and molecular mechanisms associated with the response.

## Materials and methods

### Experimental design

The following studies were carried out:

A. The effect of actinobacteria metabolites on the in vitro growth of *M. ciceri*; and enzyme activity of actinobacterial strains.
B. Germination Paper and Pot Assay 1: The effect of the inoculation of actinobacteria alone on plant growth over the initial 2 weeks in germination paper and 6 weeks in pots.
C. Pot Assay 2: The effect of the co-inoculation of actinobacteria with *M. ciceri* strain CC1192 on plant growth. Plants were harvested at 8 and 16 weeks after sowing.
D. Pot Assay 3: The effect of inoculation of actinobacteria on plant growth in two field soils with naturalised and added *M. ciceri*. Plants were harvested after 6 weeks after sowing.
E. Pot Assay 4: This experiment was set up to study the effect of actinobacterial treatments on nodule development of chickpea over time.
F. Pot Assay 5: Persistence of an endophyte treatment and the effect of treatments on the expression of flavonoid biosynthetic genes *in planta*.
G. The effect of root exudates of actinobacterial treated vs untreated plants on the chemoattraction, biofilm formation and *nod* gene expression of *M. ciceri* in vitro.

### Chickpea seeds, rhizobia and actinobacteria

Seeds of Kabuli cv. Genesis 090 and *M. ciceri* strain CC1192 were provided by the South Australian Research and Development Institute (SARDI). Rhizobia strain CC1192, the commercial inoculant used for chickpea in Australia, was grown on Yeast Mannitol Agar (YMA) medium (Kneen and Thomas 1983) and incubated at 27 °C for 3–5 days until good growth was observed. Bacterial suspensions in 0.85% saline were made and their $OD_{600}$ were measured. Serial dilutions of each rhizobial suspension (10 µL) were drop plated in duplicate onto YMA plates, and the number of colony-forming units were counted to develop a standard curve.

Actinobacterial strains *Microbispora* sp. CP56, *Actinomadura* sp. CP84B, *Streptomyces* spp. CP200B and CP21A were previously isolated from chickpea as endophytes and showed significant effects on chickpea growth and nodulation (Vo et al. 2021). These endophytic actinobacteria were grown on Mannitol Soy flour agar (MS) and incubated at 27 °C for 10–14 days until spores matured. The spores were scraped off the surface of the colonies, enumerated and stored in sterile 50% glycerol (v/v) at −80 °C.

### Effect of actinobacterial compounds on the in vitro growth of rhizobia

The *M. ciceri* was sub-cultured into 5 mL fresh sterile Yeast Mannitol Broth (YMB) in McCartney vials and incubated at 27 °C at 150 rpm overnight and the resulting suspension was inoculated into fresh YMB medium at a 1:100 dilution.

Six mm square plugs of each actinobacterium grown on ISP2 (International *Streptomyces* Project #2) agar medium for 7 days, as well as culture filtrate of actinobacteria that had been grown in ISP2 liquid medium for 5 days then filtered through a 0.22 µm filter, was added to each rhizobial culture in McCartney

bottles at a ratio of 1:10 (actinobacteria:rhizobia, v/v) and shaken at 27 °C at 150 rpm. The control only had an ISP2 plug or 10% of ISP2 medium (without actinobacteria) added. Colony Forming Units (CFU) of rhizobia in the vials was measured at 2, 4, 6, 8, and 10 h after the addition of the plugs or culture filtrate of actinobacteria. This was carried out in triplicate.

In order to rule out the possible increase in CFU due to the presence of actinobacteria on the agar plugs, it should be noted that the 10 h duration after adding the actinobacterial plugs, is too short a time for the actinobacteria to proliferate. Secondly, *M. ciceri* forms a white colony which is wet looking and soft to the touch with a nichrome wire loop. In contrast, the actinobacterial colonies were off-white to light orange to light brown and colonies are hard to the touch. As actinobacterial colonies take at least 3 days to develop the plates were kept to check for the presence of actinobacteria. In general, the growth of the rhizobia prevented the visualisation of any actinobacterial colonies.

### Indole acetic acid, Cellulase and 1-amino-cyclopropane-1-carboxylate (ACC) deaminase production

Production of Indole acetic acid was carried out using the method of Khamna et al. (2009). The IAA production was calculated on a standard curve with the known concentrations of IAA using a spectrophotometer at $OD_{530}$. Cellulase production was assessed by inoculating the actinobacterial cultures onto cellulose Congo Red agar media and incubating the plates at 27 °C for 7 days using the method of Gopalakrishnan et al. (2011). The agar plates were then observed for the presence of a halo zone around the actinobacterial colonies, which indicates cellulase production.

ACC deaminase production was measured using the method described by Penrose and Glick (2003). Actinobacteria were inoculated into Dworkin and Foster (DF) minimal liquid medium (Dworkin and Foster 1958) and shaken at 27 °C at 150 rpm for 7 days. If the growth of actinobacteria was observed, the actinobacterial mycelium was transferred to a fresh DF liquid medium to grow again. After three generations of growth was observed, the ACC deaminase production by an actinobacterium was considered positive.

### Germination assay

Chickpea seeds were surface sterilized as described previously. The actinobacterial spores were suspended in 0.3% xanthan gum at a final concentration of $10^6$ CFU $mL^{-1}$, then coated onto the surface-sterilized chickpea seeds and allowed to air dry. After spores and seeds were dry, ten seeds were placed onto moist germination paper with three replicates for each treatment. After 2 weeks at 27 °C, the germination, secondary root number and primary root length were measured and recorded.

### Pot assays: General treatments

Chickpea seeds of similar size were selected and surface sterilised as described by Coombs and Franco (2003). Briefly, seeds were immersed for 1 min in 70% (v/v) ethanol, 3 min in 4% (v/v) sodium hypochlorite solution, and then rinsed 5 times in sterilized water for 10 min each time. Seeds were removed from the water and put into petri dishes containing autoclaved wet filter paper and allowed to germinate for 36 h at 27 °C. After germination, the seedlings were placed into actinobacterial spore suspensions ($10^6$ spores $mL^{-1}$ 0.3% xanthan gum, w/v) for 12 h. Seedlings not treated with actinobacteria were added to a 0.3% xanthan gum solution for 12 h.

The treated germinated seeds with root lengths of 7–10 mm were sown into a sterilised (autoclaved twice for 30 min at 121 °C, with a 24 h interval) sand:vermiculite mix (50:50 v/v) contained in 1.25 l self-watering pots, with 100 mL MilliQ water added to each pot. The sand and vermiculite were tested to ensure that they were both N-free. After transplanting, 200 mL of McKnight's N deficient nutrient solution (McKnight 1949) supplemented with a small amount of N (15 mg $L^{-1}$ $NH_4NO_3$) was added to each pot. A thin layer of sterilised polypropylene beads was placed onto the sand surface to reduce evaporation and microbial contamination between pots. All treatment and control pots had four replicates each with four plants per pot.

All treatments and control pots were completely randomized in the glasshouse with the position of the pots changed each week. Plants grown in the glasshouse were reliant on natural light and grown during the following months in 2019: Pot assays 1 and 3 were from October to December, Pot assay 2 was

from March to June, Pot assays 4 and 5 were from June to July, with average daylight/daytime temperatures of 13 h/24 °C, 11 h/22 °C and 9.75 h/20 °C, respectively, for each period.

After placing the pots in the glasshouse for 7 days, the number of seedlings was thinned to a uniform 4 plants per pot. Then the rhizobia treatment consisting of 1 mL of *M. ciceri* CC1192 inoculant (~$10^8$ CFU $mL^{-1}$) was applied to soil at the base of each plant.

For all pot experiments: Measurements were made of shoot and root length and dry matter (DM), nodule number and DM, and leghemoglobin content; seed number and weight, and N content of the plant. Dry weights were measured after drying in a 60 °C oven (for 48 h) until constant weight.

**Pot assay 1** Chickpea plants with actinobacterial treatments in the absence of rhizobia were grown and compared to control plants (without actinobacteria or rhizobia). The experimental conditions are described above. Plants were harvested 6 weeks after sowing.

