Eco-physiological and physiological characterization of cowpea nodulating native rhizobia isolated from major production areas of Ethiopia

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Abstract: Eco-physiological and physiological characteristics are used to study native rhizobial species for their ecological and physiological adaptability to different conditions. This study aimed to assess the eco-physiological and physiological characteristics of cowpea-nodulating rhizobia isolated from major production areas of Ethiopia. Twenty-eight isolates were isolated from cowpea root nodules and evaluated for different eco-physiological and physiological characteristics. All tested isolates grew within a temperature range of 20°C and 35°C. All isolates were able to grow at pH values of between 6.0 and 8.5, with optimal growth at pH around neutral. All isolates were tolerant of NaCl concentration ranging from 0.1% to 1%. The growth of isolates decreased as the salt concentration increased. The tested isolates were able to grow on different carbon sources, such as glucose (100%).

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PUBLIC INTEREST STATEMENT
Cowpea is an important food legume that is widely cultivated and consumed in different parts of Ethiopia. Cowpea can fix atmospheric nitrogen through a symbiotic association with soil bacteria, rhizobia. Isolation of cowpea nodulating native rhizobia and their characterization using different methods is important to identify novel groups of rhizobia, enhance soil productivity and achieve maximum productivity. This article described the characterization of cowpea-nodulating rhizobia using eco-physiological and physiological characteristics. These methods mainly help to authenticate the environmental and physiological adaptation of the rhizobial isolates, ensuring the selection of superior inoculants. The study revealed that there were a variation and diversity of tolerance to different ecological conditions and utilization of different nutrients which would provide an ecological advantage to the rhizobial isolates and enhance their chance for survival.
fructose (100%), glycerol (100%), mannose (96.43%), lactose (96.43%), galactose (85.71%), arabinose (60.71%) and maltose (46.43%) showing that the majority of tested rhizobia were able to use a broad range of carbohydrates as sole carbon sources. Similarly, the isolates were able to grow on L-lysine (89.29%), L-arginine (92.86%), tyrosine (82.14%), L-tryptophan (89.29%), L-asparagine (78.57%), methionine (75%) and glutamate (85.71%) as a source of amino acids. The tested isolates showed a wide diversity for their tolerance to different eco-physiological conditions (temperature, pH and salt concentration) and had the ability to utilize a large variety of carbon and nitrogen sources. The variation and diversity of tolerance to different ecological conditions and utilization of different nutrients would provide them an ecological advantage and enhance their chance for survival.

Subjects: Agriculture & Environmental Sciences; Soil Sciences; Microbiology

Keywords: Inoculant; isolate; nutrient utilization; salt tolerance; temperature tolerance

1. Introduction
Cowpea (Vigna unguiculata (L.) Walp) is an important food legume and an essential component of cropping systems in the sub-humid tropics and dry regions across the world (Singh et al., 2002). Cowpea has the ability to fix atmospheric nitrogen through a symbiotic association with soil bacteria, commonly known as rhizobia. This symbiosis results in nitrogen replacement, as many experimental studies have demonstrated increased soil nitrogen levels following cowpea cultivation (Thies et al., 1995). Cowpea can fix about 240 kg ha⁻¹ of atmospheric nitrogen and make about 60-70 kg ha⁻¹ of nitrogen accessible for the subsequent crop ([CRI] Crops Research Institute, 2006).

Rhizobia, legume root nodule bacteria that comprise Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium and Mesorhizobium, are specific in forming an association with a particular legume and, thus, need to be studied and characterized by eco-physiological and physiological approaches. Presently, the classification of rhizobial taxonomy uses a joint assessment of morphological, physiological and different genetic tools to offer quantitative measures of differences and similarities among them. Chen et al. (2005) reported that the study of rhizobia uses molecular techniques in investigating novel groups of rhizobia capable of nodulation and nitrogen fixation in different legume crops. However, Chagas Junior et al. (2013) stated that the eco-physiological and physiological characterization of rhizobia has also been used to study and identify novel groups of rhizobia.

The eco-physiological and physiological characteristics of rhizobia are studied with the intention not only of classifying but also of authenticating their likely environmental and physiological adaptation to different conditions predominant in the environment, ensuring the selection of superior inoculants. These methods have the benefit of being fast, offering an explorative evaluation of diversity and allowing significant information for further study and classification. Deaker et al. (2004) stated that an effective understanding of the eco-physiological endurance of rhizobial species in response to numerous ecological stresses, which offer them adaptation and survival, will help in selecting elite strains capable of biofertilizer production.

