SOIL & CROP SCIENCES | RESEARCH ARTICLE

Symbiotic effectiveness of cowpea (Vigna unguiculata (L.) Walp.) nodulating rhizobia isolated from soils of major cowpea producing areas in Ethiopia

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Abstract: Isolation and evaluation of symbiotic effectiveness of native rhizobia isolates are important to develop effective inoculant and achieve maximum legume productivity. This work was initiated to authenticate and evaluate the symbiotic effectiveness of cowpea nodulating rhizobia isolates isolated from major cowpea growing areas in Ethiopia. A total of 28 rhizobia isolates were isolated, purified, authenticated for infectiveness and assessed for their symbiotic effectiveness. The inoculation of native rhizobia isolates had significantly increased nodule number per plant, nodule dry weight per plant and shoot dry weight per plant compared with the uninoculated treatment. The relative symbiotic effectiveness of the isolates ranged from 45.81% to 115.03% whereas the absolute symbiotic efficiency ranged from 32.72 to 233.25%. Besides, symbiotic effectiveness

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PUBLIC INTEREST STATEMENT

Cowpea is among pulse crops which is widely cultivated and consumed in different parts of Ethiopia. Isolation of cowpea associated rhizobial isolates and their use for inoculation purposes is important to enhance soil productivity and achieve maximum legume productivity. This article described the authentication and symbiotic effectiveness of cowpea nodulating rhizobial isolates. It elucidated the effect of native rhizobia inoculation on the growth of cowpea and the use of native rhizobial isolates which gives paramount importance for enhancement of nitrogen fixation in cowpea production. This work further helps to screen out effective native rhizobia isolates and develop biofertilizers which are cheaper and more affordable to resource-poor farmers.
showed positive and highly significant correlation with nodule number, nodule dry weight and shoot dry weight per plant. The study on symbiotic effectiveness of the isolates revealed that 10 isolates (35.72%) were highly effective, 16 isolates (57.14%) were effective and only two isolates (7.14%) were poorly effective. From the study, it was observed that the existence of naturally occurring cowpea nodulating rhizobia varied from poorly to highly effective with a predominance of effective isolates in different agro-ecological zones of Ethiopia where cowpea is under production. Further studies are recommended under varied conditions on the competitiveness, persistence, and potential of the promising isolates.

Subjects: Agriculture and Food; Agronomy; Microbiology; Soil Science

Keywords: absolute symbiotic efficiency; authentication; cowpea; rhizobia; relative symbiotic efficiency

1. Introduction
Symbiotic nitrogen fixation presents an alternative farming system that is eco-friendly, resilient to climate change, enhance soil biodiversity, and soil structure management (Adhikari et al., 2013). Reliance on superior exotic rhizobia strains as inoculants have been considered as a common approach to improve the effectiveness of symbiotic nitrogen fixation and legume productivity (Zahid et al., 2015). Studies, however, revealed that inoculation of exotic rhizobia failed to achieve the desired response in different legumes and environments (Ahmad et al., 1981; Giller, 2001). The potential for improving nitrogen fixation, in this case, can be to use native isolates that are effective as well as competitive for nodulation. These native rhizobia are more persistent and effective, well adapted to local conditions and this gives them added advantage of competing successfully than the introduced strains for nodule occupancy (Fening & Danso, 2002).

Cowpea (Vigna unguiculata (L.) Walp.) is an important source of food, income and livestock feed and forms a major component of tropical farming systems because of its ability to improve soil fertility through nitrogen fixation and as a cover crop (Sanginga et al., 2003). Among the legume crops, cowpea cultivation is known to stimulate the proliferation of rhizobia in a field due to its ability to enhance native soil rhizobia populations (Mulongoy & Ayanaba, 1986). The symbiotic effectiveness of native cowpea rhizobial isolates was comparable to plants fertilized with inorganic nitrogen fertilizer and showed superior effectiveness which suggests that these native isolates are useful for the production of inoculants (Fening & Danso, 2002).

Furthermore, many developing countries do not have inoculant factories and, therefore, native rhizobia become an important resource in their natural state. As reported by Mwangi et al. (2011), the utilization of native rhizobia as inoculants promote ecologically sustainable management of agricultural ecosystems and enhance legume production due to their growth promoting traits and adaptability to soil and environmental stress. Furthermore, crop production using native rhizobia inoculants could be cheaper and more affordable to the resource-poor smallholder farmers. Therefore, screening of native rhizobial isolates for their nitrogen fixation effectiveness and efficiencies is important to develop effective legume inoculant as a biofertilizer and to achieve maximum legume productivity.