**Pot assay 2** Three controls were included in this Assay: unlimited mineral N without rhizobia (N+), no added N or rhizobia (N-), and *M. ciceri* CC1192 only. The other treatments all had rhizobia plus seed-coated actinobacteria. The N+ treatment was 50 mL of 2.4 g $L^{-1}$ $NH_4NO_3$ solution added weekly. All treatment and control pots with four replicates each with four plants per pot. Chickpea plants were harvested at 8 and 16 weeks after sowing. The roots were gently shaken and washed with running water to remove the sand and vermiculite prior to nodulation assessment and determination of root weight. In addition, at the 16 week harvest the pods were separated and the number and fresh weight of seeds per plant were measured.

**Pot assay 3: Field soils** Chickpea seeds were surface-sterilized and coated with the four endophytic actinobacteria as described previously. Turretfield soil or Lameroo soil was added to the self-watering pots without autoclaving and the coated seeds were sown. For the Turretfield soil, which contained a naturalised community of chickpea rhizobia, no rhizobial suspension was added, but there were two controls in this pot assay with no added actinobacteria: unlimited N (N+), and no added N (N-). For Lameroo soil, all treatments and watering were the same as Assay 2. All treatment and control pots with four replicates each with four plants per pot. Six weeks after sowing, all chickpea plants were harvested, and all parameters were measured as described. The detailed information for these two field soils is listed in Supplementary Table 1.

**Pot assay 4: The nodulation process** Actinobacteria-coated chickpea seeds were sown in the sand:vermiculite mixture and treated with rhizobia as described above. The chickpea plants were harvested at 0, 3, 6, 9,12, 15,18, 21, 30 and 42 days after inoculation (DAI) of the rhizobia. The roots were carefully washed with running tap water, and excisable active nodules were counted and recorded.

**Pot assay 5** The general treatments were the same as Assay 4. The chickpea roots were collected at 0, 1, 2 and 7 days after inoculation of rhizobia in order to measure the flavonoid synthesis related gene expression levels in the roots of treated and control plants.

## Other parameters measured

### Leghemoglobin content

The content of leghemoglobin was measured according to Wilson and Reisenauer (1963): 0.5 g of the fresh nodules were crushed and ground with 3 mL of Drabkin's solution (Sigma Aldrich), then the mixture was centrifuged at 500 g for 15 min to remove residue. After this, the supernatant was collected and Drabkin's solution was added to 10 mL and the mixture was centrifuged at 2000 g for 30 min. The absorbance of the supernatant was read at 540 nm against Drabkin's solution. Bovine hemoglobin was used as a standard, and values are expressed as mg per g fresh weight of nodules.

### *Plant N content analysis*

The N content in shoots was determined using the method described by Le et al. (2016a). The shoots were dried to constant weight at 60 °C for 48 h and ground to about 1 mm in size using a mortar and pestle, then sent to Australian Precision Ag Laboratory (APAL, Hindmarsh, South Australia, www.apal.com.

au) to determine N content (%). The total N was calculated as N content×DM of plant and expressed as mg per plant.

Influence of root exudates on rhizobial chemotaxis, biofilm formation and *nod* gene expression

*Collection of root exudates*

To collect root exudates from chickpea plants inoculated, or not inoculated with actinobacteria, seedlings were grown in 50 mL tubes with sterile sand and vermiculite, with one seedling per tube. Seeds were coated with actinobacteria, as before. McKnight's solution was applied when sowing, and sterile water was added when needed. After 10 days of growth, seedlings from each treatment were washed, then transferred into tubes with 50 mL sterile water and maintained in a plant incubation room at 28 °C for 24 h to collect the root exudates. The collected root exudates were filtered through a double layer of Whatman #1 filter paper and immediately freeze dried. The lyophilized root exudate powder was dissolved in sterile water to make concentrated extracts of 1 mL per seedling for further use.

*Chemotaxis assay*

To determine the effects of the root exudates on nodulation-related biological processes on *M. ciceri* strain CC1192, the chemotaxis ability was determined as described by Zhang et al. (2014). Briefly, strain CC1192 was grown in YMB medium until log phase was reached ($OD_{600}=0.8$). The cells, collected by centrifugation, were washed twice with chemotaxis buffer (10 mM potassium phosphate buffer pH 7.0, 0.1 mM disodium EDTA) and resuspended in the same buffer. A glass tube was filled with 5 mL of the cell suspension prepared above, then 1 µL capillaries loaded with different root exudates filtered through a 0.22 µm filter were immersed in the cell suspension. After 1 h of static incubation at room temperature, the contents in the capillary were transferred into tubes. Then the suspension was diluted and plated on YMA plates. The CFU $mL^{-1}$ was counted after incubation for 48 h.

*Biofilm formation*

To determine the effects of root exudates from the actinobacteria treated and untreated plants on the biofilm formation of rhizobia, the assay was performed as described by Zhang et al. (2014). Briefly, the rhizobia were grown in YMB until $OD_{600}$ reached 1.0; then the cells were centrifuged, washed twice with YMB medium, and finally resuspended in YMB medium with the same volume. Each glass tube was filled with 1 mL YMB medium inoculated with 10 µL cell suspension, then 20 µL of each concentrated root exudate filtered through a 0.22 µm filter was added into this medium. After static incubation for 3 days, the medium and non-adherent cells were removed, and the tubes rinsed gently with distilled water. Biofilm that formed in the tubes were stained with 1 mL of 0.1% crystal violet for 30 min at room temperature. Subsequently, excess crystal violet was poured out and the tubes were washed twice with distilled water. The bound crystal violet was solubilized with 1 mL of acetic acid. Biofilm formation was quantified by measuring the $OD_{570}$.

Actinobacterial colonization assays

From each treatment shoots and roots of chickpea at 4, 8 and 16 weeks from Pot Assay 2 were harvested and washed carefully with sterilized water. The rhizosphere soil adhering to the roots after gentle shaking, and non-adhering soil (bulk soil) were collected. The plant samples were surface-sterilized before genomic DNA was extracted from the chickpea (leaf and root) and soil samples, using the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Araujo et al. 2019). Briefly, approximately 0.5 g sub-samples of actinobacterial colonies/chickpea leaves and roots/soil samples were ground with liquid nitrogen and mixed into 500 µL modified CTAB buffer along with 0.1 g glass beads. Subsequently, 500 µL of phenol: chloroform: isoamyl alcohol (25:24:1, Sigma Aldrich) was added and shaken in a bead-beater (Bio spec) for 5 min followed by incubation for 1 h at 65 °C. After that, the tubes were centrifuged at 16000 g for 5 min at 4 °C. The aqueous top layer was removed to a new tube and an equal volume of chloroform-isoamyl alcohol (24:1, Sigma Aldrich) was added and mixed. The tubes were then centrifuged at 16000 g

for 5 min at room temperature followed by removing the aqueous top layer into a new tube. Two volumes of PEG/sodium chloride solution were added and incubated for 2 h at 4 °C. The mixture was centrifuged at 16000 g for 15 min at 4 °C and the supernatant was gently poured off without disturbing the DNA pellet. Furthermore, the pellet was washed with chilled 70% ethanol and then centrifuged at 16000 g for 10 min. The DNA pellet was resuspended into 50 µL MilliQ water. DNA was quantified using a Nanodrop (ThermoFisher 2000/2000) and further dilutions were made for PCR application. The amount of actinobacteria was estimated by quantitative PCR (qPCR) system with the actinobacterial CP200B specific primers in Table 1 (Singh 2019). A standard curve was developed by plotting the logarithm of known concentrations of total CP200B DNA, whose concentration was determined using a NanoDrop spectrophotometer. The DNA concentration of actinobacteria in the chickpea and soil samples was calculated using the generated standard curve. qRT-PCR was run on the CFX96™ Real-Time System instrument (Bio-Rad, USA). Thermal cycling conditions consisted of 2 min at 95 °C, followed by 40 cycles of 95 °C for 15 s; 60 °C for 1 min; This was followed by a melt curve consisting of 5 s increments of 0.5 °C from 58 °C to 95 °C.

### RNA extraction and analysis of *nod, CHS, PAL* and *nif* gene expression

Total RNA was isolated from various samples (composite nodules from each plant in Pot Assay 2 at 8 weeks, leaf and root from Pot Assay 5, and free-living rhizobia cells in Experiment G) using Trizol reagent. Approximately 0.1 g homogenised frozen tissue was mixed with 1 mL Trizol reagent and centrifuged at 12000 g for 5 min at 4 °C. The supernatant was collected, mixed with 200 µL chloroform and incubated on ice for 3 min. Following centrifugation at 12000 g for 15 min at 4 °C, the aqueous phase was collected, mixed with 500 µL isopropanol and allowed to stand for 10 min at room temperature. The mixture was centrifuged at 12000 g for 10 min at 4 °C, and the supernatant was discarded, then 1 mL ethanol was used to rinse the pellet. After centrifugation at 12000 g for 5 min at 4 °C, the RNA pellet was dried in a laminar flow and resuspended in 30 µL DEPC-treated water. The RNA was quantified using a NanoDrop spectrophotometer.