Many developing countries including Ethiopia do not have inoculant factories and, therefore, exploitation of native rhizobia become an important resource in their natural state. The utilization of this 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. In Ethiopia, there is inadequate information on the characterization and utilization of native rhizobia nodulating cowpea. Despite dependence on native rhizobia,
little is known about native soil rhizobia that associate with cowpea. Therefore, there is a need for studying the eco-physiological and physiological characteristics of cowpea-nodulating rhizobia, followed by a selection of elite strains that can be used as inoculant and for biofertilizer production. Hence, the aim of this study was to assess the eco-physiological and physiological characteristics of cowpea-nodulating rhizobia isolated from selected major production areas of Ethiopia.

2. Materials and methods

2.1. Description of the soil collection area and soil sampling

The soil samples for the study were collected from 10 selected districts comprising the Oromia region (five woredas including Dire Dawa), Southern Nations, Nationalities and Peoples (SNNP) (three woredas) and Gambella region (two woredas) (Table 1). The sampling sites were selected in consultation with agricultural extension officers knowing farmers who grew cowpea and were

| Region      | Zone       | Woredas    | Kebeles   | Sampling sites (farms) | Isolate designation |
|-------------|------------|------------|-----------|------------------------|---------------------|
| SNNPR       | South Omo  | South Ari  | Kayisa    | 1                      | HUCR-1              |
|             |            |            |           | 2                      | HUCR-2              |
|             |            |            |           | 3                      | HUCR-3              |
|             | Segen people| Konso     | Naliya Segen | 1                      | HUCR-4              |
|             |            |            |           | 2                      | HUCR-5              |
|             |            |            |           | 3                      | HUCR-6              |
|             | Wolaita    | Humbo      | Abala Faracha | 1                      | HUCR-7              |
|             |            |            |           | 2                      | HUCR-8              |
|             |            |            |           | 3                      | HUCR-9              |
| Gambella    | Anywaa     | Aboli      | Pinkew    | 1                      | HUCR-10             |
|             |            |            |           | 2                      | HUCR-11             |
|             |            |            |           | 3                      | HUCR-12             |
|             | Abobo      | Cobo kire  |           | 1                      | HUCR-13             |
|             |            |            |           | 2                      | HUCR-14             |
|             |            |            |           | 3                      | HUCR-15             |
| Oromia      | East Hararge| Gursum    | Ilalarn   | 1                      | HUCR-16             |
|             |            |            |           | 2                      | HUCR-17             |
|             |            |            |           | 3                      | HUCR-18             |
|             | Babile     | Ifa        |           | 1                      | HUCR-19             |
|             |            |            |           | 2                      | HUCR-20             |
|             |            |            |           | 3*                     | HUCR-21             |
| West Hararge| Oda Bultum | Bakanisa   |           | 1                      | HUCR-22             |
|             |            |            |           | 2                      | HUCR-23             |
|             |            |            |           | 3                      | HUCR-24             |
|             | Miesso     | Oda Kanani |           | 1                      | HUCR-25             |
|             |            |            |           | 2                      | HUCR-26             |
|             |            |            |           | 3                      | HUCR-27             |
| Dire Dawa   | Biya Awale | Belewa     |           | 1                      | HUCR-28             |
|             |            |            |           | 2*                     | HUCR-29             |
|             |            |            |           | 3                      | HUCR-30             |

*No nodulation obtained
willing to allow soil sampling from their field. The samples were taken during the 2017/2018 cropping season from farmers’ fields having no history of rhizobial inoculation by the time the cowpea crop is still growing in the fields. The soil samples were collected randomly from surface to 20 cm depth using a zigzag pattern in the fields to ensure uniformity of the process using a hand shovel. The hand shovel was cleaned after each sampling with running tap water and dried using sterile cloth. The soil samples were mixed thoroughly to form a composite sample. The soil samples were placed in plastic polythene bags, labeled and transported to Haramaya University with their full information. All the collected soil samples (30 samples) were used for nodule-trapping experiments in the Haramaya University greenhouse.

2.2. Isolation of bacteria from cowpea root nodules

Rhizobia were isolated from the cowpea root nodules by using cowpea as a nodule trap crop. The soil from each sample was filled into a 3 kg capacity plastic pot, which had been surface sterilized with 95% alcohol. Undamaged and selected seeds of cowpea (Bole variety, released by Melkassa Agricultural Research Center in 2005) were surface sterilized briefly with sodium hypochlorite solutions for 3 minutes (Vincent, 1970). After rinsed several times with disinfected distilled water and air-dried, five seeds were planted in each pot and later thinned down to three after germination. The pots were arranged in a completely randomized design (CRD) to allow cowpea growth in a greenhouse and watered as required.