Isolation, authentication, and evaluation of native rhizobia also prosper the discovery of a new rhizobia strain which is more effective than the available commercial strains (Giller, 2001). Authentication and evaluation of native rhizobia to determine their symbiotic efficiency are, thus, required to screen out effective native rhizobia isolates. In Ethiopia, the work related to the nitrogen fixation potential of legumes is scarce and has concentrated on highland pulses such as peas, beans, chickpeas, and lentils (Akuma, 2010). Cowpea is often confounded with common
beans and, hence, the work on the evaluation of symbiotic effectiveness of rhizobia nodulating cowpea is very scarce and there is a need to isolate, authenticate and evaluate the symbiotic effectiveness of cowpea nodulating rhizobia. In line with this, the objective of this study was to authenticate and evaluate the symbiotic effectiveness of cowpea nodulating rhizobia isolated from major growing areas of Ethiopia.

2. Materials and methods

2.1. Soil sample collection

The soil sample for the study was collected from 10 kebeles of selected districts of Oromia region, Southern Nations, Nationalities and Peoples region, Gambella region and Dire Dawa (Table 1). The selection of kebeles was based on the accessibility and production status of cowpea in the area. From each selected kebele, soil samples were collected randomly from three farmers' fields from each kebele using a zigzag pattern from the soil surface to a depth of 0-20 cm while the cowpea crop is found in the farmer’s field during cropping season of 2017/2018. A total of 30 soil samples were collected from different sites and kept separately in plastic bag with its full information and transported to Haramaya University for nodule trapping experiment and rhizobial isolation.

2.2. Isolation, purification and identification of the isolates

Rhizobia isolates were directly isolated from the roots of cowpea grown on the collected soil samples by cowpea as a nodule trapping crop (Vincent, 1970). The soil from each sample was filled into 3 kg capacity plastic pots, which had been surface sterilized by swabbing with 95% alcohol. Seeds of cowpea Bole variety were surface sterilized briefly with sodium hypochlorite solutions for 3 minutes, rinsed several times with sterile water and air dried under controlled condition in the Seed Technology Laboratory of Haramaya University. Five seeds were planted in each pot and later thinned down to three after germination. The pots were arranged in a completely randomized design in the greenhouse and watered as required. During the late flowering and early pod setting stages, after 45 days of planting, the plants were carefully uprooted, washed with tap water to remove the adhering soils and pink color nodules were picked taken to Soil Microbiology laboratory, Haramaya University.

The nodules were surface sterilized in 1% sodium hypochlorite and rinsed in several changes of sterile water. The nodules, then, transferred into sterilized petridishes separately and crushed in the presence of 1 ml of sterile deionized water with a glass rod to obtain a milky suspension of bacteroides. A loop full of bacterial suspension was streaked across the surface of petridish containing yeast extract mannitol agar (YEMA) medium prepared from mannitol 10 g, K2HPO4 0.5 g, MgSO4.7H2O 0.2 g, NaCl 0.1 g, yeast extract 1 g, congo red 0.025 ml, agar 15 g, in 1000 ml distilled water, adjusted to pH 7.0 and incubated at 28 ± 2°C for 5–7 days (Somasegaran & Hoben, 1994).

Repeated sub-culturing was done until purity and uniformity were maintained. Single well isolated colonies were transferred and preserved on YEMA slants containing 0.3% (W/V) CaCO3 and stored at 4°C for further characterization (Vincent, 1970). The identification and confirmation of the isolates was checked using presumptive tests as described by Somasegaran and Hoben (1994). Accordingly, four confirmatory tests were performed viz. congo red dye absorption test, ketolactose test, growth on glucose peptone agar and gram staining to confirm isolates as Rhizobium.

2.3. Authentication of rhizobial isolates on sand culture

Each of the pure isolates was authenticated as root nodulating bacteria for infectivity and effectiveness by reinoculating the isolate on the host plant, cowpea, grown in a controlled environment using acid treated and sterilized river sand as described by Somasegaran and Hoben (1994). All the purified isolates were screened in 3 kg capacity pots containing sterilized and nitrogen-free sand under greenhouse condition. The plastic pots were surface sterilized with 95% ethanol whereas the
Table 1. Description of the area of soil samples collection and designation of cowpea rhizobial isolates

