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The influence of rhizobia and Arbuscular mycorrhizal fungi co-inoculation on growth and yield of Chickpea (*Cicer arietinum)* in the greenhouse and the field

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Abstract

The use of chemical inputs in agriculture has a negative influence on the environment. Application of chemical fertilizers (F) can be reduced by using bio-fertilizers like arbuscular mycorrhizal fungus (AMF) and Rhizobium (R). Chick pea was co-inoculated and grown in the greenhouse and in the field to investigate their effect on growth and yield. All assessed plant growth parameters were found to be highest in plants treated with AMF and Rhizobium after three months of growth. Plants treated with R+AMF+F had greater average shoot length, stem girth, shoot dry weight, root dry weight, shoot fresh weight, branch per plant, and number of pods than those treated with R+M. However, R+M had a higher number of nodules than R+AMF+F in the greenhouse, and for co-inoculation in the field, indigenous rhizobium and AMF with fertilizer application had the highest shoot length (82.0±2.3), number of pods (75.0±2.6), dry weight (109.5±11.2), 100 seed weight (14.2±0.2), and husk weight (12.06±0.2) when compared to the control. Furthermore, root colonization ranged from 63.7±2.9% to 85.0±8.1% for all AMF infected treatments. In the green house, the maximum spore density (560.0±17.3 spore/50g dry soil) was recorded for solitary mycorrhiza inoculated chickpea. As a result, it is determined that dual inoculation of the pulse with AMF and Rhizobium, both with and without NPK treatment, increased growth and production. This suggests that smallholder farmers may be able to use bio-inoculants to boost chickpea output.

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Introduction

Chickpea is a versatile crop that offers numerous advantages for integrated crop-livestock smallholder farming systems. It is a good source of protein and micronutrients; it improves soil fertility and health through nitrogen fixation; it has a low carbon footprint and contributes to climate change mitigation; and in Ethiopia, it is easily incorporated into crop rotations with wheat (Triticum aestivum) and teff (Eragrostis tef) to ensure the sustainability of farming systems. It is also a significant contributor to household income. When compared to cereals, chickpea (*Cicer arietinum*) is a less labor-intensive leguminous crop that requires fewer external agricultural inputs. Because chickpeas is a leguminous plants, it have a natural ability to fix biological nitrogen by converting atmospheric nitrogen into ammonia. *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* form nodules when they come into contact with chickpea roots (Nandwani and Dudeja, 2009).

Chickpea's nitrogen-fixing ability increases not only chickpea production but also the residual nitrogen that is left after harvesting chickpea; it can grow on residual moisture, allowing farmers to engage in double cropping, where chickpea is sown at the end of the rainy season following the harvest of the main crop; (Egamberdieva *et al.*, 2014). Chickpea is a good source of protein, vitamins, and carbohydrates, which can help with human nutrition (Botir *et al.,* 2016).

Although chickpeas fix nitrogen, it is insufficient to produce the desired product; therefore, farmers use inorganic fertilizer to increase chickpea production. There is no doubt that inorganic fertilizer increases production, but it has drawbacks in terms of affordability, accessibility, and environmental impact. For example, smallholder farmers could not afford the high cost of inorganic fertilizer, and due to transportation delays, farms could not get the inorganic fertilizer at the precise time of application. Furthermore, the irrigation of this inorganic fertilizer, particularly phosphorous, harms the environment by polluting the water body.

Continuous use of inorganic fertilizers causes plants to absorb and accumulate heavy metals, reducing crop nutrition and quality (Abdiani *et al.*, 2019).

As a result, the use of organic fertilizer (Arbuscular mycorrhizal fungi (AMF) and Rhizobium) is critical for mitigating the negative effects of this inorganic fertilizer. Organic fertilizers increase organic matter, which is critical for soil fertility (Shurigin *et al.*, 2015). Organic fertilizers such as rhizobia and arbuscular mycorrhiza fungi (AMF) can help plants grow by fixing atmospheric nitrogen; on the other hand, arbuscular mycorrhiza fungi help plants grow by extending the plant roots deep into the soil, releasing the bind phosphorous (Malina and Sharma, 2013). The purpose of this study therefore is to determine the role of rhizobia and arbuscular mycorrhiza fungi in chickpea growth in a greenhouse and in the field.

Materials and methods

Description of the study area

Hawassa University is situated in the Ethiopian rift valley, 275 kilometers south of Addis Abeba, between 7° 3′1.35′′N latitude and 38° 29′43.81′′E longitude, at an elevation of approximately 1736 meters above sea level. The greenhouse and the Hawassa University research village, where the research was conducted, are located on the main campus's eastern edge.

Table 1. Treatment and experimental design.

1. Chickpea(Cp) inoculated with Rhizobium
2. Chick pea inoculated with Arbuscular mycorrhizal fungi (AMF)
3. Chick pea (Cp) with NPK application
4. Chick pea (Cp) + Rhizobium (R) + NPK
5. Chick pea (Cp) + AMF + NPK
6. Chick pea (Cp) + Rhizobium R + AMF + NPK
7. Chick pea (Cp) + Rhizobium R + AMF
8. Control
9. MBI

Key: 1) R=Rhizobia isolates alone: Two inoculants of individual isolates of Rhizobia (commercial and elite indigenous isolates of chickpea; 2) M=AMF alone: one inoculant of AMF, mixture of Gigaspora rosea and Rhizophugus clarus; 3) P sources (NPK); 4) AMF + P sources (NPK); 5) Rhizobia + P sources (NPK) ; 6) AMF + Rhizobia + P sources (NPK; 7) Co-inoculation (AMF + Rhizobia); 8) Non-inoculated control. 9) MBI. The NPK used in this treatment was (50, 25, and 25) which is 3.57×10^{-6kg} N/pot and 1.78×10^{-6kg} per pot.

Treatments and experimental design

Experiments in plastic pots (2kg soil carrying capacity) and in the field were carried out in the greenhouse and research village at Hawassa University in 2019. The experiments were conducted in the field using a Complete Randomized Block Design (RCBD) and in the greenhouse using a completely randomized design (CRD), each with three replications.

Inoculums Preparation

Preparation of AMF Inoculum

Gigaspora rosea and *Rhizophugus clarus morphospecies* previously isolated (Beyene *et al.*, 2016) from the rhizospheric soil of agroforestry shade trees and crops by wet sieving and decanting technique (Gedman and Nicolson, 1963) and multiplied in the roots of Sorghum (Sorghum bicolor) plants were used as AMF inoculate. After three months of growth, the substrate containing root fragments, mycelium, and spores was collected, air-dried, and used as a crude inoculum. Chickpea seeds were surface sterilized with 0.3 percent hypochlorite solution for 30 seconds before being washed with sterile distilled water (Utobo *et al.*, 2011). The seeds were then germinated in a sterilized soil-sand mixture in the dark. The seedlings were allowed to grow for 8-10 days. Seedlings were transplanted in plastic pots containing a 2kg oven sterilized mixture of sand and soil in a 1:1 ratio. A mycorrhizal inoculum containing sorghum root fragments, mycelium, and spores was applied to seedlings (100g per pot). The treatments were replicated three times.

Isolation, authentication, and inoculation of indigenous Rhizobia

Soil sample collection and analysis of physicochemical properties

The first step in trapping and identifying rhizobia isolates was to collect soil and seed samples. Using an auger, twenty (20) soil samples of 3.5kg each were collected from 10-15cm depth on a cultivated land with no previous leguminous plant production. Soil samples were collected from 10 villages in the Shalla, Wolaita Sodo, and Boricha areas with favorable temperature, soil type, and altitude for chickpea cultivation (Table 1) and separated into two parts: 3kg for trap culturing and 500g for soil physicochemical property analysis.