During the late flowering and initial pod setting stages, the plants were carefully uprooted and washed with tap water to remove the adhering soils. Healthy and pink color nodules were selected and enclosed in a disinfected absorbent paper and taken to the Soil Microbiology Laboratory, Haramaya University. The nodules were surface sterilized in 95% alcohol and washed several times with sterile distilled water. The disinfected nodules were, then, transferred into sterilized Petri dishes and separately crushed in the presence of 1 ml of sterile deionized water with a flaming glass rod to obtain a milky suspension of bacteroids. A loop full of crushed bacterial suspension was streaked on yeast extract mannitol agar (YEMA) medium, which is the most commonly used medium for the culture of rhizobia. The YEMA was prepared using 10 g mannitol, 0.5 g K₂HPO₄, 0.2 g MgSO₄.7H₂O, 0.1 g NaCl, 1 g yeast extract, 0.025 ml congo red, 15 g agar, in 1000 ml distilled water, adjusted to pH 7.0 and incubated at 28 ± 2°C for 5–7 days (Somasegaran & Hoben, 1994).

2.3. Identification and confirmation of the isolates

It is well known that all the bacteria that are isolated and grown on media (particularly YEMA which is used for rhizobial culture) may not be rhizobia. In this experiment, identification and confirmation of the isolates were done to check whether the isolates were categorized under the rhizobia taxonomy or not based on the standard procedures described by Somasegaran and Hoben (1994), Lupwayi and Haque (1994) and Kucuk et al. (2006). 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 and not the Agrobacterium or other bacteria, which frequently come as a contaminant.

2.4. Purification, preservation and designation of the isolates

The purification of isolates was done by picking a single well-grown rhizobial colony with a disinfected inoculating loop and streaking on freshly prepared sterile YEMA and incubated at 28 ± 2°C for 5–7 days. For further purification, repeated sub-culturing was done until purity and consistency were preserved. The presumptive test was done to check the purity of the rhizobia by re-culturing the isolates on YEMA containing congo red, peptone glucose agar, and lactose medium, and examining their gram stain reaction using microscopy as described by Somasegaran and Hoben (1994).

For preservation and further characterization of the isolates, purified and single well-isolated colonies were transferred and preserved on YEMA slants containing 0.3% (W/V) CaCO₃ and stored
at 4°C (Vincent, 1970). All isolates were named as HUCR (Haramaya University Cowpea Rhizobia) followed by different numbers for each isolate.

2.5. Eco-physiological characteristics test of the isolates
All purified isolates were evaluated in vitro for the following eco-physiological characteristics in the laboratory. The test was carried out in three replicates and the plates streaked with isolates were arranged in a completely randomized design. The development of the colony was inspected and the result was recorded qualitatively either as “+” for the presence of growth or “−” for the absence of growth after 5–7 days of incubation (Somasegaran & Hoben, 1994).

2.5.1. Temperature tolerance test
To verify the temperature tolerance ability of the isolates, a loop full of purified cultures incubated for 5–7 days was streaked on YEMA media plates and incubated at 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C and 45°C. The tolerance of each rhizobial isolate was determined by evaluating the development of distinct colonies on media. To make comparisons among the isolates and determine the development of the isolates at each temperature level, continuous growth at 28 ± 2°C was used as a control (Alexandre & Oliveira, 2011).

2.5.2. pH tolerance test
The characterization of cowpea nodulating rhizobia isolates to produce a colony at different pH levels was tested on YEMA adjusted to different pH levels. The pH levels used for the study were 4.0, 4.5, 5, 5.5, 6.0, 6.5, 7, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0 and 10.5 using sterile 0.1 N (normal) HCl and 1 N NaOH as described by Bernal and Graham (2001). All isolates were tested for the development of distinct colonies at each pH level by incubating at 28 ± 2°C for 5–7 days (Somasegaran & Hoben, 1994). Three replicates per isolate per pH level were executed.