| Region      | Zone            | Woredas       | Kebeles     | Sampling sites | Isolate designation | Altitude (masl) | Latitude   | Longitude   |
|-------------|-----------------|---------------|-------------|----------------|----------------------|-----------------|------------|-------------|
| SNNPR       | South Omo       | South Ari     | Kayisa      | 1              | HUCR-1               | 1387            | 05° 41’ 26” | 36° 44’ 04” |
|             |                 |               |             | 2              | HUCR-2               | 1378            | 05° 31’ 71” | 36° 37’ 90” |
|             |                 |               |             | 3              | HUCR-3               | 1362            | 05° 31’ 93” | 36° 37’ 97” |
|             | South Ari       | Konso         | Naliya Segen| 1              | HUCR-4               | 1148            | 05° 14’ 31” | 37° 31’ 24” |
|             |                 |               |             | 2              | HUCR-5               | 1167            | 05° 14’ 46” | 37° 31’ 29” |
|             |                 |               |             | 3              | HUCR-6               | 1162            | 05° 14’ 27” | 37° 30’ 71” |
|             | Konso           | Naliya Segen  |             | 1              | HUCR-4               | 1148            | 06° 39’ 51” | 37° 50’ 07” |
|             |                 |               |             | 2              | HUCR-5               | 1167            | 06° 39’ 09” | 37° 49’ 55” |
|             |                 |               |             | 3              | HUCR-6               | 1162            | 06° 39’ 13” | 37° 48’ 52” |
|             | Wolaita         | Humbo         | Abala Faracho| 1              | HUCR-7               | 1390            | 08° 14’ 43” | 34° 29’ 49” |
|             |                 |               |             | 2              | HUCR-8               | 1418            | 08° 14’ 42” | 34° 29’ 44” |
|             |                 |               |             | 3              | HUCR-9               | 1383            | 08° 14’ 40” | 34° 29’ 47” |
|             | Gambella        | Aboli         | Pinkew      | 1              | HUCR-10              | 436             | 07° 53’ 17” | 34° 34’ 32” |
|             |                 |               |             | 2              | HUCR-11              | 415             | 07° 53’ 23” | 34° 34’ 32” |
|             |                 |               |             | 3              | HUCR-12              | 437             | 07° 52’ 99” | 34° 34’ 11” |

(Continued)
| Region            | Zone                | Woredas         | Kebeles | Sampling sites | Isolate designation | Altitude (masl) | Latitude   | Longitude   |
|-------------------|---------------------|-----------------|---------|----------------|---------------------|----------------|------------|-------------|
| Oromia            | East Hararge        | Gursum          | Ilalam  | 1              | HUCR-16             | 1906           | 09° 19’ 98” | 42° 25’ 41” |
|                   |                     |                 |         |                |                     |                |            |             |
|                   |                     |                 |         | 2              | HUCR-17             | 1912           | 09° 19’ 91” | 42° 25’ 36” |
|                   |                     |                 |         | 3              | HUCR-18             | 1904           | 09° 19’ 34” | 42° 25’ 34” |
|                   |                     | Babile          | Ifa     | 1              | HUCR-19             | 1730           | 09° 14’ 88” | 42° 18’ 53” |
|                   |                     |                 |         |                |                     |                |            |             |
|                   |                     |                 |         | 2              | HUCR-20             | 1633           | 09° 14’ 04” | 42° 18’ 96” |
|                   |                     |                 |         | 3              | HUCR-21             | 1642           | 09° 14’ 06” | 42° 19’ 01” |
| West Hararge      | Oda Bultum          | Bakanisa        |         | 1              | HUCR-22             | 1671           | 08° 54’ 49” | 40° 43’ 06” |
|                   |                     |                 |         | 2              | HUCR-23             | 1709           | 08° 53’ 67” | 40° 43’ 27” |
|                   |                     |                 |         | 3              | HUCR-24             | 1738           | 08° 53’ 69” | 40° 43’ 29” |
|                   |                     | Oda Kanani      |         | 1              | HUCR-25             | 1435           | 09° 10’ 91” | 40° 39’ 60” |
|                   |                     |                 |         | 2              | HUCR-26             | 1474           | 09° 10’ 29” | 40° 40’ 50” |
|                   |                     |                 |         | 3              | HUCR-27             | 1464           | 09° 10’ 51” | 40° 40’ 85” |
| Dire Dawa         | Biya Awale          | Belewa          |         | 1              | HUCR-28             | 1693           | 09° 31’ 88” | 42° 01’ 78” |
|                   |                     |                 |         | 2              | HUCR-29             | 1704           | 09° 31’ 65” | 42° 01’ 17” |
|                   |                     |                 |         | 3              | HUCR-30             | 1704           | 09° 31’ 63” | 42° 01’ 16” |

*No nodulation obtained; HUCR: Haramaya University Cowpea Rhizobia.
river sand was treated with concentrated sulphuric acid (H$_2$SO$_4$) and sterilized in an autoclave as indicated by Somasegaran and Hoben (1994).

Five surfaces sterilized, undamaged and uniform sized cowpea seeds (Bole variety) were planted into the pots. As all pots received 1% KNO$_3$ at a rate of 5 ml/pot applied at planting as starter nitrogen because the growth medium often lacks sufficient nitrogen to sustain the legume after seed reserves are exhausted, and before nitrogen fixation begins (Howieson & Dilworth, 2016). Cowpea rhizobial isolates were grown in 10 ml yeast extract mannitol broth (YEMB) on rotary flask shaker at 150 rev/min for 72hrs at room temperature. One week after planting, the seedlings were thinned down to three seedlings per each pot and each seedling was inoculated with 1 ml broth culture containing the isolate (about 10$^8$ cells) which is sufficient for nodulation as described by Howieson and Dilworth (2016). An un-inoculated control treatment consisting nitrogen treated control (with chemical fertilizer, 100 ml of 0.05% KNO$_3$(w/v) solution once a week for 4-weeks and without inoculation) and the control check (no chemical fertilizer and uninoculated) were included. The experiment was laid down in a completely randomized design with three replications in Haramaya University greenhouse.