500g of each soil sample collected was analyzed for its physicochemical properties. The soil samples were air-dried at room temperature for two weeks, grounded, homogenized, and passed through a 2mm sieve and preserved at 4°C for analysis of soil physicochemical properties. Soil analysis was undertaken at Debrezeite Agricultural Research Center following standard procedures and methods: Soil textural fractions were analyzed following the hydrometric method after removing organic matter using H_2O_2 and dispersing the soils with sodium hexameta-phosphate (Black *et al.*, 1965).

Table 2. Geographical locations from where soil samples were collected for Rhizobia isolation.

	Sampling site	Longitude	Latitude	Altitude
1	Shalla, Bekele Daya farm	38° 25′ 20.8″	7 °17′ 24.3″	1671
2	Halaba Zone, Gedeba village(FTC)	38° 8′ 41.6″	7° 18′ 24.7″	1782
3	Halaba, 1st Chorko village	38° 5′ 56.4″	7° 21′ 0.6″	1787
4	Damot Gale, Hagaza Doge village	37° 52′ 22.2″	6° 58′ 28.7″	1971
5	Damot gale, Ade Koisha village	37° 52′ 57.3″	6° 59′ 55.1″	1894
6	Damot Gale, Chocha village	37° 50′ 43.1″	6° 56′ 05.0″	2076
7	Damot Gale, Fate village	37° 50′ 14.0″	60 55’18.5”	2104
8	Damot Woide, Mayo Kole village	37° 51′ 16.4″	6° 53′ 18.2″	2092
9	Damot Woide, Kindo Koyo kebele	37° 52′ 51.2″	6° 53′ 43.3″	1993
10	Boricha, Shello Abore kebele	38° 14′ 01.8″	6° 56′ 45.9″	1840

Soil pH was determined by potentiometric methods using 1:2.5 soil: water ratio. Soil organic carbon (SOC) was determined by the Walkley-Black oxidation method (Schnitzer, 1982). Total nitrogen (TN) was determined using the Kjeldahl distillation method (Bremner and Mulvaney, 1982), and available phosphorous (AP) was determined using Olsen's extraction method (UV/visible Spectrometer, Lambda EZ 201) (Olsen and Dean, 1965). Available potassium (Av. K) was determined by Sodium Acetate (flame photometer) method (Jones, 2001).

The exchangeable bases (Ca^{2+} and Mg^{2+}) were measured by atomic absorption spectrophotometer (NOV AA 400) after extraction with ammonium acetate at pH_7 (Black *et al.*, 1965).

Chickpea Trap Culturing

Healthy Chickpea seeds of the same size were sterilized by rinsing with 95 percent alcohol for 10 seconds, followed by 3 percent Sodium hypochlorite for 4 minutes, and the sterilant was then drained off. Following that, the seeds were rinsed eight times with sterile water. A chickpea trap culture (10 soil samples) was established in the Hawassa University greenhouse in accordance with (Somasegaran and Hoben, 1994).

Trapping of Nodules

Nodulation was induced in greenhouse conditions using Vincent's 'plant trap' method (1970). The soil samples were placed in surface-sterilized plastic pots weighing 2kg (using 70 percent alcohol for 5sec.). Similar-sized chickpea seeds were surface sterilized for 5 seconds with 70% ethanol and 3 minutes with a 3% (v/v) solution of sodium hypo-chlorate before being thoroughly washed with five changes of sterile distilled water. Six seeds were then planted in each pot in the greenhouse. The seedlings were thinned to two after germination. For 45 days, the plants were watered every two days. At the flowering stage of the plants, the pink and undamaged nodules were collected after 45 days. The collected nodules were then preserved on Silica-gel until analyzed.

Preparation of Culture Media

Yeast Extract Mannitol Agar (YEMA) (Vincent, 1970) was prepared to isolate rhizobia with the following ingredients: Mannitol (10g/l), K_2HPO_4 (0.5g/l), $MgSO_4.7H_2O$ (0.2g/l), NaCl (0.1g/l), Yeast Extract (0.5g/l), Agar (15g/l), Distilled Water (1000ml), pH ((Somasegaran and Hoben, 1994). Finally, the media was autoclaved for 15 minutes at 121 0C degrees Celsius.

Isolation of Rhizobia from Nodules

Surface sterilized nodules were treated with 95 percent ethanol for 10 seconds before being transferred to a 3 percent (v/v) solution of sodium hypo-chlorate for 3-4 minutes. The surface-sterilized nodules were then rinsed five times with sterile distilled water to remove all of the sterilizing chemicals (Lupwayi and Haque, 1994). The nodules were then transferred from each pot into separate sterile Petri dishes and crushed with an alcohol-flamed sterile glass rod in a drop of normal saline solution (0.85 percent NaCl) inside a laminar airflow hood. The suspensions were then streaked on a plate containing Yeast Extract Mannitol Agar (YEMA) +0.2 percent Congo red indicator and incubated at 280C for 3-5 days.

Purification and preservation of isolates

After 3-5 days of growth, single dome-shaped colonies were picked with a sterile inoculating loop, streaked on sterile YEMA plates, and incubated at 2820 degrees Celsius. Through repeated re-streaking, the purity and uniformity of colony types were carefully examined, and a single well-isolated colony was picked and transferred to YEMA slant containing 0.3 percent (W/V) CaCO3 in a culture tube and incubated at 2820C. When enough growth was observed, the culture was transferred to be stored at 40 degrees Celsius for future use (Vincent, 1970). According to Jordan, the native isolates were then characterized based on morphological and physiological/ biochemical characteristics (1982).

Authentication of rhizobial isolates

Sterile growth plastic pots filled with acid-washed sterile sand (river sand washed with sulfuric acid (38%; 5 L/20kg sand) to reduce organic matter that could be a N source for the plants) (Lupwayi and Haque, 1994). The sand was then rinsed with distilled water until its pH was neutral, autoclaved, and used to test the isolate rhizobia's ability to form nodules on the homologous legume species from which it was obtained, as specified by Somasegaran and Hoben (2012). To activate the rhizobia, a loopful of material from a preserved slant was transferred aseptically to a 100ml flask containing 60ml of YEM broth, and the flasks were shaken on a rotary shaker until the liquid became cloudy.

In triplicate, one surface sterilized seed (as described above) was placed in a small polyethylene pot containing washed and sterilized sand. The seed was then pipetted with 1ml of the broth culture (with a bacterial density of 109 cells/ml). After that, the pots were placed in the greenhouse.

Following Koch's postulates, *Cicer arietinum* grown in nitrogen-free media were inoculated with pure rhizobia isolates (rhizobia isolates of *Cicer arietinum* grown in sampled soils) under sterile conditions, while other *Cicer arietinum* were grown for 45 days in rhizobia free media and remained non-inoculated as a control. Isolates that nodulated *Cicer arietinum* grown in sterile media were found to be rhizobia, while those that did not nodulate were thought to be non-rhizobia for *Cicer arietinum*.

Morphological and Biochemical studies of the bacteria

The shape, color, size, elevation, margin, and texture of Rhizobia colonies on YEMA were studied morphologically (Vincent, 1970). Cultures were then struck on YEMA and incubated at 30°C. Rhizobia shape, color, size, elevation, margin, and texture were recorded after 4 days. Finally, the isolated rhizobia were subjected to a series of biochemical tests in order to characterize them.