2.5.3. Salt tolerance test
All isolates were tested for their tolerance to salinity on YEMA supplemented with sodium chloride (NaCl) at concentrations of 0.1%, 0.4%, 0.8%, 1%, 2%, 3%, 4%, 5%, 6% and 7% (w/v). The isolates were characterized for the development of distinct colonies after 5–7 days of incubation at 28 ± 2°C. Three replicates per isolate per NaCl concentration were executed following the procedure presented in Lupwayi and Haque.

2.6. Physiological characteristics test of the isolates
All isolates were assessed in vitro for their physiological characteristics (carbohydrate and amino acid utilization) in the laboratory. A 5–7 days old rhizobial isolate was inoculated on a basal medium containing different carbon and nitrogen sources. The tests were conducted in triplicates per isolate per each physiological characteristic and a completely randomized design was used to arrange the plates as described by Farissi et al. (2014). As a positive control, isolates were inoculated on to YEMA containing congo red (YEMA plates). The growth of the isolate was observed and the result was recorded qualitatively either as “+”, for the presence and “−”, for the absence of growth by comparing with the positive control after 5–7 days of incubation at 28 ± 2°C (Legesse, 2016; Somasegaran & Hoben, 1994).

2.6.1. Carbohydrate utilization test
The isolates were streaked on media supplemented with eight carbon sources (glucose, fructose, galactose, lactose, maltose, arabinose, mannose, and glycerol) to characterize their utilization capability after inoculating on freshly prepared sterile media (Somasegaran & Hoben, 1994). Each carbon source was added at a final concentration of 1 g/liter to the basal medium containing (per liter) 1 g of K₂HPO₄, 1 g of KH₂PO₄, 0.01 g of FeCl₃, 6H₂O, 0.2 g of MgSO₄.7H₂O, 0.1 g of CaCl₂, 1 g of H(N₄)₂SO₄, and 15 g of agar which was autoclaved at 121°C for 15 minutes, and pH was adjusted to 7.
2.6.2. Amino acid utilization test
Amino acid utilization was tested using seven nitrogen sources (L-lysine, L-arginine, tyrosine, L-tryptophan, L-asparagine, glutamate, and methionine). Each nitrogen source was added at a concentration of 0.5 g/l to the basal medium used for carbohydrate utilization from which ammonium sulfate was absent and to which mannitol was added at a concentration of 1 g/liter as reported in Amarger et al. (1997).

3. Data analysis
Analysis of data was performed using range and percentage on the basis of the presence of growth (+) or absence of growth (-) as described by Legesse (2016). The tolerance range was determined for eco-physiological characteristics (temperature, pH and salt tolerance) of the isolates based on the data obtained on the growth of isolates. The utilization percentage was calculated for physiological characteristics (carbohydrate and amino acid utilization) for each isolate and substrate based on the data obtained on the growth of isolates on the media.

4. Results and discussions

4.1. Identification of rhizobial isolates
Thirty soil samples were used for nodule-trapping experiments in the greenhouse. Two of the soil samples failed to nodulate and 28 cowpea nodulating rhizobia were isolated and identified under laboratory using standard procedures (Somasegaran & Hoben, 1994). All the 28 isolates were presumptively identified as root-nodule bacteria, rhizobia, following the procedures described by Somasegaran and Hoben (1994), Lupwayi and Haque (1994) and Kucuk et al. (2006). The confirmatory tests on morphological parameters of the isolates revealed that all the isolates were confirmed to be the members of the genus Rhizobium. Consequently, the morphological study of the isolates on media has 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). Microscopic observation has also shown that the isolates were gram-negative, rod-shaped and non-spore forming which is a distinct feature of Rhizobium species as described by Somasegaran and Hoben (1994).

4.2. Eco-physiological characteristics test of the isolates
4.2.1. Temperature tolerance
The temperature tolerance test revealed that the isolates grew and were able to develop a colony at a temperature range between 20°C and 35°C (Figure 1). However, the growth of isolates was progressively decreased when the temperature increased. Usha and Archana (2015) indicated that

Figure 1. Tolerance of rhizobia nodulating cowpea at different range of temperatures.
cowpea rhizobial isolates exhibited growth at temperatures 10°C, 15°C and 30°C whereas only one strain was able to resist temperature up to 40°C. It has been reported that high temperature decreases the survival, establishment, and growth of the rhizobia colony (Hungria & Vargas, 2000). Florentino et al. (2010) also reported that rhizobia isolated from cowpea were able to tolerate temperatures of 28°C and 36°C, and none of the strains grew at 45°C. The higher temperature is reported to alter the permeability of the membrane and leading to the denaturation of enzymes/proteins causing the death and/or poor growth of the rhizobia (Bhargava et al., 2016).