All pots were fertilized once a week with the full strength of Broughto and Dilworth (1970) N-free medium for four consecutive weeks at a rate of 100 ml/pot as described by Somasegaran and Hoben (1994). The nutrient consisted of 5 stock solutions containing in g/L of 0.1 CaCl$_2$, 0.12 MgSO$_4$7H$_2$O, 0.1 KH$_2$PO$_4$, 0.15 Na$_2$HPO$_4$2H$_2$O, 0.005 ferric citrate, and 1.0 mL of trace elements stock solution. The trace elements stock solution contained 2.86 H$_3$BO$_3$, 2.03 MnSO$_4$7H$_2$O, 0.22 ZnSO$_4$7H$_2$O, 0.08 CuSO$_4$5H$_2$O, and 0.14 NaMoO$_2$2H$_2$O in g/L. The pH of the solution was adjusted to 6.8 using NaOH (1.0 M) or HCL (1.0 M). All solutions were sterilized by autoclaving at 121°C for 15 minutes. Regular checking of levels of the moisture content of the sand was carried out and water was applied to ensure that the seedlings were adequately moistened. After 45 days of planting, the plants were carefully uprooted, root and shoot fractions were separated. Nodule number were counted, nodule dry weight and shoot dry weight were recorded after drying the nodules and shoot at 70 ºC for 48 hours (Kawaka et al., 2014).

### 2.4. Determination of symbiotic effectiveness indices of rhizobia isolates

**Relative symbiotic effectiveness percentage (RSE %)** of the isolates for atmospheric nitrogen fixation was calculated using the methods of Purcino et al. (2000) by comparing the inoculated plant with the N-fertilized positive control by using the following formula:

\[
\% \text{RSE} = \frac{\text{Inoculated shoot dry matter}}{\text{N fertilized shoot dry matter}} \times 100
\]

Nitrogen fixing efficiency being classified as: highly effective (SE %> 80%), effective (SE % = 50-80%), poorly effective (SE % = 35-50%) and ineffective (SE % <35%) (Purcino et al., 2000).

**Absolute symbiotic effectiveness percentage (ASE %)** was calculated using the method of Dos Santos et al. (2011) by comparing the inoculated plant with the uninoculated and unfertilized negative control by using the following formula:

\[
\% \text{ASE} = \frac{\text{Inoculated shoot dry matter} - \text{Shoot dry matter without N}}{\text{Shoot dry matter without N}} \times 100
\]

### 2.5. Data analysis

Data collected (nodule numbers, nodule dry weight and shoot dry weight) were statistically analyzed by subjecting to the analysis of variance (ANOVA) by using the GLM procedure of SAS software Version 9.2. Mean separation was done using the least significant difference (LSD) and Pearson correlation analysis was carried out to study the nature and degree of relationship between selected parameters.
3. Results and discussions

3.1. Identification of rhizobial isolates

Thirty soil samples were used for nodule trapping experiments in the greenhouse of Haramaya University, Ethiopia. Two of the soil samples failed to nodulate and twenty-eight cowpea nodulating rhizobia were isolated and identified under laboratory. All twenty-eight isolates were presumptively identified as root nodule bacteria (Kucuk et al., 2006; Lupwayi & Haque, 1994; Somasegaran & Hoben, 1994). Gram staining test further showed that the isolates were gram-negative, rod-shaped and non-spore forming. All isolates did not grow on the peptone glucose agar medium. Besides, all the isolates were found to be negative for the production of 3-ketolactose from lactose on the ketolactose medium. Depending on the colony color on YEMA containing bromothymol blue, 22 (78.57%) isolates were slow growers whereas 6 (21.43%) isolates were fast growers. The morphological study of the isolates on media has also confirmed that the result is standard culture and morphological characteristics of *Rhizobium* species as described by Somasegaran and Hoben (1994), Howieson and Dilworth (2016), and Legesse (2016).

3.2. Authentication of rhizobial isolates on sand culture

Whenever a rhizobial isolates achieves nodulation with a legume, the association may have one of several possible outcomes for nitrogen fixation, varying from no nitrogen fixation to maximum nitrogen fixation (Terpolilli et al., 2008). Determination of infectivity, nodulation and symbiotic effectiveness of native rhizobial population is, thus, an important parameter for the selection of isolates for inoculant production. In this study, the rhizobial isolates were tested in a pot experiment using sterilized sand culture to assess their infectivity and effectiveness on cowpea from which they were trapped previously under greenhouse condition. Accordingly, all the tested isolates formed nodules on cowpea upon reinoculation indicating all isolates in this study were true rhizobia infecting their host (Table 2). The results confirmed that all cowpea rhizobial isolates considered in this study were authenticated to be cowpea nodulating rhizobia that infected their host as described by Subba Rao (1988) and Giller (2001). Based on the host plant specificity for infection and nodulation, these rhizobial species could also generally assumed to be cowpea-miscellany *Rhizobium* (Van Berkum et al., 1995). Similar to this finding, Akuma (2010) tested isolates of groundnut from Eastern Ethiopia and reported that all isolates formed nodules and obtained a 100% infection of *Bradyrhizobium* isolates upon reinoculation on groundnut.