Gram's staining

Gram's staining was used to study the gram reaction, and the staining was done according to the standard Gram's procedure (Somasegaran and Hoben, 1994). To calcify bacteria based on gram staining results, four different reagents in the order of Gentian violet, Gram's iodine, alcohol distaining reagents, and safranin were used.

Catalase Test

On a clean glass slide, a drop of rhizobial culture suspension was placed. The culture was given a few drops of hydrogen peroxide. Positive results are indicated by the evolution of air bubbles from the suspension.

Triple Sugar Iron Agar Test

A triple sugar iron agar medium was prepared (TSI agar slants contain 1% lactose, sucrose, and 0.1 percent glucose).

To detect acid production from carbohydrate fermentation, the pH indicator phenol red is also added to the medium. The medium was poured into sterile test tubes and allowed to settle. After inoculating the rhizobial cultures into the tubes and incubating them at 28°C for 24 hours, the results were recorded. The fermentation of glucose results in yellow butt (A) and red slant (K). Because of the limited glucose in the medium, the slant remains red (alkaline) (K). A yellow butt (A) and slant (A) due to lactose and/or sucrose fermentation (yellow slant and butt due to the high concentration of these sugars), resulting in excessive acid formation in the entire medium. Splitting the agar revealed the gas formation. The blackening of the agar indicates gas formation (H_2S). Red butt (K) and slant (K) indicate that no sugars were fermented, and no gas or H_2S was produced.

Methyl Red Tests

Methyl Red Voges Proskauer broth was made (Glucose Broth, Methyl Red indicator for MR test Voges Proskauer reagents A: 5 percent AlphaNaphthol & ethanol, B: Potassium Hydroxide; (3:1 ratio), and Deionized Water). In sterile test tubes, 5ml of broth was poured. Separate rhizobial isolates were inoculated into tubes and incubated at 28°C for two days. Following the incubation period, each test tube received 5ml of methyl red indicator. The broth's red color indicates a positive result; however, turning methyl red to yellow indicates a negative result.

Citrate Utilization Test

Simon's citrate agar medium was ready (adjust the pH to 6.9, add agar and Bromothymol blue, gently heat to boiling the agar is dissolved, dispense 4 to 5ml into tubes, Autoclave at 1210c for 15 minutes, and cool in slanted position). Individual Rhizobial isolates were then inoculated into test tubes and incubated at 28°C for 4 days. After the incubation period, the green color changed to blue, indicating that the results were positive.

Starch Hydrolysis

Agar medium with starch was made. The medium was placed in sterile Petri plates and allowed to solidify. The rhizobial cultures were incubated separately in

Petri plates for 4 days at 28°C. Following the incubation period, 5ml of iodine solution was added and observed: a clear zone of hydrolysis surrounding the organisms' growth indicates positive results.

Evaluation of symbiotic effectiveness of new rhizobia isolates

Preparation of sterile media

The river sand was collected, and all debris was removed before it was washed, dried, and autoclaved at 121⁰ degrees Celsius for 30 minutes to kill any inherent rhizobia. The autoclaved sand was then allowed to cool for 24 hours. After cooling, the sterile sand was placed in 1/5kg plastic cups/pots to be used for growing *Cicer arietinum*.

Preparation of rhizobia isolates for inoculation

The authenticated isolates of indigenous rhizobia were re-grown on plates containing Yeast Extract Mannitol Agar (YEMA) with Congo red (CR) and incubated at 28⁰C. After three days, a loopful of rhizobia isolates was inoculated into 100ml sterile beakers containing 60ml of Yeast Extract Mannitol Broth (YEMB). The inoculated beakers of YEMB were covered with autoclaved foil paper and kept in a shaker incubator for 72 hours at 28⁰C and 120 rpm (rotation per minute) until broth color changed from colorless to the milky indicating presence of rhizobia isolates. Then 1ml of YEMB containing (10^9 CFU/ml) rhizobia were applied in 1/5kg plastic cups containing sterile sand and germinating seeds *Cicer arietinum*.

Seed sterilization and planting

Cicer arietinum seed of the same size was sterilized by rinsing in 95 percent alcohol for 10 seconds, followed by 3 percent Sodium hypochlorite for 4 minutes, and then the sterilant was drained off. Following that, the seeds were rinsed eight times with sterile water. Following that, the seeds were immersed in sterile water and placed in a refrigerator at 4°C for 4 hours to allow the seeds to imbibe. After 4 hours, the seeds were rinsed three times with sterile water and left at room temperature for 24 hours before being sown in sterilized sand pots. The rhizobia isolates were then inoculated into 20 pots, while 2 were inoculated with "MBI" isolates to compare and N-fertilizers as a positive control, and the remaining pots were left uninoculated as a negative control. The plants were grown in a greenhouse and were watered every 15 days with both N-free and N-fertilizer. Growth parameters such as nodule number, stem girth, number of leaves, number of branches, and shoot length were measured and compared after 5 weeks, as described by Bala *et al.* (2010) and Mhango *et al.* (2013).

Harvesting the plants and assessing symbiotic effectiveness

Five weeks after inoculation, the plants were harvested. The shoots were collected by cutting the plants at the sand level. Shoots from each growth unit were then placed in paper bags and dried at 70 0C for 48 hours as described by Somasegaran and Hoben (1994), and their dry weight was calculated. The roots and adhering sand were sifted through a coarse sieve (0.76mm), washed with gentle tap water, and examined for nodules. The nodules were gathered and counted. The relative effectiveness of isolates in accumulating plant shoot dry matter was calculated in the same way that Somasegaren and Hoben (1994) did:

$$SE = \frac{\text{Inoculants plant D.M. X 100}}{\text{N-fertilized plant D.M.}}$$

Where, D.M. = dry matter, S.E. = symbiotic effectiveness

The rate of nitrogen fixing effectiveness is evaluated as: Highly effective > 85%, Effective 55-85%, Lowly effective 35-54% and Ineffective <35%.

Greenhouse and Field Experiments

Experimental Design and Sample Collection

In May 2019, the experimental site in Hawassa University's research village was prepared for field trials. The treatments were set up in a fully randomized (RCBD) layout of field plots (quadrats), with each treatment being replicated three times (Table 1). Equivalent treatments for indigenous AMF and Rhizobia were also established in comparison to (MBI) obtained from Menagesha Biotechnology Industry. Surface sterilized seeds were sown in 3 X 2M quadrants in the field and inoculated with rhizobia (109cell/ml) and/or AMF (100g crude inoculum) inoculum based on treatment. The control treatments received no rhizobia or AMF inoculation.

Similar treatments were also established in the Hawassa University research village greenhouse in May 2019. However, using 2kg polyethylene pots in a completely randomized (CRD) design with three replications.

Following germination, seedlings in both the field and the greenhouse were thinned and allowed to grow for 180 days. Throughout the growing season, the plants were monitored on a daily basis, with weeds removed and plants watered as needed.

Plant and Soil Sampling Procedures

Plants were harvested by removing them completely from the field and greenhouse pots. Plants were divided into fractions. From the greenhouse before dry matter determination of roots, 0.5mg fine root segments (1cm in length) below the upper 2cm of the roots were sampled to estimate root colonization by AMF and stored in a 50% ethanol solution until analyzed. Soil samples from each pot which is inoculated with AMF were collected during harvesting after the plants had been removed and the representative sample was put in a plastic bag separately. Then the soil samples were dried at room temperature for 15 days and preserved at 4°C until analyzed.