RNA was treated with DNase I (Sigma Aldrich), prior to cDNA synthesis, following the manufacturer's protocol. A High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) was used for reverse-transcription to make the DNase treated RNA into cDNA. A reaction mix containing: 2 µL 10×RT Buffer; 0.8 µL 25×dNTP Mix; 1 µL Reverse

**Table 1** Primers used in this study (5′ ~ > 3′)

| Primer | Forward Sequence (5′-3′) | Reverse Sequence (5′-3′) | Reference |
|---|---|---|---|
| CP200B | TCCACTTCATCCCCGCCATGCT | ACGTCGAGCAGGTCGCGGAAGT | Singh (2019) |
| GAPDH | CCAAGGTCAAGATCGGAATCA | CAAAGCCACTCTAGCAACCAAA | Garg et al. (2010) |
| PAL | ACGCATGGTGGAAGAGTACC | GCACCACCCTGTITTGTTCT | Singh and Gaur (2017) |
| CHS | GGCTATTGGCACTGCTAACC | CAGGCATGTCAACACCACTT | Kavousi et al. (2009) |
| 16S rRNA | CTGCGGCAAAGCGTTAAGAG | CACCGCGTTTGTCAACCTTA | This study |
| *nifH* | CATCCTCAAATATGCCCATTC | GTGGATCTTCTCGGCCAGAG | Esfahani et al. (2014) |
| *nifD* | GCATACTGCTTGAGGAGATAG | TTGGCAATGACCTTTTCGGTC | Esfahani et al. (2014) |
| *nifK* | AGTCATGTCGACGGCTATGAC | ATCGAACTGGTCAGAGGCATC | Esfahani et al. (2014) |
| *nifA* | TCAACGTCGTCACTTTACGC | CGCAGTTCATTAGCACCTCA | da Silva et al. (2019) |
| *fixA* | GCAGACTCCCTTGCCTCATT | CTGCAGTCCACTTCACGACT | This study |
| *nodA* | CCGAATGTCGAGTGGAAGTT | CTCGCCAACTTTGATGAAGC | This study |
| *nodB* | CGAAGGGCAGAGTGACAAT | TGCGCCAGCACATTGAGTA | This study |
| *nodC* | TCTGGTTGACGACGGTTCTG | TTGCCAACATTTTCGCGGAG | This study |
| *nodD* | TAAGAGACGMTCGAGGICG | TCTCGGTGAATGTGGGAAAT | da Silva et al. (2019) |

Transcriptase; 2 µL 10×RT Random Primers; 4.2 µL Nuclease free water. The reaction mixture was added to 10 µL DNase-treated RNA then placed in the thermal cycle under the following cycling conditions: 25 °C for 10 min, 37 °C for 120 min and then 85 °C for 5 min. The cDNA product was diluted 1:10 in DEPC-treated water and stored at −20 °C.

The gene expression of 4 *nod* (in free-living rhizobial cells), *CHS*, *PAL* (in chickpea roots), *fix* and 4 *nif* (in nodules) genes was conducted by qRT-PCR using Power SYBR™ Green PCR Master Mix (ThermoFisher scientific). Each reaction contained: 5 µL SYBR-Green mix; 1 µL cDNA; 1 µL forward/reverse primer; and 2 µL water. qRT-PCR was run on the CFX96™ Real-Time System instrument (Bio-Rad, USA). Thermal cycling conditions consisted of 5 min of 95 °C, followed by 45 cycles of 95 °C for 10 s; 60 °C for 20 s. This was followed by a melt curve consisting of 5 s increments of 0.5 °C from 65 °C to 95 °C. Ct values were compared against that of the standard curve and normalized against the reference genes (*GAPDH* for chickpea, *16S rRNA* for rhizobia and nodules). All samples and standards were run in duplicate. The primer pairs used in this study are listed in Table 1 and were synthesized by Sigma Aldrich.

### Statistical analyses

The data was entered and collated in a MS Excel spreadsheet and analyzed using the IBM SPSS Statistic. All statistical analysis of data was performed using one-way ANOVA, and Duncan's multiple range test was performed to detect statistical significance between treatments. $P < 0.05$ was considered statistically significant.

## Results

### Effect of actinobacteria on rhizobia growth in vitro

The effect of metabolites of the four endophytic actinobacterial strains, CP56, CP84B, CP200B and CP21A2, on the growth of rhizobia in broth culture was tested. None of the actinobacterial strains negatively affected the growth of rhizobial strain CC1192 (Fig. 1); on the contrary, both the actinobacterial agar plug and culture filtrate increased the rate of growth of rhizobia compared to the control treatment. The agar plug samples, which were lower in volume would have contained a lower amount of metabolite, gave slightly lower increases but indicated that similar types of growth promoting metabolites are present in both the agar plug and the culture filtrate.

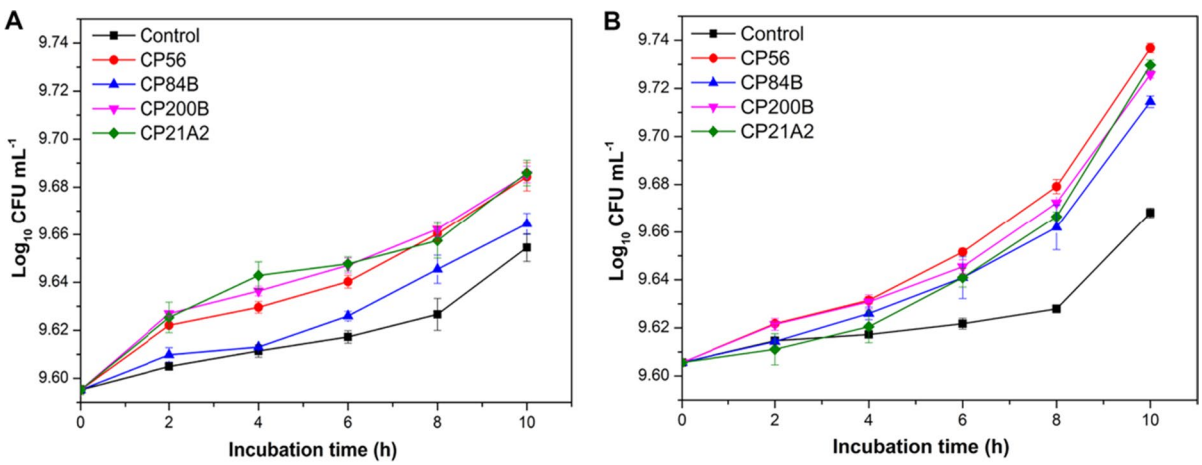


**Fig. 1** Growth (CFU) in Yeast Mannitol Broth medium of *Mesorhizobium ciceri* CC1192 in the presence of actinobacteria plugs grown on ISP2 agar plates for 7 days (**A**) and culture filtrate in ISP2 broth for 5 days (**B**). Data and error bars represent the mean ± SD of three replicates

### Indole acetic acid, Cellulase and ACC deaminase production of actinobacteria

The four actinobacterial endophytes showed the ability to produce between 16 to 54 µg/ml Indole Acetic acid (Vo 2017). In addition, all four actinobacterial strains produced cellulase and ACC deaminase activity (data not shown).

### Evaluation of actinobacteria on chickpea growth in the absence of rhizobia

In the absence of chickpea rhizobia, the effects of actinobacterial strains on the germination and early growth of chickpea was measured. All actinobacterial inoculation treatments increased the shoot length. Strains CP84B and CP200B also increased the number of secondary roots and root length at 14 days (Table 2). Increases in germination were also noted for CP200B.

In addition, three strains of actinobacteria increased the total DM production of chickpea grown in sand and vermiculite media, after six weeks under N limited conditions (Table 3), the result of increased root production rather than shoot production. CP21A2 increased total dry matter by 39%, compared to untreated plants, followed by CP84B and CP200B at 31% and 28%. Corresponding increases in root DM were 161%, 106% and 117%.