3.3. Effect of native rhizobial inoculation on nodule number and nodule dry weight

The study showed that nodule number and nodule dry weight per plant varied significantly in response to native rhizobial inoculation (P < 0.0001) (Table 2). The parameters also displayed significant variability among rhizobial inoculated plants. Nodule dry weights increased in line with the nodule number and could be indicative of the development of nodules. The nodule number recorded ranged from 16/plant to 102/plant. The highest number of nodules was recorded from cowpea plant inoculated with isolate HUCR-3 whereas the lowest number of nodules was recorded from cowpea plant inoculated with isolate HUCR-1 (Figure 1). The enhancing effects of inoculation on nodule number per plant were also supported by the finding of Manish and Kumawat (2011) who reported that an increased number of root nodules and nodulation by inoculating soybean varieties with native *Bradyrhizobium* isolates compared with uninoculated treatments.

The nodule dry weight recorded ranged from 0.15 gm/plant to 2.50 gm/plant (Table 2). The highest and lowest nodule dry weight was recorded from cowpea plant inoculated with rhizobia isolate HUCR-3 and HUCR-1, respectively (Figure 2). The difference between the nodule dry weight per plant obtained from inoculated plants may be attributed to the size and number of the nodules. Isolates having better infective capacity and effectiveness formed a greater number of nodules than those having the least effectiveness since effective rhizobial isolates are competitive and able to initiate nodulation with cowpea roots which agrees with the report of Chiamaka (2014). A similar promoting effect of inoculation on the dry weight of nodules per plant has also been reported by Nyoki and N.d.akidemi (2014).
Table 2. The effect of inoculation of native rhizobia on nodule number, nodule dry weight and shoot dry weight per plant of cowpea using sand culture

| Isolates    | NN ±SE     | NDW±SE(g)  | SDW±SE(g)  | RSE (%) | SE rating | ASE (%) |
|-------------|------------|------------|------------|---------|-----------|---------|
| HUCR-1      | 16.00 ± 1.53 | 0.15 ± 0.02 | 5.34 ± 0.23 | 48.63   | PE        | 40.90   |
| HUCR-2      | 54.00 ± 4.73 | 1.08 ± 0.15 | 8.77 ± 0.52 | 64.75   | E         | 131.40  |
| HUCR-3      | 102.00 ± 4.58 | 2.50 ± 0.19 | 12.61 ± 0.81 | 115.03  | HE        | 233.25  |
| HUCR-4      | 31.00 ± 5.13 | 0.70 ± 0.15 | 6.42 ± 0.49 | 58.47   | E         | 69.39   |
| HUCR-5      | 87.67 ± 6.39 | 1.82 ± 0.14 | 11.23 ± 0.66 | 102.28  | HE        | 196.31  |
| HUCR-6      | 27.00 ± 3.06 | 0.98 ± 0.14 | 7.14 ± 0.38 | 65.03   | E         | 88.39   |
| HUCR-7      | 48.00 ± 1.73 | 1.13 ± 0.02 | 9.66 ± 0.68 | 87.98   | HE        | 154.88  |
| HUCR-8      | 28.67 ± 6.69 | 0.80 ± 0.22 | 7.11 ± 0.55 | 70.22   | E         | 103.43  |
| HUCR-9      | 25.00 ± 1.53 | 0.69 ± 0.13 | 6.62 ± 0.50 | 60.29   | E         | 74.67   |
| HUCR-10     | 75.33 ± 4.81 | 1.55 ± 0.18 | 8.95 ± 0.96 | 81.51   | HE        | 136.15  |
| HUCR-11     | 73.33 ± 7.23 | 1.42 ± 0.11 | 10.87 ± 0.78 | 99.00   | HE        | 186.81  |
| HUCR-12     | 33.00 ± 2.08 | 0.84 ± 0.10 | 8.24 ± 0.79 | 75.05   | E         | 117.41  |
| HUCR-13     | 44.67 ± 5.36 | 0.85 ± 0.12 | 7.44 ± 0.96 | 67.76   | E         | 96.31   |
| HUCR-14     | 22.33 ± 3.18 | 0.45 ± 0.08 | 5.69 ± 0.43 | 51.82   | E         | 50.13   |
| HUCR-15     | 97.33 ± 6.39 | 1.82 ± 0.23 | 10.47 ± 0.72 | 95.36   | HE        | 176.25  |
| HUCR-16     | 66.67 ± 6.74 | 1.06 ± 0.14 | 7.84 ± 0.73 | 71.40   | E         | 106.86  |
| HUCR-17     | 32.33 ± 5.21 | 0.63 ± 0.10 | 7.08 ± 0.57 | 64.48   | E         | 86.81   |
| HUCR-18     | 53.33 ± 3.84 | 1.03 ± 0.28 | 8.79 ± 0.47 | 80.05   | HE        | 131.93  |
| HUCR-19     | 37.00 ± 3.79 | 0.65 ± 0.09 | 5.78 ± 0.75 | 52.64   | E         | 52.51   |
| HUCR-20     | 54.67 ± 3.48 | 1.09 ± 0.14 | 8.81 ± 0.77 | 80.24   | HE        | 132.45  |
| HUCR-21     | 65.67 ± 6.74 | 1.31 ± 0.15 | 6.47 ± 0.46 | 58.93   | E         | 70.71   |
| HUCR-22     | 51.33 ± 4.33 | 0.96 ± 0.05 | 6.69 ± 0.41 | 60.93   | E         | 76.52   |
| HUCR-23     | 47.67 ± 4.26 | 0.84 ± 0.12 | 6.91 ± 0.60 | 62.93   | E         | 82.32   |
| HUCR-24     | 81.33 ± 10.27 | 1.87 ± 0.13 | 11.58 ± 0.53 | 105.46  | HE        | 205.54  |
| HUCR-25     | 40.33 ± 5.61 | 0.80 ± 0.11 | 6.07 ± 0.29 | 55.28   | E         | 60.16   |