Mycorrhizal Colonization and Spore Density

The roots were cleared with a 10% KOH solution in a water bath at 90^{o}C for 1hr and stained with a 0.05% trypan blue or methylene blue solution and disdained using acidified glycerol solution. Percent root colonization was estimated using a magnified intersection method, hairline graticule was inserted into the eyepiece acting as the line of intersection with each root at X200 magnification under the compound light microscope (McGonigle *et al*., 1990).

To study the spore density in the pot experiments 50g soil samples were collected (as in 3.5 above) and mixed in a two-liter capacity beaker containing 1.5 liters of water. The soil in the water was agitated by stirring vigorously by hand and left to settle down. The suspension was then be sequentially sieved with sieves having mesh sizes of 500, 100, 50 & 38µm in diameter, following the wet sieving and decanting method (Gerdemann and Nicolson, 1963).

The last pellet (38µm) was suspended in 60% sucrose solution and was thoroughly mixed and centrifuged at 2000rpm for 1 minute and the spores were rinsed carefully with tap water and transferred into plastic Petri-dishes. The AMF spores and sporocarps of each sample were counted under a 4x stereomicroscope. The spore density was expressed as the number of spores and sporocarps per 50g of the dry substrate (soil plus sand mixture from the pot experiment).

Rhizobial Infection

On two harvests, two plants were taken from each pot, shaken free of superficial soil, and all nodules present were carefully removed. The numbers of nodules per plant were counted independently and the mean value was calculated for the two harvests.

Dry Matter Production

Plants were divided into shoots and roots. Root samples were washed with water carefully in a sieve (250µm mesh size) and were dried at 105 °C for 24 hr and amounts of dry matter were measured gravimetrically. Thereafter, shoot samples were ground to determine nutrient content.

Nutrient Content

Plant tissue analyses were undertaken at Debrezeite Agricultural Research Center following standard procedures. The concentration of nitrogen in the samples was determined using an element analyzer based on the Dumas principle (LECO CN). For the plant tissue concentration of other elements, the dried sample (500mg) was digested in a tri-acid (HNO_3 + $HClO_4$ + H_2SO_4) mixture (Nabrzyski and Gajewska, 1998). K content in the digest was measured using atomic absorption spectrometry (Varian SpektrAA 300) (Beaty and Kerber, 1993). The P content in the plant tissues was analyzed by the vanadium phosphomolybdate method after the wet digestion followed by photometry (Varian DMS 200) (Cavell, 1955).

Statistical Analysis

Statistical analysis for comparison of all growth parameters (plant height, stem diameter, root and

shoot dry matter yield, number of nodules, branch per plant, and number of the pod) among treatments and control plants were carried out using the SPSS 20.0 version for Windows software package. Mycorrhizal dependency (MD) of the two pulses was calculated according to Plenchette *et al.* (1983) as follows: MD (%) = [(M-NM)/M] X 100; *where*: M is the total dry biomass of mycorrhizal plant; NM is the total dry biomass of the non-mycorrhizal plant. The chemical and microbiological (root colonization, spore density, and the number of nodules) data were analyzed by one-way analysis of variance, and treatment means were compared using Duncan's multiple range tests.

Results

Isolation and Authentication of Rhizobia

Soil Aanalysis

500g of each soil sample collected was analyzed for its physicochemical properties. The soil analysis results show that pH values ranged between 6.1±0.4c to 7.7±0.4 (Table 1). Organic carbon was above critical levels (0.40%) and ranged from moderate (0.7±0.1) in a soil sample from DGH to high (4.9±4.2) in HG and (4.9±0.1) in DWK sampling sites. So, there is a moderate to a high level of organic matter in the study area. Total nitrogen was ranged from the lowest (0.1%) in SB to 4.9% in HG. Phosphorus concentration was low in all soil samples ranging from lowest (1.9±0.2mg/kg) in DGF to (6.7±0.2 mg/kg) in DGcho soil samples. Soil texture was clay-loam throughout, except SB and BSA of which their soil texture was clay. The results of the analyzed soil components are presented in Table 3.

Table 3. Physicochemical properties of soils collected from sampling sites in Halaba, Wolaita Sodo, and Boricha districts (Woredas).

Parameters	SB	HG	Hcho	DGH	DGA	DGcho	DGF	DWM	DWK	BSA
pH	6.8±0.9g	6.8±4.2a	6.2±0.7d	6.6±0.2e	6.2±0.5d	6.7±0.7f	6.2±0.6d	6.1±0.4c	6.0±0.1b	7.7±0.4
OC(%)	1.0±0.2ab	4.9±4.2d	1.4±0.5ab	0.7±0.1a	2.2±0.0bc	1.9±0.3b	2.5±0.1c	1.9±0.2b	4.9±0.1	2.1±0.1bc
TN(%)	0.1±0.0a	4.9±4.2d	0.2±0.3b	0.1±0.0a	0.2±0.3b	0.2±0.0b	0.3±0.0c	0.3±0.0c	0.2±0.1b	0.2±0.1b
P(mg/kg)	2.7±1.2b	4.9±0.9bc	5.6±2.9c	4.4±0.2bc	4.6±0.1bc	6.7±0.2de	1.9±0.2a	5.4±0.2c	6.2±0.1d	4.4±0.2bc
Mgcmol(+)/kg	5.2±.8cd	5.5±0.4cd	4.27±2.1b	3.6±0.3ab	3.9±0.1ab	5.1±0.3cd	3.3±0.1a	4.8±0.0bc	3.3±0.0a	3.6±0.3ab
Cacmol(+)/kg	2.3±0.1bc	2.4±0.2bc	3.3±0.0c	2.2±0.0bc	3.4±0.1c	2.5±0.1bc	1.5±0.1ab	1.2±0.1a	2.1±0.1bc	3.3±0.1
Kcmol(+)/kg	3.2±0.2bc	2.4±0.2ab	3.4±0.1bc	3.3±0.0bc	4.4±0.1cd	3.3±0.4bc	2.4±0.1ab	2.1±0.1a	2.9±0.4bc	4.1±0.1c
Texture	SCL	CL	CL	CL	CL	CL	CL	CL	CL	SCL

Key: SB=Shalla Bekele Daya Kebele;HG= Halaba Zone Gedeba K(FTC); Hcho= Halaba 1st Choroko Kebe; DGH= Damot Gale Hagaza Doge K; DGA=Damot gale Ade Koisha Kebele; DGcho= Damot Gale Chocha Kebele; DGF= Damot Gale Fate Kebele; DWM= Damot Woide Mayo Kole Kebele; DWK= Damot Woide Kindo Koyo; BSA= Boricha Shello Abore Kebele; SCL=silt clay loam; CL= clay loam.

Isolated rhizobia isolates

A total of 10 rhizobia were isolated from the nodules of *Cicer arietinum* (*10 isolates)* grown in soils collected from sampling sites in Shalla, Halaba, Wolaita Sodo, and Boricha Woredas of Southern Ethiopia. All rhizobia isolates were fast growers having taken 2-5 days to grow in Yeast Extract Mannitol Agar (YEMA) after inoculation. All isolates were authenticated and have been proved to be rhizobia by inducing nodulation after inoculating in *Cicer arietinum* grown in Jensen nitrogen-free media. Representative examples are shown in tables 4 and 5 below.

Table 4. Morphological Characteristics of the Isolated Indigenous Rhizobia isolates from Chickpea.

Isolates	Morphological characteristics								
	Shape	Color	Opacity	Surface	Texture	Congo red absorption	Elevation	Margin	Size
IR 10cp	circular	milky white	translucent	smooth	mucoid	not absorbing	raised	Entire	medium

Table 5. Biochemical characteristics of the rhizobial isolates (representative).