### Evaluation of endophytic actinobacteria on chickpea inoculated with rhizobia

The results of the actinobacterial treatments with rhizobia at the 8-week harvest (Table 4) showed an increase in the total chickpea plant DM from 115 to 176%, compared to the rhizobia only treatment. Chickpea nodules from plants co-inoculated with rhizobia and CP200B had the highest leghemoglobin content, followed by CP56.

At 16 weeks harvest (Table 5), for co-inoculation treatments there was a significant further increase compared to the rhizobia only treatments of between 60 and 136% in number of nodules, between 69 and

**Table 2** Effect of endophytic actinobacterial (CP strains) on the growth and germination of chickpea plants after 2 weeks in moist germination paper. Chickpea seeds were coated with different actinobacteria in 0.3% xanthan gum. Values are Means ± SD ($n=3$). Within a column, values not containing the same letter are significantly different (One-way ANOVA and Duncan's multirange tests, $p<0.05$)

| | Number of secondary roots/ plant | Root length/plant (cm) | Shoot length/ plant (cm) | Percentage of seeds germinated |
|---|---|---|---|---|
| Control | 11.5 ± 0.7 c | 13.4 ± 1.6 bc | 3.3 ± 0.8 e | 61.7 ± 11.9 b |
| CP56 | 18.0 ± 4.2 bc | 14.9 ± 4.1 abc | 5.3 ± 1.9 c | 69.1 ± 19.9 ab |
| CP84B | 36.9 ± 6.4 a | 16.9 ± 4.9 ab | 8.3 ± 0.8 a | 73.9 ± 4.2 ab |
| CP200B | 34.2 ± 8.5 a | 17.1 ± 5.2 ab | 7.3 ± 1.4 b | 85.2 ± 9.5 a |
| CP21A2 | 22.0 ± 4.2 bc | 10.8 ± 0.5 c | 4.1 ± 1.4 d | 71.4 ± 16.3 ab |

**Table 3** Effect of endophytic actinobacteria (CP strains) on the growth of chickpea plants grown in a N limited sand/vermiculite medium, 6 weeks after sowing. Chickpea seeds were coated with actinobacteria in 0.3% xanthan gum. ($n=4$ pots, 4 plants per pot). DM: dry matter. Values are Means±SD. Different letters within a column indicate significant differences among treatments (One-way ANOVA and Duncan's multirange tests, $p<0.05$).

| Treatments | Shoot length (cm/plant) | Shoot weight (mg DM /plant) | Root weight (mg DM/plant) | Total weight (mg DM/plant) |
|---|---|---|---|---|
| Control | 33.1 ± 4.2 c | 730.1 ± 67.8 ab | 197.9 ± 40.0 b | 928.0 ± 97.3 b |
| CP56 | 35.1 ± 2.9 abc | 732.5 ± 35.6 ab | 423.3 ± 122.7 a | 1155.8 ± 136.1 ab |
| CP84B | 37.7 ± 5.3 a | 804.9 ± 38.3 a | 407.9 ± 40.2 a | 1212.8 ± 68.9 a |
| CP200B | 35.7 ± 3.1 abc | 756.4 ± 58.1 a | 429.9 ± 1.4.7 a | 1186.3 ± 124.2 a |
| CP21A2 | 34.2 ± 4.6 bc | 773.9 ± 96.1 a | 517.7 ± 48.1 a | 1291.6 ± 131.5 a |

**Table 4** Effect of endophytic actinobacteria (CP strains) on the nodulation and the growth of chickpea co-inoculated with *Mesorhizobium ciceri* strain CC1192, harvested 8 weeks after sowing. Chickpea seeds were coated with different actinobacteria in 0.3% xanthan gum. R = *M. ciceri* strain CC1192. ($n = 4$ pots, 4 plants per pot). DM: dry matter. Values are Means±SD. Different letters within a column indicate significant differences between treatments (One-way ANOVA and Duncan's multirange tests, $p < 0.05$)

| Treatments | Nodules/plant | Nodule weight (mg DM/plant) | Leghemoglobin (mg/g fresh weight) | Shoot weight (mg DM/plant) | Root weight (mg DM/plant) | Total weight (mg DM/plant) | Total N (mg/plant) |
|---|---|---|---|---|---|---|---|
| N + control | 0 | / | / | 540.2 ± 152 a | 192.9 ± 35e | 733.1 ± 167 c | / |
| N- control | 12 ± 2.7 c | 7.8 ± 1.2 c | 13.4 ± 0.9 f | 238.3 ± 51 d | 237.5 ± 56 de | 475.8 ± 108 e | / |
| R only | 20.6 ± 6.8 bc | 13.7 ± 3.5 bc | 33.9 ± 5.6 c | 300.3 ± 114 cd | 336.8 ± 81 cd | 637.1 ± 141 d | 332.4 ± 16 b |
| R + CP56 | 20 ± 5.8 bc | 20.6 ± 2.1 b | 43.0 ± 5.6 a | 430.6 ± 35 ab | 427.8 ± 60 bc | 858.4 ± 65 b | 453.4 ± 18 a |
| R + CP84B | 43.5 ± 10.4 a | 29.1 ± 5.7 a | 18.5 ± 0.8 e | 422.7 ± 76 ab | 392.5 ± 68 bc | 815.2 ± 85 b | 307.1 ± 13 c |
| R + CP200B | 21.4 ± 6.9 bc | 17.1 ± 1.6 b | 46.8 ± 10.8 a | 485.0 ± 59 ab | 639.7 ± 119 a | 1124.7 ± 142 a | 384.5 ± 48 ab |
| R + CP21A2 | 12.6 ± 1.7 c | 13.3 ± 0.4 bc | 29.9 ± 6.7 d | 397.5 ± 85 bc | 340.7 ± 70 cd | 738.2 ± 133 c | 342.7 ± 21b |

**Table 5** Effect of endophytic actinobacteria on the nodulation and the growth of chickpea co-inoculated with *Mesorhizobium ciceri* strain CC1192, harvested 16 weeks after sowing. Chickpea seeds were coated with different actinobacteria in 0.3% xanthan gum. R = *M. ciceri* strain CC1192. ($n = 4$ pots, 4 plants per pot). DM: dry matter. FW: Fresh weight. Values are Means±SD. Different letters within a column indicate significant differences among treatments (One-way ANOVA and Duncan's multirange tests, $p < 0.05$)

| Treatments | Nodule no./plant | Nodule weight (mg DM/plant) | Leghemo globin FW (mg/g) | Shoot weight (mg DM/plant) | Root weight (mg DM/plant) | Seeds /plant | Seeds FW (mg/plant) | Individual seed biomass (mg/seed) | Total N (mg / plant) |
|---|---|---|---|---|---|---|---|---|---|
| R only | 41.3 ± 8.2 d | 83.2 ± 5.6 d | 32.8 ± 7.6 b | 843 ± 13 e | 1094.8 bc | 1.0 ± 0.0 b | 786 ± 36 c | 786 ± 36 a | 1188 ± 85 c |
| R + CP56 | 73.6 ± 21.3 bc | 136.7 ± 42.0 cd | 36.3 ± 3.4 a | 2050 ± 280 b | 1166 ± 116 abc | 3.0 ± 1.2 ab | 1579 ± 176 b | 790 ± 59 a | 2511 ± 154 b |
| R + CP84B | 95.3 ± 26.3 a | 197.6 ± 17.5 ba | 28.2 ± 2.1 c | 2817 ± 267 a | 1238 ± 101 ab | 4.25 ± 1.9 a | 2499 ± 190 a | 515 ± 38 b | 3873 ± 99 a |
| R + CP200B | 86.5 ± 12.5 ab | 348.2 ± 64.2 a | 40.2 ± 6.9 a | 1829 ± 136 bc | 1299 ± 47 a | 3.0 ± 0.7 ab | 2381 ± 220 a | 794 ± 37 a | 2716 ± 46 b |
| R + CP21A2 | 66.1 ± 11.3 c | 233.8 ± 28.4 b | 37.8 ± 2.6 a | 1426 ± 121 cd | 1059.8 ± 85.8 c | 3.2 ± 1.0 ab | 2275 ± 129 a | 719 ± 61 a | 1284 ± 20 c |

234% for shoot dry weight, and 128–209% for total plant dry weight. The total N content of the plants treated with actinobacteria and rhizobia was higher by 111 to 226% compared to the rhizobia only treatment. Of note, for all co-inoculation treatments the fresh seed weight was between two and three times higher than for rhizobia alone.