Continued...
| Isolates    | NN ±SE  | NDW±SE(g) | SDW±SE(g) | RSE (%) | SE rating | ASE (%) |
|------------|---------|-----------|-----------|---------|-----------|---------|
| HUCR-27    | 36.00 ± 4.62 f | 0.74 ± 0.14 h | 5.68 ± 0.56 m | 51.73   | E         | 49.87   |
| HUCR-28    | 61.67 ± 6.98 g | 1.34 ± 0.22 e | 9.45 ± 0.55 o | 86.07   | HE        | 149.34  |
| HUCR-30    | 21.00 ± 5.57 p | 0.45 ± 0.07 k | 5.03 ± 0.29 q | 45.81   | PE        | 32.72   |
| +ve control| 0.00 ± 0.00     | 0.00 ± 0.00  | 10.98 ± 0.72 d |         |           |         |
| -ve control| 0.00 ± 0.00     | 0.00 ± 0.00  | 3.79 ± 0.36   |         |           |         |
| Mean       | 47.14 ± 2.83    | 0.99 ± 0.06  | 7.94 ± 0.25   |         |           |         |
| CV (%)     | 18.56            | 13.37       |            |         |           |         |
| LSD        | 14.28            | 3.79        |            |         |           |         |
| P-V        | 0.0001           | 0.0001      |            |         |           |         |

Means within a column of the same factor followed by the same letter(s) are not significant at p < 0.05. CV: Coefficient of variation, E = Effective, HE = Highly effective, LSD: Least significant difference, NDW: Nodule dry weight (g), NN: Nodule number, PE = Poorly effective, SDW: Shoot dry weight, SE: Standard error, ASE (%): Absolute symbiotic effectiveness percentage, RSE (%): Relative symbiotic effectiveness percentage SE (%): Symbiotic effectiveness percentage.
There were no nodules formed on the root of cowpea treated with nitrogen (+ve control) and negative control indicating the absence of contamination. Due to the absence of rhizobia in the sand used for cowpea growth in both positive and negative control treatments, plants were having no nodules which in turn leads to the accumulation of less biomass (Fatima et al., 2007; Van Noorden et al., 2016).

3.4. Effect of native rhizobial inoculation on shoot dry weight
Native rhizobial inoculation significantly affected the shoot dry weight of cowpea and shoot dry weight displayed significant variability among rhizobial inoculated plants. The highest mean shoot dry weight observed was 12.63 gm/plant which was recorded from isolate HUCR-3 and showed pronounced improvement in shoot dry weight i.e., 233.25 and 15.03% over negative and N-treated plants, respectively (Figure 3). This improvement of shoot dry weight could be attributed to the fact that rhizobia increase plant growth and improved the plant biomass by providing products of nitrogen fixation. These could also be due to the fact that isolates of rhizobia have produced plant growth promoting hormones (Gulati et al., 2007). These results imply that plants that were able to
form effective nodules accumulated higher shoot biomass compared to negative control that did not nodulate. The mean shoots dry weight of nitrogen treated plants is higher than some plants inoculated with native rhizobia. This may be due to nitrogen enhanced plant growth and biomass production.