Isolates HUCR-16, HUCR-20, HUCR-22, and HUCR-25 tolerated and developed colony at the lowest temperature of 10°C. In contrast, isolate HUCR-3, HUCR-5, HUCR-7, HUCR-10, HUCR-11, HUCR-15, and HUCR-28 tolerated and able to grow at the highest temperature of 45°C (Table 2). Such rhizobial isolates tolerating low and high temperatures have also been reported to nodulate legumes (Bansal et al., 2014). However, the ideal temperature that allows the growth of most rhizobia has been reported to be between 25°C and 30°C (Zhang et al., 1995), and nevertheless, there is no universal temperature that can be appropriate to all rhizobial species (Alexandre and Oliveira, 2013).

Isolates HUCR-5, HUCR-7, HUCR-10, HUCR-11, and HUCR-28 showed growth at a broader range of incubation temperature which is 15–45°C (Table 2). These isolates were categorized as having a wider temperature tolerance range. Besides, the isolates can be used as potential inoculants to realize higher yields under a wide range of environmental conditions. A study by Degefu et al. (2018) also reported that rhizobial isolates which tolerated a broader range of temperature are often used as inoculant and/or for production of biofertilizer under wider ecological conditions.

4.2.2. pH tolerance
Rhizobial strains of particular species usually differ in their pH tolerance (Zahran, 1999). Evaluation of cowpea nodulating rhizobia isolates relative to their tolerance to pH levels showed a wider tolerance to pH levels ranging between 5 and 10.5. All isolates grew well at pH values ranging between 6.0 and 8.5 with optimum growth density detected at pH around neutral (7.0), but limited growth at pH 5 and 10.5 (Figure 2). Kaur et al. (2012) indicated that the ideal rhizobial growth on media is with pH around neutral. Mensah et al. (2006) showed that the optimal pH for colony growth of cowpea rhizobia isolates is between 6.0 and 7.0. Besides, Del papa et al. (1999) showed that rhizobia species can grow at pH ranges between 5.5 and 8.5, with a pH of around 7 being optimal for growth. Bhargava et al. (2016) also reported that pH around neutral is ideal for the uptake of a proper amount of nutrients and allows optimal growth of the rhizobia isolates. These authors also showed that the growth of rhizobia isolates decreases with an increase in acidity or alkalinity.

Eight isolates (28.57%) were able to tolerate and grow at pH 5 indicating these isolates were more tolerant of acidic conditions (Table 3). Kenasa et al. (2018) reported that most of the cowpea rhizobial isolates from Ethiopian soils were tolerant to acidic pH up to 4.5. Florentino et al. (2010) also evaluated cowpea nodulating rhizobia for tolerance to pH levels and reported that all isolates were able to grow at pH levels 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0.

As a result, rhizobial isolates that showed growth at pH 5 are often very important candidates as inoculant and for biofertilizer production in the acidic soils of cowpea producing areas to realize higher yields. Previous studies revealed those cowpea rhizobia species are much more tolerant of acidic conditions than other legume nodulating rhizobial species (Graham et al., 1994) and strains of cowpea rhizobia able to tolerate even a pH as low as 4 (Mpepereki et al., 1997). In their study on rhizobia from cowpea and mungbean grown in different regions of China, Zhang et al. (2006) reported that rhizobial isolates of cowpea and mungbean could grow at pH values varying between 5.0 and 11.0.
Table 2. Tolerance of rhizobia nodulating cowpea at different temperature levels