## Effect of actinobacteria on chickpea grown in field soils

In the Turretfield soil where chickpea was reliant on the naturalised population of rhizobia, inoculation with actinobacteria promoted nodulation, compared to untreated plants which did not produce nodules. Most nodules were produced with CP84B, followed by CP200B (Table 6).

CP200B also increased total plant weight by 57% compared to the no N control. There was no difference in these soils between the growth of the plants with or without added N which suggests that N was not the limitation to plant growth in this experiment.

In the Lameroo soil, plants co-inoculated with rhizobia and actinobacteria CP56 and CP84B produced most nodules, but plants inoculated with CP200B showed a significant increase of at least 60% in total plant weight compared to both the rhizobia only and the No N control (Table 6).

## The kinetics of nodule development

To further study how endophytic actinobacteria improved chickpea nodulation, the kinetics of nodule development was examined. Nodules were clearly

**Table 6** Effect of endophytic actinobacteria (CP strains) on the nodulation and growth of chickpea in Turretfield soil that contains rhizobia and in Lameroo soil that has been co-inoculated with *M. ciceri* strain CC1192. Chickpea seeds were coated with actinobacteria in 0.3% xanthan gum. Seeds for N+ and N- control plants and Rhizobium only treatments were coated with 0.3% xanthan gum. Plants were harvested 6 weeks after sowing. Values are Means ± SD (n = 4 pots, 4 plants per pot). R = *M. ciceri* strain CC1192. Different letters within a column indicate significant differences among treatments (One-way ANOVA and Duncan's multirange tests, $p < 0.05$).

| Treatments | Nodules /plant | Nodule weight (mg DM/plant) | Shoot weight (mg DM/plant) | Root weight (mg DM/plant) | Total weight (mg DM/plant) |
|---|---|---|---|---|---|
| Turretfield soil | | | | | |
| N + control | 0.0 ± 0.0 d | 0.0 ± 0.0 c | 684.6 ± 73.5 bc | 298.5 ± 79.4 b | 983.1 ± 142.5 c |
| N- control | 0.0 ± 0.0 d | 0.0 ± 0.0 c | 666.3 ± 69.8 c | 301.5 ± 49.9 b | 967.8 ± 89.7 c |
| CP56 | 1.3 ± 0.4 c | 1.3 ± 0.5 b | 895.2 ± 142.5 abc | 547.8 ± 38.9 a | 1443.0 ± 158.1 a |
| CP84B | 10.7 ± 1.7 a | 5.6 ± 1.0 a | 819.7 ± 118.8 abc | 458.9 ± 51.7 b | 1278.6 ± 157.9 b |
| CP200B | 7.3 ± 1.6 b | 4.1 ± 0.8 a | 987.0 ± 135.8 a | 551.5 ± 83.5 a | 1538.5 ± 185.4 a |
| CP21A2 | 1.2 ± 0.4 c | 1.2 ± 0.3 b | 951.1 ± 157.5 ab | 507.4 ± 84.9 a | 1458.5 ± 195.8 a |
| Lameroo soil | | | | | |
| N + control | 0.0 ± 0.0 d | 0.0 ± 0.0 c | 684.6 ± 73.5 bc | 298.5 ± 79.4 b | 983.1 ± 142.5 c |
| N- control | 0.0 ± 0.0 d | 0.0 ± 0.0 c | 666.3 ± 69.8 c | 301.5 ± 49.9 b | 967.8 ± 89.7 c |
| CP56 | 1.3 ± 0.4 c | 1.3 ± 0.5 b | 895.2 ± 142.5 abc | 547.8 ± 38.9 a | 1443.0 ± 158.1 a |
| CP84B | 10.7 ± 1.7 a | 5.6 ± 1.0 a | 819.7 ± 118.8 abc | 458.9 ± 51.7 b | 1278.6 ± 157.9 b |
| CP200B | 7.3 ± 1.6 b | 4.1 ± 0.8 a | 987.0 ± 135.8 a | 551.5 ± 83.5 a | 1538.5 ± 185.4 a |
| CP21A2 | 1.2 ± 0.4 c | 1.2 ± 0.3 b | 951.1 ± 157.5 ab | 507.4 ± 84.9 a | 1458.5 ± 195.8 a |

All the other actinobacterial treatments also showed an increase of at least 27% in total plant weight.

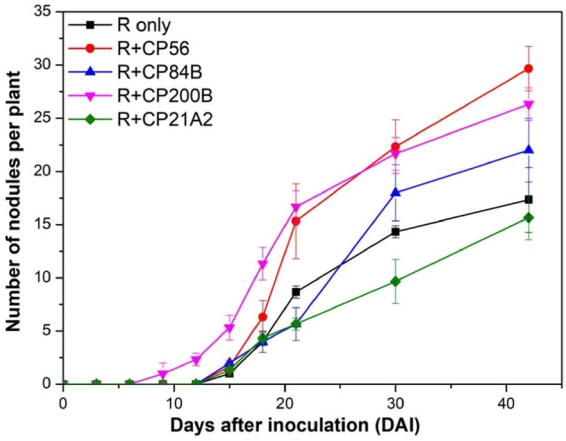


**Fig. 2** Number of nodules on chickpea roots inoculated only with rhizobia or combined with actinobacteria, measured after inoculation of rhizobia. Data and error bars represent the mean ± SD of four replicates

visible as white swellings on roots co-inoculated with rhizobia and CP200B at 9 DAI, whereas in the CP56, CP84B, CP21A2 and rhizobia only treatments nodules were not visible until 15 DAI (Fig. 2). Roots co-inoculated with CP56 and CP200B produced at least 50% more nodules compared to the rhizobia-only treatment, between 20 and 42 DAI.

## Colonization by endophytic actinobacteria CP200B

Based on qPCR, CP200B had colonized chickpea leaves and roots endophytically at 4 and 8 weeks and persisted in root material at 16 weeks (Fig. 3A). The amount of CP200B genomic DNA reached a maximum at 4 weeks in co-inoculated roots, then decreased, but still remained in roots and leaves at 8 weeks, and in roots and shoots 16 weeks after sowing. The presence of CP200B was also detected in chickpea seeds at 16 weeks. CP200B was also found at significant levels in rhizosphere and sand samples collected from the bottom of the pots at 4 weeks, persisting through to the 16-week harvest (Fig. 3B).

## Flavonoid synthesis-related genes expression

*PAL* gene expression in all actinobacteria co-inoculated roots showed the highest increase on Day 1 compared to the rhizobia-only treatment (Fig. 4). Co-treatments with CP84B and CP200B resulted

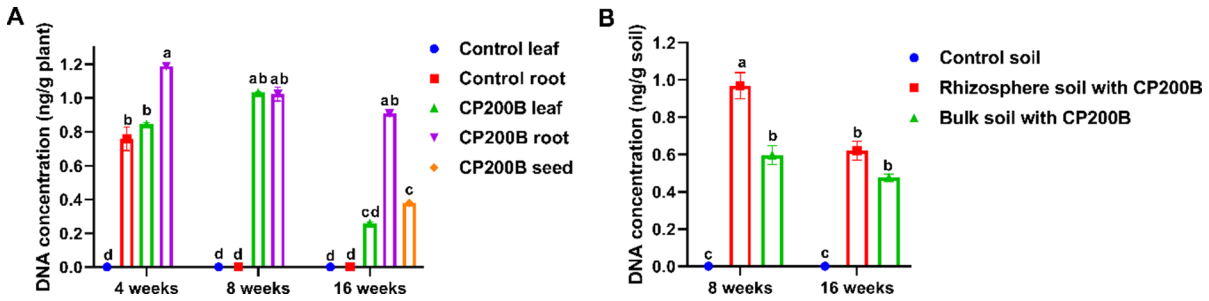


**Fig. 3** DNA concentration of actinobacterial endophyte CP200B in (A) chickpea leaf, root and seed (seed tested only at 16 weeks) samples, and (B) in rhizosphere and bulk soil with chickpea plants. Plants were uninoculated control and co-inoculated with rhizobia and CP200B from Pot Assay 2. Soil without actinobacterial inoculation were used as control soil. Data and error bars represent the mean ± SD of three replicates. Different letters indicate significant differences among treatments (One-way ANOVA and Duncan's multirange tests, $p < 0.05$)