Isolates	Biochemical characteristics						
	Gram stain	Catalase	Oxidase	TSI	Methyl Red	Citrate	Starch Utilization
IR10cp	-Ve	+++	+Ve	ALK/AB	+Ve	+Ve	-Ve

Key: ALK/AB: Alkaline slant acid butt

Symbiotic effectiveness of the isolated indigenous rhizobia isolates

Isolated rhizobia showed significant influence on various growth parameters. They have shown a significant influence on the average number of nodules (32.6 for chickpea inoculated with CpIR10) (Table 6). Chickpea CpIR10 isolate showed significantly higher ($p<0.05$) in stem girth (SG) and a number of the nodule (NN) than other isolates but there is no significant difference ($p>0.05$) than MBI in stim girth (value of SG for R: 4.1±0.1 and for MBI: 3.7±0.0) and the number of the nodule (value of NN for R: 32.6±0.1 and for MBI: 32.5±0.1) (Table 6). CpIR10 showed no significant difference ($p>0.05$) with other isolates of rhizobia concerning shoot length (SL), shoot dry weight (SDW), and branch per plant (BPP) parameters (Table 6).

Table 6. Influence of isolated indigenous rhizobia on growth parameters of chickpea.

Isolated Indigenous rhizobia/control	Growth parameters (Mean ± SEM)					
	SL(cm)	SG(mm)	SDW(g)	NN	BPP	LPP
CpIR1	41.0 ±0.5fg	3.5 ±0.0c	1.2 ±0.0bc	14.8 ±0.1c	1.6 ±0.1d	1.1 ±0.2ab
CpIR2	41.4 ±0.1fg	3.2 ±0.0b	1.2 ±0.0bc	30.0 ±5.0g	1.5 ±0.5d	0.9 ±0.2a
Cp IR3	35.5 ±4.6de	3.9 ±0.1cd	1.0 ±0.2b	28.0 ±1.0f	0.8 ±0.3bc	12.0 ±2.0bc
Cp IR4	32.3 ±2.8c	3.9 ±0.1cd	0.8 ±0.3a	29.4 ±0.9g	0.8 ±0.1bc	11.0 ±1.0b
CpIR5	29.6 ±0.5bc	3.5 ±0.1c	1.3 ±0.1bc	13.5 ±0.1b	1.0 ±0.2c	11.2 ±0.0b
CpIR6	40.5 ±0.5f	4.0 ±0.1d	1.3 ±0.0bc	25.9 ±0.4d	1.5 ±0.5d	11.1 ±0.5b
CpIR7	33.3 ±0.8cd	4.3 ±0.0e	1.3 ±0.1bc	27.5 ±0.5e	1.3 ±0.0cd	11.0 ±1.0b
CpIR8	28.1 ±0.5b	3.8 ±0.2cd	1.3 ±0.0bc	27.0 ±3.0e	0.3 ±0.0a	12.5 ±0.0c
CpIR9	34.2 ±0.2d	3.7 ±0.0cd	1.3 ±0.0bc	28.5 ±1.5f	0.5 ±0.1b	11.0 ±1.0b
CpIR10	43.2 ±1.0g	4.1±0.1de	1.5 ±0.0c	32.6 ±0.1h	1.8 ±0.1de	13.5 ±0.5cd
Negative control	20.3 ±0.6a	2.4 ±0.1a	0.7 ±0.1a	0.0 ±0.0a	1.1 ±0.0cd	10.2 ±0.0ab
Positive control	37.4 ±0.0e	3.5 ±0.1c	1.0 ±0.1c	0.0 ±0.0a	1.3 ±0.3cd	12.5 ±0.3c
MBI	41.9 ±0.1fg	3.7 ±0.0cd	1.6 ±0.0d	32.5 ±0.1h	2.8 ±0.3e	14.7 ±2.5d

Key: SL-shoot length; SG-Stem girth; SDW-shoot dry weight; NN-number of nodules; BPP- branches per plant; LPP-leaves per plant. Similar letters in columns show no significant difference between treatments at α 0.05 (n=10).

Results recorded in table 7 below show the effectiveness of the rhizobia isolates which ranged from ineffective to highly effective (Highly effective >85%, Effective 55% - 85%, Lowly effective 35% - 54%, and Ineffective < 35%). Based on these results, though all isolates display effective performance in authentication and effectiveness tests, only CpIR10 has been selected as elite isolates for further greenhouse and field experiments and their inoculums was mass-produced.

Soil analysis results of greenhouse pot experiment

The soil sample collected from Fate Kebele of Wolaita zone was analyzed for its physicochemical properties with an emphasis on nitrogen, phosphorus, organic matter, and soil texture the result showed available nitrogen 0.12%, phosphorus 6mg/kg (Olson), soil organic matter 0.82% and soil texture to be clay.

Table 7. Symbiotic effectiveness.

Sn	Cp%	Status
1	120	Highly Effective
2	120	Highly Effective
3	100	Highly Effective
4	80	Effective
5	130	Highly Effective
6	130	Highly Effective
7	130	Highly Effective
8	130	Highly Effective
9	130	Highly Effective
10	140	Highly Effective

Effect of sole and co-inoculation of AMF and Rhizobia on growth and biomass yield of chickpea in the greenhouse

The greenhouse experiment on inoculation of the mixture of indigenous arbuscular mycorrhizal fungi (AMF) and indigenous rhizobia (CpIR10), showed no significant difference $p>0.05$ in all parameters between single R and single M treatments (Table 8a, 8b). There was no significant difference $p>0.05$ between treatments F, R+F, and M+F in all parameters. Co-inoculation of Chickpea showed a significant difference $p<0.05$ when compared with single inoculation treatment (Table 8a, 8b). But single inoculation of R showed a significant difference in the number of the nodule (NN) when compare with co-inoculation. The value of NN for R is (57.6±1.5) whereas for M+R+F was (39.7±0.9) (Table 8b).

Table 8a. Sole and co-Inoculation of *Cicer arietinum* with commercial rhizobia (MBI) and AMF isolates in the greenhouse.

Treatments	(Mean±SEM) of Growth parameters							
	SL (cm)		SG (mm)		SFW (g)		SDW (g)	
	IR	MBI	IR	MBI	IR	MBI	IR	MBI
R	23±0.3b	22.0±0.9a	1.8±0.0b	2.2±0.0b	6.3±0.4b	6.5±0.6a	1.5±0.0b	1.8±0.1a
M	24.0±1.2b		1.8±0.1b		6.5±0.3bc		1.8±0.1bc	
F	26.2±0.1c		2.1±0.0c		10.1±0.6e		2.4±0.0cd	
R + F	26.0±0.9c	25.9±0.7b	2.2±0.1c	2.2±0.0b	10.0±1.2e	7.3±0.4b	2.3±0.0cd	2.5±0.0b
M + F	26.0±0.5c		2.1±0.0c		9.9±0.4de		2.2±0.0c	
R+M+F	28.0±0.0e	28.2±0.9d	2.3±0.0de	2.3±0.0bc	11.0±0.1g	11.6±0.7d	2.7±0.1de	2.8±0.0cd
R+ M	27.0±0.0d	27.0±0.1c	2.05±0.1d	2.1±0.0a	10.5±0.7ef	10.6±0.1c	2.6±±0.1d	2.6±0.0c
Control	9.0±0.4a		0.9±0.0a		4.0±0.0a		0.8±0.0a	

Key: SL-shoot height; SG-stem girth; SFW-shoot fresh weight; SDW- shoot dry weight. Similar letters in columns show no significant difference between treatments at α 0.05.