Higher shoot dry weight in plants inoculated with rhizobial isolates might be ascribed to more nitrogen supply to the crop through nitrogen fixation provided by the inoculation of native rhizobial isolates. Kyei-Boahen et al. (2017) reported that the efficiency of rhizobial isolates in fixing nitrogen is demonstrated in the production of higher shoot dry matter at flowering. Ampomah et al. (2008) reported a significant effect (P < 0.05) of rhizobial inoculation on shoot dry weight produced on cowpea. Kawaka et al. (2014) also reported that inoculated common beans had higher shoot dry weight compared to the control indicating that inoculation with native isolates improved the growth of plants and are, therefore, efficient in nitrogen fixation.

Most of the native isolates showed superior nodulation indicating that these native rhizobia isolates compete better for nodulation. Particularly, three isolates, HUCR-3 from Kayisa, HUCR-5 from Naliya Segen, and HUCR-25 from Oda Kanani, showed the highest nodule number, nodule dry weight and shoot dry weight than other isolates tested (Table 2). This could be due to the fact that the study areas are the major cowpea growing region of the country thereby harboring symbiotically effective isolates of rhizobia. Moreover, isolate HUCR-3 scored the highest nodule number, nodule dry weight and shoot dry weight per plant than all other tested isolates showing its superiority in nodulation and dry matter accumulation. This result supports the finding reported by Martins et al. (1997) who found that some of the groups of cowpea isolates showed higher nitrogen fixation effectiveness in Brazil. Onyango et al. (2015) also reported that native isolates showed better competence for nodule occupancy in Bambara groundnuts under controlled greenhouse conditions.

3.5. Correlation of selected parameters on sand culture
Symbiotic effectiveness showed a positive correlation with all measured parameters. Symbiotic effectiveness was positively correlated with nodule number, nodule dry weight and shoot dry weight (Table 3). Shoot dry weight was also positively correlated with nodule number and nodule dry weight indicating isolates with higher fitness and nodulation capacity provide a better benefit to their host plants as described by He et al. (2011). The same finding was reported by Fening and
Danso (2002) who reported that shoot dry weight of cowpea was positively correlated with nodule number and nodule dry weight, and symbiotic effectiveness positively correlated with nodule number, nodule dry weight and shoot dry weight. This implies that plants inoculated with rhizobia fixed atmospheric nitrogen in higher proportion than uninoculated plants, thus, their tissue N contents are increased which in turn improves biomass accumulation in the plant. Nodulation was also positively correlated with symbiotic effectiveness as was reported by Denton et al. (2000).

Although high nodulation was significantly correlated to symbiotic effectiveness, not all isolates that formed more nodule numbers and nodule dry weight showed the highest effectiveness. In this study, 13 (46.43%) of the isolates formed more than 50 nodules per plant while only 9 (32.14%) of the isolates that recorded more than 50 nodules had a highly effective association (Table 2). Likewise, one isolate (HUCR-7) formed as low as 48 nodules but had 87.98% symbiotic effectiveness percentage which is rated as highly effective. Similarly, Fening and Danso (2002) reported a significant correlation between high nodulation of cowpea and symbiotic effectiveness although not all isolates that formed a high number of nodules exhibited high effectiveness.

Despite such positive and highly significant correlation between the nodulation and symbiotic effectiveness expressed by shoot dry weight, previous results have found that nodule number and nodule dry weight are not an appropriate trait for selection of the most effective nitrogen fixing Rhizobium-legume association (Deli et al., 1997; Hefny et al., 2001). This could be attributed to the fact that nodule number and nodule dry weight includes nonfunctional nodules and may not as valid an indicator of nitrogen fixation as shoot dry weight. This suggests that apart from nodule number and nodule dry weight, other nodule factors such as nodule efficiency may be more important in estimating the amount of nitrogen fixed and play a crucial role in influencing the amount of total accumulated dry matter. However, these positive and highly significant associations between all parameters confirm the dependence of cowpea nodule numbers, nodule and shoot dry weight on nodulation of the crop. Ampomah et al. (2008) reported that strong positive correlation ($r = 0.961$, $p < 0.001$) between shoot dry weight and nodule dry weight was observed which indicated that the isolates that produced high shoot dry weights were more effective and fixed more nitrogen on the legume host. This may imply that nitrogen fixation is a function of photosynthate availability, translocation and/or interactions between fixed and soil nitrogen.

3.6. Symbiotic effectiveness of cowpea rhizobia isolates

The efficient exploitation of biological nitrogen fixation to improve agricultural productivity requires that the symbiotic effectiveness of native rhizobia is adequately characterized. Osei et al. (2018) indicated that the ability to form nodules (infectivity) along with the subsequent capacity of fixing nitrogen (symbiotic effectiveness) are widely used as means of evaluating the inherent links between rhizobia and respective hosts. The symbioses between legumes and rhizobia, thus, must be effective for enhanced BNF and subsequent yield improvement to be realized.