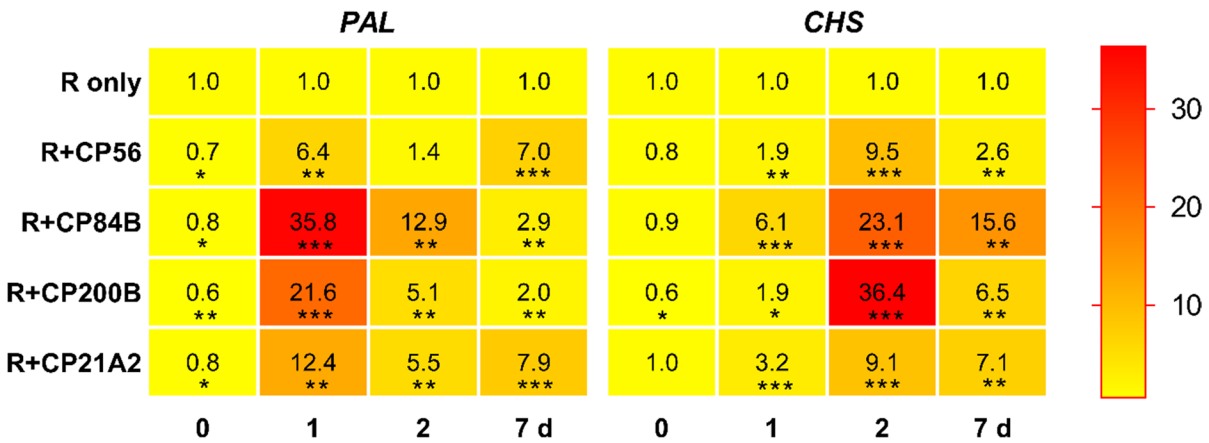


**Fig. 4** Effect of actinobacterial endophytes colonization on flavonoids synthesis-related gene expression. Chickpea roots were collected at 0, 1, 2 and 7 days after inoculation of rhizobia with rhizobia (R only) only or rhizobia and actinobacteria co-inoculation (R + CP). The qRT-PCR results are expressed in relative transcript fold increase over the R only control and presented as a heatmap (n = 3). Asterisk (*) within a column indicate significant differences with the R only control (One-way ANOVA and Duncan's multirange tests, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). PAL: Phenyalanine ammonia lyase; CHS: chalcone synthase

in a 36-fold and 22-fold increase, respectively, 24 h after inoculation with rhizobia. Increases in *CHS* transcript level were highest at 2 DAI and for CP84B and CP200B co-treatments were 36-fold and 23-fold higher than the rhizobia only control, respectively.

Effect of root exudates from plants inoculated with actinobacteria

Root exudates from chickpea inoculated with CP56 and CP200B were found to have a stronger chemotactic attraction toward rhizobial cells than exudates from roots without actinobacteria inoculation or from the other two actinobacteria (Fig. 5A). As well, rhizobia biofilm formation was increased by the root exudates inoculated with the same actinobacterial strains (Fig. 5B).

### *Nod* gene expression

In comparison to the root exudates from untreated plants, all the exudates from the plants treated with actinobacteria increased then expression of at least

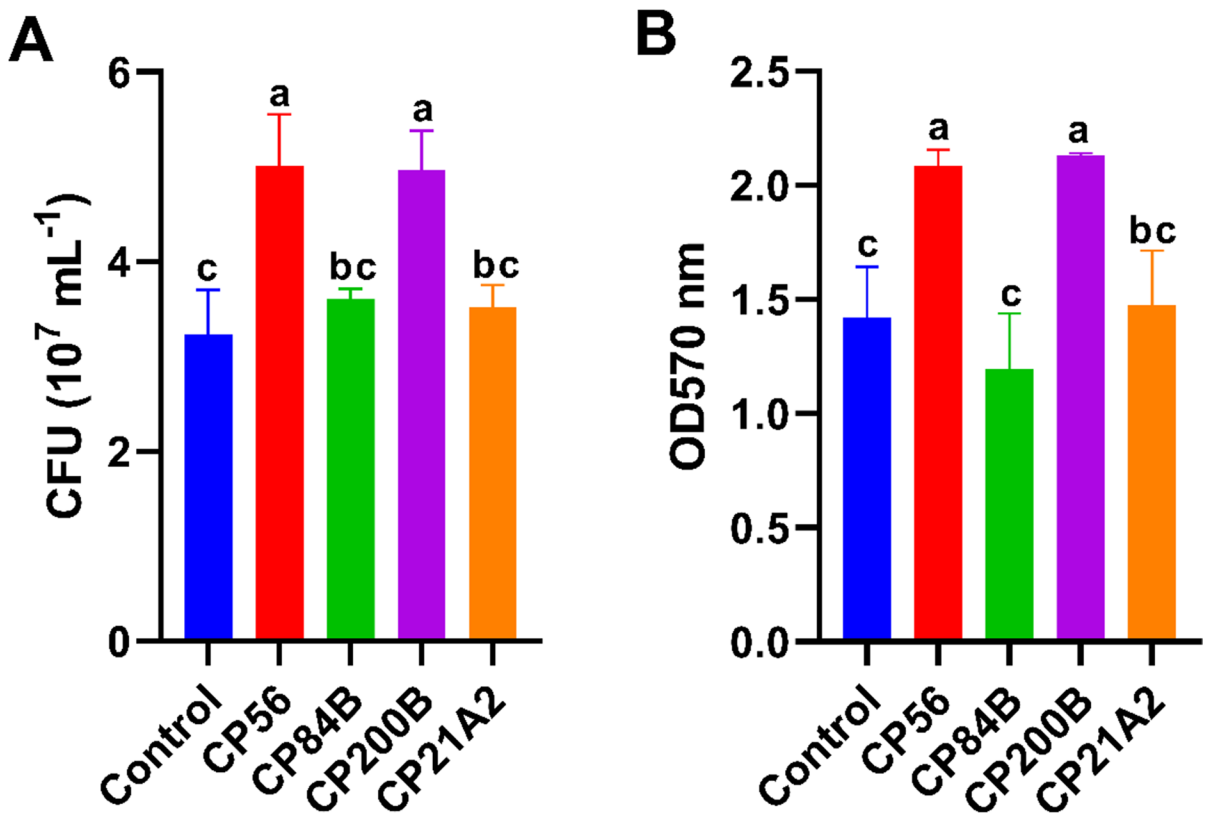


**Fig. 5** Effect of chickpea root exudates on rhizobial chemotaxis (**A**) and biofilm formation (**B**). Chickpea root exudates were collected after 10 days growth with inoculation with actinobacteria or without as control. Chemotactic response of rhizobia towards these root exudates were evaluated by capillary assay. The biofilm formation was presented as the $OD_{570}$ of the formed biofilm staining with crystal violet. Values are means±SD of three replicates. Different letters indicate significant differences among treatments (One-way ANOVA and Duncan's multirange tests, $p < 0.05$)

2 of the 4 *nod* genes tested in rhizobia free-living cells. Exudates of CP84B and CP200B increased the expression of all 4 genes and CP200B elicited the highest expression with a 7.95-fold, 10.8-fold and 12.5-fold increase for *nod*A, *nod*C and *nod*D genes, respectively (Fig. 6A).

### Effect of actinobacteria on nitrogen fixation related gene expression

To reveal the mechanisms of action involved in $N_2$-fixing enhancement, the effect of endophytic actinobacteria on the expression of nitrogenase genes, i.e., *nif*HDK, *nif*A and *fix*A in chickpea nodules from the 8-week harvest in Pot Assay 2 was determined by qRT-PCR. Transcription levels of *nif*H and *nif*K, that encode subunits of nitrogenase, exhibited more than 5-fold of increase in the CP56 treatment, while *nif*H and *fix*A showed more than 10-*fold* increase in the CP200B treatment compared to rhizobia alone (Fig. 6B). However, the *nif*HDK genes in CP84B treated chickpea nodules were expressed at lower levels; these results are consistent with leghemoglobin content level with these different treatments.