Table 8b. Sole and co-Inoculation of *Cicer arietinum* with commercial rhizobia (MBI) and AMF isolates in the greenhouse.

Treatments	(Mean±SEM) of Growth parameters							
	NN		B/P		RDW (g)		NP	
	IR	MBI	IR	MBI	IR	MBI	IR	MBI
R	57.6±1.5d	57.4±1.6d	3.0±0.0c	3.1±0.3a	0.3±0.0b	0.3±0.1a	3.0±0.3b	3.0±0.6a
M			3.2±0.0cd		0.6±0.1bc		3.2±0.7bc	
F			3.7.±0.3d		0.76±0.1d		4.1±0.9cd	
R + F	34.7±3.8a	35.0±2.6a	3.5±0.0cd	3.5±0.0b	0.69±0.0^{c}	0.5±0.0b	3.9±0.6c	4.0±0.7b
M + F			3.6±0.3cd		0.75±0.0d		4.0.±0.3cd	
R+M+F	39.7±0.9b	40.0±0.3b	4.3±0.3df	4.4±0.3cd	0.81±0.0e	0.8±0.1c	4.8±0.3d	4.9±0.3cd
R+ M	52.3±1.2c	52.5±2.0c	4.0±0.6d	4.0±0.0c	0.79±0.1de	0.8±0.0c	4.0±0.3cd	4..5±0.3c
Control			1.7±0.3a		0.09±0.0a		1.1±0.3a	

Key: NN-number of nodules; B/P-branches plant-1; RDW- root dry weight; NP-number of pods. Similar letters in columns show no significant difference between treatments at α 0.05.

Effectiveness of the AMF inoculants mixtures - Root colonization and spore density

All Chickpea treatments inoculated with sole AMF, AMF + fertilizer, AMF + rhizobia, and AMF + Rhizobia + fertilizer treatments were found colonized with AMF in the greenhouse. But those treatments inoculated with rhizobia alone (R), R+F, only fertilizer (F), and the control were found not colonized (Tables 9). When comparing sole and co-inoculation treatments with commercial and indigenous rhizobia, the highest root length colonization (80.0±2.9) was recorded for R+M+F (R-commercial) treatments. Similarly, the highest root length (85.0±8.1) was recorded for R+M (IR-indigenous) treatments (Tables 9). These treatments were found not colonized because the soil-sand substrate used was sterilized from inherent rhizobia and AMF.

The same spore density/50g dry soil was recorded by R+M+F (520.0±2.9) and R+M (520.0±2.9) treatments were found in chickpea inoculated with MBI whereas 560.0±17.3 and 520.0±23.1 spores/50g dry soil was recorded in IR inoculated chickpea for R+M+F and M treatments respectively. In these experiments, for R, F, R+F, and the control treatments were not recorded spore formation (Table 9).

Mycorrhizal Dependency (MD) of Chickpea in the Greenhouse

The highest MD values (70%) were recorded for co-inoculation and fertilizer application treatments in the chickpea, followed by co-inoculation without fertilizer application (M+R) 69%. The least values 55% were recorded in sole mycorrhizal treatments (Table 10).

Table 9. AMF root colonization and spore density in *Cicer arietinum*.

Treatment	(Mean±SEM) of Growth parameters (MBI)				(Mean±SEM) of Growth parameters (IR)			
	A	V	RLC%	SD/50g dry soil	A	V	RLC%	SD/50g dry soil
R	-	-	-	-	-	-	-	-
M	-	-	-	-	14.7±1.5a	34.7±3.8b	63.7±2.9a	520.0±23.1c
R+M	20.0±2.9a	40.0±2.9a	75.3±2.0a	520.0±2.9a	25.0±0.6c	35.0±4.0c	85.0±8.1d	440.0±5.8b
F	-	-	-	-	-	-	-	-
R+F	-	-	-	-	-	-	-	-
M+F	-	-	-	-	20.7±0.3b	40.0±5.2d	70.0±2.9b	360.0±11.5a
R+M+F	25.3±1.5b	40.0±1.2a	80.0±2.9b	520.0±5.8a	30.0±2.9d	30.0±2.9a	80.0±2.9c	560.0±17.3d
Control	-	-	-	-	-	-	-	-

Key: A-arbuscules; V-vesicles; RLC- Root length colonization; SD-Spore density. Similar letters in columns show no significant difference between treatments at α 0.05.

Table 10. Mycorrhizal dependency of chickpea in the greenhouse (MD%)= M-NM/M*100).

Treatment	Chickpea%
M	55
M + F	63
M + R + F	70
M + R	69

Key: M-mycorrhiza; R- rhizobia; F-fertilizer

Nutrient Content

All sole inoculated, co-inoculated and sole-fertilized-applied treatments showed an increase in chickpea tissue nutrient uptake as compared with the control (Table 11). Plant tissue nitrogen, phosphorus and potassium concentrations were higher in the co-inoculated (M+R+F) treatments with fertilizer application, while the next better nutrient uptake was recorded for co-inoculation treatment (M+R) without fertilizer application (Table 11). Besides, for sole mycorrhizal inoculation treatments better nutrient uptake was recorded as compared with sole rhizobia inoculation (Table 11). Also, for sole mycorrhizal inoculums with fertilizer application was recorded better performance as compared with sole rhizobia inoculation with fertilizer application.

Field Experiment

Effect of sole and co-inoculation of AMF and Rhizobia on growth and biomass yield of chickpea in the field

The Chickpea field result showed that treatment R showed significantly higher $p<0.05$ shoot length (SL), pod, total yield g/plot, and 100 seed weight than M.

Their value for both R and M respectively were SD (70±0.6 R): (65.7±2.3 M), pod (69±0.6 R): (55±2.4), total yield g/plot (66±1.4 R): (60±0.6 M), 100 seed weight (12.6±0.2 R): (9.3±0.2 M) and husk (10.2±0.9 R): (9.6±0.1 M) (Table 12a & 12b). Treatment M also showed significantly higher fresh weight (115±3.1 R and 156±9.1 M) and in dry weight (82.3±3 R and 86.2±9.7 M) than R (Table 12a & 12b).

Table 11. Effect of AMF and rhizobia inoculation on nutrient (N, P, K) uptake of chickpea in the greenhouse.

	Chickpea					
	Nutrient uptake (MBI)			Nutrient uptake (IR)		
K%	N%	P%	K%	N%	P%	K%
R	0.22c	0.13b	0.14b	0.14b	0.14b	0.17c
M	0.43de	0.15b	0.28d	0.23c	0.19bc	0.19c
R+M	0.22b	0.24c	0.22c	0.19b	0.13b	0.14b
F	0.31d	0.27d	0.24c	0.20bc	0.21c	0.14b
R+F	0.42de	0.20c	0.28d	0.23c	0.21c	0.16bc
M+F	0.88f	0.18c	0.58e	0.31d	0.25c	0.33d
R+M+F	1.77g	0.37e	0.60e	1.21e	0.45d	0.35d
R	0.6a	0.2a	0.3a	0.2a	0.1a	0.4a

Key: R-rhizobia; M-mycorrhiza; F-fertilizer; N-nitrogen; P-phosphorus; K-potassium; IR-indigenous rhizobia; MBI- menagesha biotechnology industry. Similar letters in columns show not significantly different relationships between treatments at α 0.05.

The F, R+F, and M+F treatments showed significantly higher $p<0.05$ seed number than single R or M treatment. The co-inoculation of R+M+F showed a significantly higher $p<0.05$ number in all parameters when compared with that of single inoculation (Table 12a & 12b).