**Table 3. Correlation coefficients among nodule number, nodule dry weight, shoot dry weight and symbiotic effectiveness**

|       | NN   | NDW(g) | SDW(g) | SE (%) |
|-------|------|--------|--------|--------|
| NN    | 1.00000 |       |        |        |
| NDW(g) | 0.94120** | 1.00000 |        |        |
| SDW(g) | 0.67869** | 0.73056** | 1.00000 |        |
| SE (%) | 0.73982** | 0.75070** | 0.93378** | 1.00000 |

** highly significant at p < 0.0001, NN: Nodule number, NDW: Nodule dry weight (g), SDW: Shoot dry weight, SE (%): Symbiotic effectiveness.
The shoot dry weight of plants harvested after significant plant biomass accumulation is an accepted criterion for nitrogen fixing effectiveness in systems that are free of mineral nitrogen (Howieson & Dilworth, 2016). In this study, the high correlation of shoot dry weight with all of the tested parameters also confirmed its reliability as an indicator of efficiency in N fixation. Estimated values for relative symbiotic effectiveness ranged from 45.81% (isolate HUCR-30) to 115.03% (isolate HUCR-3) for all 28 cowpea rhizobial isolates. Similarly, the absolute symbiotic efficiency of the isolates ranged from 32.72% (isolate HUCR-30) to 233.25% (isolate HUCR-3) (Figure 4). Based on the relative shoot dry matter accumulation of inoculated plants with nitrogen-fertilized control, 10 isolates (35.72%) were highly effective, 16 isolates (57.14%) were effective and only two isolates (7.14%) were poorly effective based on Purcino et al. (2000) classification (Table 2). The occurrence of rhizobial isolates that are highly effective in nitrogen fixation indicates the potential benefits of native isolates from natural environments.

Based on the effectiveness percentage, the isolates varied from poorly effective to highly effective with a predominance of isolates being ranked as effective (57.14%). This suggests that the presence of most effective cowpea nodulating rhizobia in Ethiopian soils with the possibility of selecting potential isolates that can nodulate the host abundantly and effectively, thus, used as a biofertilizer. Similarly, Temesgen (2017) reported that selected isolates from Ethiopian soils were either effective or highly effective, indicating native rhizobia were capable of establishing highly effective symbiosis. Previous studies have also revealed that rhizobial isolates that nodulate cowpea in Africa have generally been described as promiscuous with varying degrees of effectiveness on cowpea and other compatible hosts (Fening & Danso, 2002; Singleton et al., 1992). A study on cowpea *Bradyrhizobium* isolates in soils across the different ecological zones of Ghana indicated that a minority (26%) of the isolates are effective in fixing nitrogen with cowpea, a majority (68%) moderately effective and the remaining (6%) ineffective (Fening & Danso, 2002). Furthermore, the absence of ineffective rhizobia that nodulate cowpea without fixing nitrogen in this study indicates that cowpea in the various soils used in the study is nodulated primarily by rhizobia that exhibited optimal symbiotic effectiveness.

The first criterion for a rhizobial strain used as inoculant or biofertilizer is it must be superior and highly effective in nitrogen fixing ability forming a symbiotic association with the host legume (O’Hara et al., 2002). This is required to achieve high nitrogen fixation together with the presence of adequate numbers of highly effective native rhizobia in the soil. In this study, five isolates identified with cowpea showed high effectiveness ranging from 95.36% to 115.03% as compared to the nitrogen treated plants. These isolates were HUCR-3, HUCR-25, HUCR-5, HUCR-11, and HUCR-15 having symbiotic
effectiveness of 115.03%, 105.46%, 102.28%, 99.00%, and 95.36%, respectively (Table 2). Therefore, these native isolates maybe a useful source of isolates to resolve practical problems in the field inoculation of cowpea production.

4. Conclusions

The symbiotic effectiveness study of rhizobia isolates is important for efficient exploitation of biological nitrogen fixation and to improve agricultural productivity. In this study, authentication of cowpea rhizobia isolates confirmed that all tested isolates were true rhizobia that infect their host and formed nodules on cowpea upon reinoculation. Native rhizobia inoculation significantly affected nodule number, nodule dry weight and shoot dry weight of cowpea, and all parameters displayed significant variability among rhizobial inoculated plants. Symbiotic effectiveness showed positive a correlation with nodule number, nodule dry weight and shoot dry weight. The symbiotic effectiveness study also revealed the existence of naturally occurring effective cowpea rhizobia in different agro-ecological zones of Ethiopia where cowpea is under production. The results of this study indicated that native rhizobial isolates give paramount importance for the enhancement of nitrogen fixation in cowpea production. However, the isolates that showed different symbiotic characteristics and proven to be highly effective in nodulating cowpea should be used to determine their persistence and potential for achieving inoculation success under varied conditions.

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Competing interest
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