## Discussion

Legume plants are increasingly recognised to form symbiotic associations with microbes other than rhizobia, however, to what extent they can be used to improve symbiotic efficiency is still to be demonstrated (Sathya et al. 2017). The majority of studies with actinobacteria have focused on their growth promoting traits with cereals, but less so in legumes. Even less is known about the impact of endophytic

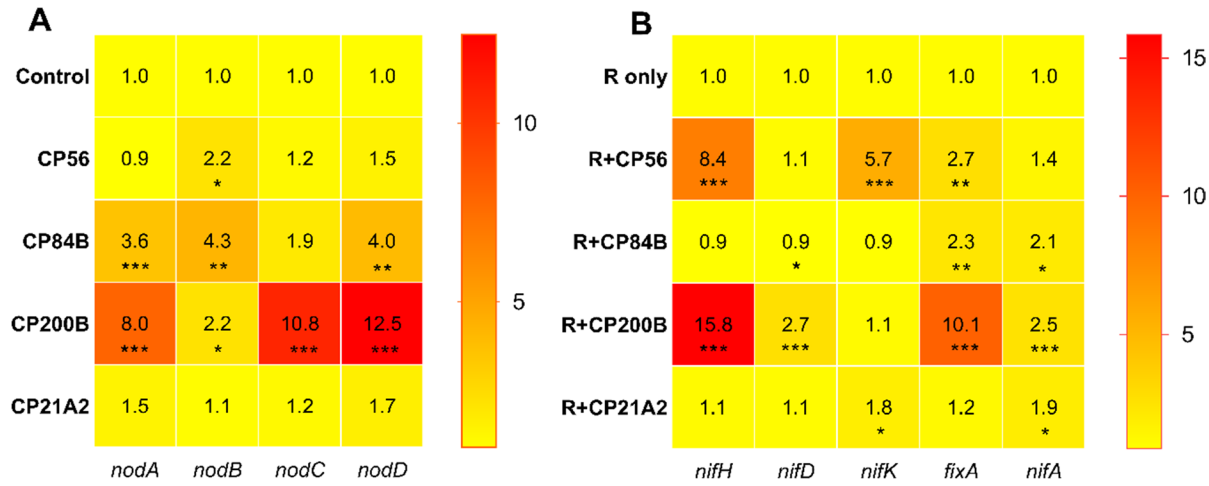


**Fig. 6** Effect of chickpea root exudates on *nod* gene expression (**A**) and Effect of actinobacteria on the nitrogen fixation (*nif* and *fix*) gene expression in chickpea nodules (**B**). Chickpea root exudates were collected after 10 days growth in a sand: vermiculite system with inoculation with actinobacteria or without as control. The qRT-PCR results are expressed in relative transcript fold increase over the control and presented as a heatmap ($n=3$). Chickpea nodules were collected after 8 weeks growth with rhizobia only or rhizobia and actinobacteria co-inoculation in Pot Assay 2. The qRT-PCR results of nodules are expressed in relative transcript fold increase over the R only control and presented as a heatmap ($n=3$). Asterisk (*) within a column indicate significant differences with the control (One-way ANOVA and Duncan's multirange tests, where * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

actinobacteria on the nitrogen fixing symbioses that form between legumes and rhizobia bacteria. In the present study, we investigated the tripartite interactions between endophytic actinobacteria, isolated from chickpea roots, rhizobial inoculant *M. ciceri* strain CC1192 and Kabuli chickpea cv. Genesis 090.

## Non-symbiotic impacts

In the absence of rhizobia, the four actinobacterial strains increased shoot growth and root development of chickpea, measured at 2 and 6 weeks after sowing. Increases were greatest with CP84B, occurred early in plant development and were independent of the rhizobia symbiosis. Chickpea seedlings inoculated with actinobacterial endophytes developed longer roots and more lateral roots in the germination paper assay (Table 2) and increased root dry matter in pots (Table 3). Lateral root formation is a complex process, with increased endogenous and exogenous addition of IAA is known to increase the formation of lateral roots (Malamy 2018). Microbial associations have been shown to induce modifications in root morphology in plants (Wang et al. 2011) and some studies found that endophyte colonization enhanced root branching (Egamberdieva et al. 2018; Krell et al. 2018). Increased lateral roots would provide more initial infection sites for rhizobia, which might be one of the reasons for more nodules being formed in actinobacteria treated chickpea roots (Schiessl et al. 2019).

The four actinobacteria are able to produce IAA (Vo 2017), cellulase and ACC deaminase; that might play a role in the observed changes to root morphology. Cellulase is considered to be essential for primary symbiotic infection of host roots by rhizobia, therefore, degradation of the root cell wall by actinobacteria-produced cellulase can also be responsible for the increase in nodule number (Martínez-Hidalgo and Hirsch 2017; Poole et al. 2018). Auxins are known to induce cell wall changes during cell expansion, thus may be beneficial for the rhizobial invasion (Nafisi et al. 2015). Indeed, auxins are involved in three main functions involved in nodule formation-control of the cell cycle, vascular tissue differentiation and rhizobial infection (Kohlen et al. 2018). Other mechanisms involved in plant growth promotion not measured in this study includes phosphate solubilization and iron acquisition (Palaniyandi et al. 2013). Ethylene has been shown to inhibit nodulation at its early stage by regulating threshold concentration of

Nod factors required for nodule initiation during the process of nodulation (Mulder et al. 2005). Hence, ACC deaminase produced by actinobacteria might also play a role in enhanced nodulation by controlling ethylene levels in chickpea (Ferguson and Mathesius 2014; Glick 2014).

### Impacts on the chickpea growth, nodule development and function

In the pot assays, co-inoculation with rhizobia and endophytic actinobacteria CP84B and CP200B significantly increased chickpea DM after both 8 and 16 weeks indicating both early and enduring benefits. Similar results have been reported for soybean co-inoculated with rhizobia and *Streptomyces griseoflavus* P4 (Soe and Yamakawa 2013) or endophytic *Bacillus* (Subramanian et al. 2015).

The application of actinobacteria with rhizobia increased both nodule number and total plant weight. In addition to effects on nodule number, numerous aspects important to nodule function were measured. First, the potential for nodules to fix $N_2$ was assessed by quantifying leghemoglobin content (Medeiros-Silva et al. 2014). Leghemoglobin content was increased by CP200B but reduced by CP84B at both harvests. Nodulation is an energy-consuming processes, therefore more nodules mean increased carbon demand (Xie et al. 2019b). In our case, it is possible that the reduced leghemoglobin associated with CP84B suggests that this strain has a reduced symbiotic function, and that the plant has compensated by continuing to make more, but smaller, nodules to satisfy its N demand. This treatment is linked to the higher plant flavonoid gene expression and reduced Nif gene expression. Overall plant N for this treatment was lowest at the 8-week harvest indicating a poor initial performance. However, by week 16 it appears to overcome this limitation producing the highest DM and equal highest seed yield. In contrast CP200B, with fewer, more efficient nodules, and highest leghemoglobin levels and possibly lower C demand, achieved the highest plant growth at 8 weeks. As noted for CP84B treated plants, the total N content or the total plant weight did not correlate with either the nodule mass or leghemoglobin content at either harvest time. It is reported that under N limited growth condition, the increase in total shoot N is usually associated with improved $N_2$ fixation (Carter and Tegeder 2016), which is more related to the increase of nodule function rather than more root nodules. Another explanation might be that at 16 weeks most of the nodular fixed N in CP200B treated plants was transported from shoot to pod, which supported chickpea seed formation at the pod bearing period (Zhang et al. 2016).

All four actinobacterial strains increased total grain weight by between 2 and 3-fold per plant when co-treated with rhizobia compared to the rhizobia only treatment. CP56 was the least effective actinobacterium in this regard with the other three producing higher seed yields. Similar studies also highlighted the positive effects of chickpea co-inoculation with *Mesorhizobium* and *Pseudomonas aeruginosa* (Verma et al. 2013). We also noted that CP84B and CP200B treated chickpea accumulated the highest seed mass and shoot dry matter which is in accordance with the report that shoot dry matter can often be considered as a valid yield indicator (El-Akhal et al. 2013).

### Evaluation in field soils

Considering that the experiments in a sterile sand-vermiculite system do not necessarily reflect the impacts under field conditions, evaluation of the actinobacterial treatments were carried out at 6-weeks using natural field soils. In the Turretfield soil that contained a community of chickpea rhizobia, compared to the no N control, only the actinobacterial treated plants were able to attract rhizobia and form nodules which were most abundant in CP200B and CP84B treated plants. All four actinobacteria significantly increased total plant weight with the highest increase of 59% with CP200B treated plants. In the Lameroo soil with *M. ciceri* CC1192 added, nodulation was similar to the rhizobia only control but once again all the actinobacterial co-treatments significantly increased total plant dry weights, with CP200B treated plants increased by 64%. This suggests that the actinobacteria have a strong potential for application in the field, especially strain CP200B.