Table 12a. Sole and co-inoculation of *Cicer* arietinum with commercial rhizobia (MBI) and AMF isolates in the field.

Treatment	(Mean±SEM) of Growth parameters							
	Shoot length (cm)		Pod		Fresh weight (g)		Dry Weight (g)	
	IR	MBI	IR	MBI	IR	MBI	IR	MBI
R	70.0±0.6cd	65.0±3.6a	69.0±0.6cd	69.0±2.6b	115.0±3.1b	139.3±55.4a	82.3±3.0b	111±39.5a
M	65.7±2.3b		55.0±2.9b		156.0±9.1c		86.2±9.7c	
F	80.0±5.6e		69.0±2.9cd		156.53±9c		86.7±0.9c	
R+F	74.3±2.0d	70.3±2.0b	68.0±3.5c	66.3±2.3a	181.0±11.6e	192.0±24.4b	103.0.±4.2e	143.6±22.0c
M+F	69.1±0.5c		58.0±1.5bc		178.2±22.9d		90.0±18.9d	
R+M+F	82.0±2.3de	73.3±2.6d	75.0±2.6de	70.0±6.0c	219.0±19.2g	219.9±30.7d	109.5±11.2f	165.5±24.2d
R+M	78.8±5.1dc	71.0±3.8c	71.3±5.7d	70.0±1.5c	191.7±6.0f	200.0±34.4c	105±8.2ef	122.0±1.8b
Control	58.9±1.8a		15.3±1.8a		70.0±6.9a		41.2±1.4a	

Key: R-rhizobia; M-mycorrhiza; F-fertilizer; IR-indigenous rhizobia; MBI-Menegasha biotechnology industry. Similar letters in columns show no significant difference between treatments at α 0.05.

Table 12b. Sole and co-inoculation of *Cicer* arietinum with commercial rhizobia (MBI) and AMF isolates in the field.

Treatment	(Mean±SEM) of Growth parameters					
	Total yield g/plot		100 seed weight (g)		Husk (g)	
	IR	MBI	IR	MBI	IR	MBI
R	66.0±1.4c	103.9±2.4a	12.6±0.2d	14.4±0.2b	10.2±0.9c	10.0±0.1b
M	60.0±0.6b		9.3±0.2b		9.6±0.1b	
F	70.0±0.6d		13.0±0.1e		10.9±0.2cd	
R+F	72±0.8de	158.8±1.5c	13.7±0.7ef	13.9±0.5a	11.5±0.3d	9.1±3.7a
M+F	60.0±2.3b		11.43±0.8c		10.1±0.2c	
R+M+F	6.3±2.0g	173.4±3.6d	14.2±0.2f	15.8±0.1c	12.06±0.2e	13.9±0.1cd
R+M	90.0±1.4f	105.0±1.9b	13.5±0.3ef	14.9±0.5bc	11.7±0.2d	13.3±0.4c
Control	39.6±1.8a		2.83±0.6a		3.6±0.3a	

Key: R-rhizobia; M-mycorrhiza; F-fertilizer; IR-indigenous rhizobia; MBI-Menegasha biotechnology industry. Similar letters in columns show no significant difference between treatments at α 0.05.

Discussion

Mycorrhiza-Rhizobia-plant interactions of legumes have a bigger implication to maintain sustainable agriculture. Most of the previous research literature focused on studying interactions between legumes and one of the symbionts, either AMF or rhizobial bacteria (Solomon and Fasil, 2014: Zelalem, 2018). However, an understanding of legume with only one of the symbionts at a time does not provide enough information about the dynamics of the nutrient exchanges process between symbiotic partners, as a legume in natural conditions forms symbiotic relations simultaneously with AMF and rhizobial bacteria forming tripartite interactions.

The main goal of this investigation was to study the effects of sole and dual inoculation of Arbuscular mycorrhizal fungi and rhizobia inoculants on growth, yield, and yield components of chickpea in the greenhouse and the field. For these activities indigenous AMF species and Rhizobia were isolated, authenticated and their symbiotic effectiveness was tested from southern Ethiopia.

Isolates of Mycorrhiza and Rhizobia

The mixture of *Gigaspora rosea* and *Rhizophugus clarus* morphospecies, used as an inoculum in this study were isolated from rhizospheric soils of agroforestry trees and crops and were tested for their effectiveness on *Sorghum bicolor* as a model trap plant. Beyene *et al.* (2016) have also tested and proved their effectiveness on leguminous trees and perennial crops in the greenhouse. The isolates have increased all growth parameters in sole and dual inoculation with fertilizer application in the greenhouse and the field. These morphospecies could be potential bio-inoculants in the production of pulses and other crops. The characteristics of the rhizobia isolates for chickpea presented in this study shows that all isolates were rhizobia. These findings are in line with the study done by Muthini *et al.* (2014) and Simon *et al.* (2014) who isolated the indigenous rhizobia from nodules of haricot bean and chickpea and the colony characteristics was similar to those found in this study. These findings gave hopeful indicators towards the production of inoculants for chickpea, since all of the isolated rhizobia showed the ability to induce nodulation on the roots of chickpea.

Symbiotic effectiveness of isolated rhizobia

The recorded results show that isolated rhizobia have a positive influence on shoot length (cm), stem girth (mm), and shoot dry mass (g), the number of nodules plant^{-1}, branches plant$^{-1,}$ and the number of leaves plant^{-1}. The isolated rhizobia CpIR10 showed better performance in symbiotic effectiveness exceeding or equaling with the (MBI) isolates in various growth parameters.

Based on the number of nodules, CpIR10 rhizobia are considered to be more effective in nitrogen fixation than or equal to the MBI isolates. These findings agree with Rodriguez-Navarro *et al.* (2000), Muthini *et al.* (2014), and Simon *et al.* (2014) whereby all of them found that some of the indigenous isolates had a higher ability to form effective nodules in the roots of haricot bean and chickpea than the commercial isolate. Also, this study demonstrated that nodule can be the best measure of the symbiotic effectiveness of rhizobia since all highly nodulated plants in this study showed higher shoot length and shoot dry mass as opposed to Sharma and Kumawat (2011) who demonstrated that nodule number is not an appropriate measure of effectiveness in rhizobia-legume symbiosis.

Effect of AMF and Rhizobia inoculation on chickpea growth parameters in the greenhouse

In the current greenhouse study, co-inoculation of a mixture of AMF morpho-species with indigenous and commercial rhizobia isolates showed a significant increase $p<0.05$ in all growth parameters except the number of nodules in the greenhouse trials when compared with single inoculation. These shows that inoculation with effective Rhizobia in combination with AMF had a better effect on plant growth enhancement and N_2 fixation. Various studies are describing many significant findings in the synergistic interaction between AMF and symbiotic N_2-fixing bacteria (Sylvia *et al.*, 1994; Barea, 2000; Azcon-Aguilar *et al.*, 2002; Kathiresan and Selvam, 2006).

The role of AMF as a phosphorus supplier to legume root nodules is of great relevance. The strain *Glomus intraaridices* was found to be more effective with Rhizobia spp. NR 4, whereas *Glomus coronatum* was effective when co-inoculated with Rhizobia spp. NR9 strain (Kathiresan and Selvam, 2006). Research has provided evidence that the genetic pathway of AM symbiosis is shared in part by other root-microbe symbioses such as N_2-fixing rhizobia (Spaink *et al.*, 1998). Such specific interactions between AMF, Rhizobia, and plant growth-promoting rhizobacteria (PGPR) have provided an insight into specific functional compatibility relationships between AMF and PGPR and their management when used as bio-fertilizers or bio-control agents.