### Impacts on rhizobia growth in vitro

Actinobacteria are well known for their antagonistic activity against other bacteria and fungi and have been shown to have inhibitory effects on the growth

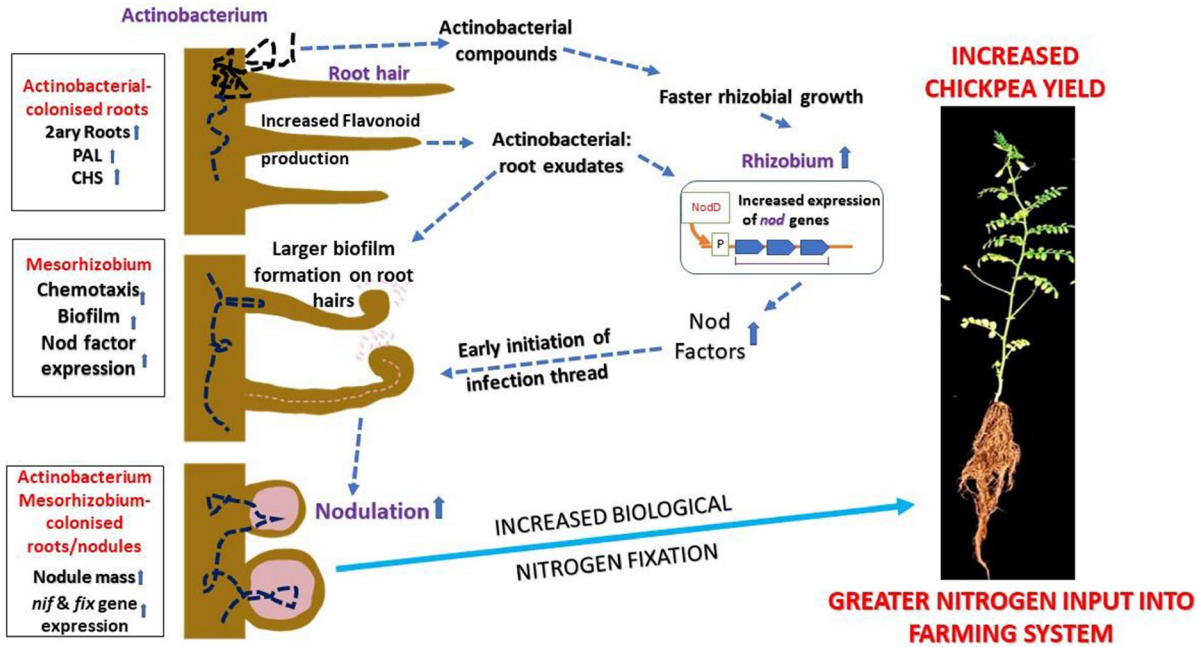


**Fig. 7** A model explaining how treatment with endophytic actinobacteria enhances the performance of the chickpea-rhizobia symbiosis with increased chickpea nodulation and $N_2$ fixation leading to improved growth and grain yield. The Up arrow indicates significant upregulation. Adapted from Laranjo et al. (2014)

of some rhizobial strains, potentially limiting nodulation if applied as an inoculant on legume seed (Lima et al. 2017). In contrast, the compounds produced by the actinobacteria tested here stimulated the growth of *M. ciceri*, which would aid faster colonisation of the rhizosphere and root hairs.

Rhizobial chemotaxis and colonization are two primary elements for the subsequent legume-rhizobium nodule initiation (Poole et al. 2018). Chemotaxis towards root exudates is supposed to be the first step of rhizobia attraction, then biofilm formation on root hairs (Poole et al. 2018). Metabolites from both CP56 and CP200B increased chemotaxis and biofilm formation of rhizobia which would contribute to increased colonization and nodulation by rhizobia.

## Colonisation and survival of actinobacteria

The colonization and survival of the most potent strain, CP200B, indicated that CP200B can reside within the chickpea for the full growth cycle (Fig. 3), and this should confer the ability of CP200B to continuously produce long-term impacts on chickpea metabolic and physiological activities. We also noted that CP200B could be detected in non-inoculated chickpea roots at 4 weeks. CP200B was previously isolated from chickpea roots, so that it may be part of the normal endophytic microflora. Nevertheless, there are still chances that the primers amplified non-specific products in other similar strains. Unlike our findings, others reported that the population of exogenous microorganisms decreased after a relative short period of time after inoculation (Araujo et al. 2019; Bonaldi et al. 2015; Wang et al. 2014). In addition, the presence of CP200B was detected at 16 weeks in chickpea seeds, suggesting CP200B could travel to the seed from root and shoot, which meant CP200B could be transmitted to the next generation. Further tracking of CP200B in the chickpea seedlings germinated from these seeds would be useful to understand how long the effect exerted by CP200B will last.

## Possible mechanisms to enhance symbiosis

Several of the measured actinobacteria effects occurred before nodule initiation, associated with the complex molecular dialogue between the legume host and rhizobia (Deakin and Broughton 2009; Wang

et al. 2012) that leads to an abundance of rhizobia in the rhizosphere, a fundamental element in nodule initiation (Masson-Boivin and Sachs 2018). Rhizobial accumulation in the rhizosphere soil undergo changes in response to phenolic and flavonoid compounds secreted by roots. PAL and CHS enzymes play a pivotal role in flavonoid and phenolic compounds synthesis (Yu et al. 2000), and beneficial microorganisms can elevate PAL activity and promote phenolic and flavonoid synthesis (Palma-Tenago et al. 2017). For instance, inoculation with *Bradyrhizobium japonicum* elevated PAL and CHS mRNA levels in soybean (Pregelj et al. 2010) and peanut (Xie et al. 2019a). Our study showed that the use of actinobacteria increased the expression of *PAL* and *CHS* genes (Fig. 4), which might ultimately contribute to a rapid increase in the biosynthesis and secretion of flavonoid compounds into the rhizosphere.

The release of Nod factors in the rhizobia are induced by flavonoids, which are in turn recognized by plant specific receptors, and give rise to a nodule primordium (Xie et al. 2019b). Our results demonstrated that exudates from actinobacteria-colonized roots significantly increased the chemotaxis, biofilm formation and *nod* gene expression of the chickpea rhizobia strain CC1192, especially for CP200B colonized plants. Further research is needed to analyse the role of indole acetic acid and the composition of root exudates in response to colonization by endophytic actinobacteria.

Nodulation kinetics showed that nodulation occurred earlier on plants treated with strain CP200B which also showed an increased nodulation. A similar phenomenon was noted with actinobacterial inoculation with lucerne (Le et al. 2016a). As a consequence, actinobacterial inoculation might lead to earlier nodulation and N-fixation to support early plant growth.

Increased *nif* gene expression (*nif*H and *fix*A) over the rhizobium only control was also detected in nodules from plants inoculated with actinobacterial strain CP200B, and to a lesser extent strain CP56, indicating a higher nitrogenase activity and better $N_2$ fixation (Esfahani et al. 2014; Lindström and Mousavi 2020; Wongdee et al. 2018). Other strains (e.g., CP84B) reduced a number of measures associated with nodule function, highlighting the specific nature of actinobacterial effects and that careful strain selection will be needed to optimise symbiotic improvement.

Additionally, actinobacteria conferred growth promotion in the absence of rhizobia, and are expected to contribute to the enhanced growth of chickpea seen in the tripartite symbiosis.

## Conclusion

Endophytes have been shown to play a significant role in promoting host plant growth. However, the underpinning mechanisms have remained poorly understood, particularly for legumes. Here, the co-inoculation of rhizobia and strains of endophytic actinobacteria was found to consistently increase the nodulation, growth and grain yield of chickpea in a largely Nitrogen-free sand -vermiculite system. As displayed in the conceptual model (Fig. 7), adapted from Laranjo et al. (2014), multiple mechanisms may have contributed to the responses due to the treatment with actinobacteria. These include changes in the root architecture both in the absence and presence of rhizobia, faster and increased growth of the rhizobia in response to actinobacterial compounds and increased secretion of specific root exudates including flavonoids, increased attraction of rhizobia to the roots, increased biofilm production, and the higher levels of expression of *nod* genes leading to earlier nodulation and increased nodule mass. Together with a higher level of expression of *nif* and *fix* genes for some actinobacteria strains, their application resulted in higher grain yields. If duplicated in the field, the application of actinobacteria should increase nitrogen inputs into the farming system.

**Authors' contributions** CMMF, RAB and YZ designed the experiments and QATV, SJB and TX carried out the experimental work. SJB assisted with the statistical analysis and TX and CMMF wrote the manuscript with assistance from RAB and SJB.

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**Declarations**

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