Effect of AMF and Rhizobia inoculation on chickpea growth parameters in the field

In contrary to the controlled experimental studies in the greenhouse, this study was also done in the field exposing the inoculants to the natural factors and soil inherent characteristics (soil nutrients, fungal and bacterial densities, and other physical and chemical properties) to study the effectiveness of the bio-inoculants on chickpea growth and productivity under natural conditions. The results showed that despite the influence of natural environmental factors the inoculants promoted all tested growth parameters. In the greenhouse study, for sole AMF inoculation better performance was recorded and enhanced almost all growth parameters. However, in the field study, the opposite result was recorded for sole inoculation of rhizobia that showed better growth in shoot length, fresh weight, seed number, pod, total yield g/plot, and 100 seed when compared with sole AMF inoculation.

Most of the legumes possess two types of microbial symbionts namely mycorrhizal fungi and nitrogen-fixing bacteria thereby establishing the triple association, capable of supplying N and P contents to the plants (Silveira and Cardoso, 2004). Dual inoculation with both microorganisms results in a tripartite mutualistic symbiosis and generally increases plant growth to a greater extent than inoculation with only one (Chalk *et al.*, 2006). Inoculation alone or in combination with beneficial microorganisms including AMF, rhizobia, PGPR, and PSB (Phosphate Solubilizing Bacteria) have been observed to increase production in chickpea, nitrogen fixation, and nutrient uptake (Singh and Singh, 2010; Thenua *et al.*, 2010). Murat *et al.* (2011) reported that AMF inoculation, alone or in combination with rhizobial inoculation, increased yield, root colonization, and phosphorus content of the seed and shoot.

Biomass yield in the greenhouse and the field

Tripartite (AMF-R-legume) interactions can have a synergistic effect on plant biomass, particularly under low N conditions. In the greenhouse study, the result showed that plants in tripartite interactions (M+R+Cp) had significantly higher root and shoot biomass and N and P tissue concentrations than plants that were only colonized by AMF or rhizobia. Statistical data analysis regarding yield-related traits of chickpea indicated that considerable differences were recorded among different inoculated treatments. The co-inoculated Plants had recorded a substantially higher number of shoot lengths, number of pods, and shoot dry weight when compared with sole inoculation either with AMF and rhizobia in the greenhouse. This results in line with the work of (Alireza *et al.*, 2011)

Mycorrhizal root colonization and spore density in the greenhouse

Mycorrhizal root colonization and spore density was studied only for the greenhouse trials to study the capacity of the inoculants' mycotrophic association with the pulse. In this study, all chickpea treatments inoculated with sole AMF, AMF + fertilizer, AMF + rhizobia, and AMF + Rhizobia + fertilizer treatments were found colonized with AMF in the greenhouse. The rate of AM colonization is normally attributed to the tree and crop species and environmental factors. Smith *et al.* (1979) reported that the extent to which typical AMF colonizes root systems varies with plant species.

The extent of AM infection in root systems is also known to be influenced by environmental conditions; the most important being the age of the plants, the level of phosphate (P) in the soil relative to the

requirements of the plant, and the capacity of the population of AMF propagules in the soil to form AMF. In this study, the percentage colonization was ranged from 63.7% (M) to 85% in R+M in chickpea showing that dual inoculation is more favored root colonization in both pulses. The present study disagrees with the work of (Yinsuo *et al.,* 2004) which indicates that single inoculation of AMF showed a higher result than dual inoculation of R + M in *Vicia faba*. In the current study, the spore density ranged from 60/50g (M+F) dry soil to 560/50g (R+M+F) dry soil in chickpea.

Mycorrhizal dependency of chickpea

Though more than 80% of global plant species are dependent on the mycorrhizal association, the extent to which they depend on the association varies with specific plant species. In this study, chickpea showed similar dependency levels. The highest MD values (70%) were recorded for co-inoculation and fertilizer application treatment, followed by co-inoculation without fertilizer application (M+R) 69%. The least value 55% was recorded in sole mycorrhizal treatment. The present result in line with the work of (Arumugam *et al.,* 2010) their work showed that the co-inoculation of rhizobia and AMF increase mycorrhizal dependence than sole inoculation of AMF.

Nutrient uptake

Sole and tripartite interactions can have a synergistic effect on plant biomass, particularly under low N and P conditions. The present result found that plants in tripartite(M+R+CP) interactions had significantly higher root and shoot biomass and N and P contents than plants that were only colonized by rhizobia or AMF. Khan *et al.* (2008) also found that the dry weight of shoot and root were improved in dual inoculation while the nutrients uptake (NPK) was also higher due to dual inoculations which are in line with the present result. Bhattacharjee & Sharma (2012) also suggested that dual inoculation can increase the nutrients and chlorophyll content of pigeon pea. Synergistic responses in tripartite interactions have also been described by other authors especially under low P and N supply conditions (Larimer *et al.*, 2014; Bournaud *et al.*, 2018).

The dual inoculation with rhizobia and AMF can lead to higher photosynthetic rates and improve the harvest index (proportion of seed yields concerning the total plant biomass) of legumes (Kaschuk *et al.,* 2009). In this study, the positive impact of tripartite interactions on plant growth was mainly the result of a higher biological nitrogen fixation BNF activity of the nodules and the improved plant N nutrition. Higher BNF rates in tripartite interactions have mainly been attributed to an improved P supply by the colonization with AMF (Mortimer *et al.*, 2008; Püschel *et al.*, 2017). Root nodules act as very strong P sinks, and P deficiency can cause lower BNF rates of root nodules and inhibit nodule growth (Kossmann and Valentine, 2014). According to Mensah *et al.* (2015), the capability of some AMF to deliver N can even lead in legumes, such as Medicago sativa, to a strong growth response.

Conclusion

This study has clearly demonstrated that the mycorrhiza-rhizobia-plant interaction has significantly improved plant growth through phosphate and nitrogen uptake of the plants. The result suggests that the nutrient demands of the host and the fungal access to nutrients are important factors that control the nutrient (photosynthetic carbon) allocation to individual root symbionts in mycorrhiza-rhizobia-plant interactions. Mycorrhiza-rhizobia-plant interactions have a synergistic effect on the host plant growth response as AMF delivers phosphate from soil beyond root access and rhizobial bacteria provide nitrogen through the biological nitrogen fixation process to the host plant. The effects of indigenous AMF, indigenous and commercial inoculant of rhizobia on plant growth and seed yield of chickpea cultivars in greenhouse and field conditions shows that application of AMF and rhizobia inoculums increased all studied plant growth parameters including biomass and seed yield except the number of nodules in greenhouse and field conditions as compared with the control and sole fertilizer applications. Responses of AMF and rhizobia inoculant on plant growth and seed yield were notably higher in limited soil nutrient conditions.

Taken together, the application of indigenous rhizobia, commercial rhizobia, and AMF inoculant have positive effects on plant productivity and seed yield especially in a limited supply of nutrients which could be an alternative option against chemical fertilizers for chickpea production.

The results also indicated that AM colonization promotes plant growth by enhancing nutrient uptake. The absence of AM and rhizobia inoculants in controls and fertilizer treatments showed no root colonization and spore density. Lower dry biomass, height growth, root number, and length were also recorded for un-inoculated treatments. In conclusion, these results clearly showed that sole AMF and rhizobia and dual-inoculation with and without fertilizer have increased all studied yield and yield contributing characteristics in the greenhouse and the field condition as compared with the control.

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