| Isolates | Temperature Range (°C) | Tolerance range(°C) |
|----------|------------------------|---------------------|
|          | 5   | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 |
| HUCR-1   | -   | -  | +  | +  | +  | +  | +  | +  | -  | 15–40 |
| HUCR-2   | -   | -  | +  | +  | +  | +  | +  | +  | -  | 15–40 |
| HUCR-3   | -   | -  | -  | +  | +  | +  | +  | +  | +  | 20–45 |
| HUCR-4   | -   | -  | +  | +  | +  | +  | +  | +  | -  | 15–40 |
| HUCR-5   | -   | -  | +  | +  | +  | +  | +  | +  | +  | 15–45 |
| HUCR-6   | -   | -  | +  | +  | +  | +  | +  | +  | -  | 15–40 |
| HUCR-7   | -   | -  | +  | +  | +  | +  | +  | +  | +  | 15–45 |
| HUCR-8   | -   | -  | +  | +  | +  | +  | +  | +  | -  | 15–40 |
| HUCR-9   | -   | -  | +  | +  | +  | +  | +  | +  | -  | 15–40 |
| HUCR-10  | -   | -  | +  | +  | +  | +  | +  | +  | +  | 15–45 |
| HUCR-11  | -   | -  | +  | +  | +  | +  | +  | +  | +  | 15–45 |
| HUCR-12  | -   | -  | +  | +  | +  | +  | +  | +  | -  | 15–40 |
| HUCR-13  | -   | -  | +  | +  | +  | +  | +  | +  | -  | 15–40 |
| HUCR-14  | -   | -  | +  | +  | +  | +  | +  | +  | -  | 15–40 |
| HUCR-15  | -   | -  | -  | +  | +  | +  | +  | +  | +  | 20–45 |
| HUCR-16  | -   | +  | +  | +  | +  | +  | +  | -  | -  | 10–35 |
| HUCR-17  | -   | -  | +  | +  | +  | +  | +  | +  | -  | 15–40 |
| HUCR-18  | -   | -  | +  | +  | +  | +  | +  | +  | -  | 15–40 |
| HUCR-19  | -   | -  | +  | +  | +  | +  | +  | +  | -  | 15–40 |
| HUCR-20  | -   | +  | +  | +  | +  | +  | +  | -  | -  | 10–35 |
| HUCR-22  | -   | +  | +  | +  | +  | +  | +  | -  | -  | 15–35 |
| HUCR-24  | -   | -  | +  | +  | +  | +  | +  | -  | -  | 15–35 |
| HUCR-25  | -   | +  | +  | +  | +  | +  | +  | -  | -  | 10–35 |

(Continued)
Table 2. (Continued)

| Isolates | Temperature Range (°C) |          |          |          |          |          |          |          |          |
|----------|------------------------|----------|----------|----------|----------|----------|----------|----------|----------|
|          | 5          | 10        | 15        | 20        | 25        | 30        | 35        | 40        | 45        |
| HUCR-26  | -          | -         | +         | +         | +         | +         | -         | -         | 15–35     |
| HUCR-27  | -          | -         | +         | +         | +         | +         | -         | -         | 15–35     |
| HUCR-28  | -          | -         | -         | +         | +         | +         | +         | +         | 15–45     |
| HUCR-30  | -          | -         | +         | +         | +         | +         | +         | -         | 15–40     |
| Total    | 0          | 4         | 25        | 28        | 28        | 28        | 20        | 7         |          |
| % tolerated | 0          | 14.29     | 89.29     | 100       | 100       | 100       | 71.43     | 25        |          |

*presence of growth; absence of growth
Six isolates (HUCR-3, HUCR-7, HUCR-11, HUCR-15, HUCR-20, and HUCR-25) were able to grow at pH as high as pH 10.5 (Table 3). Abdelnaby et al. (2015) reported that higher pH has very little effect on the growth of most rhizobial isolates of cowpea as a majority of the isolates tolerate pH as high as 10 and suggested that tolerance to higher pH is often associated with the calcareous and dry condition from which the isolates were originally isolated. However, Kenasa et al. (2018) reported that cowpea nodulating isolates were sensitive to pH 8 contrasting the present finding and indicating that their isolates had a narrow range of pH tolerance.

In this study, it was observed that some isolates were sensitive to highly acidic and alkaline pH. A study by Farissi et al. (2014) indicated that highly acidic and highly alkaline conditions limit rhizobia from growing and succeeding formation of viable nitrogen fixation with a host legume. The adverse effect of the higher pH of the soil conditions is reported as unavailability of essential mineral nutrients for rhizobia. Therefore, it makes a good agronomic advantage to select rhizobial isolates that can tolerate both acidic and alkaline pH conditions.

Isolates HUCR-7 (Wolaita), HUCR-11 (Aboli) and HUCR-15 (Abobo) showed growth on a broader range of pH levels of 5–10.5 (Table 3). These isolates might have a practical advantage in the selection of a broader range of pH tolerant isolates that can enhance soil fertility, achieve a higher yield of cowpea and promote sustainable agricultural production systems under acidic, neutral and alkaline soil conditions. The ability of the rhizobial isolates which survived at acidic pH could be due to physiological and biochemical mechanisms of rhizobial adaptation to acidic conditions as reported previously. These mechanisms include exclusion and expulsion of protons H+ (Kurchak et al., 2001), accumulation of polyamines, high content of potassium and glutamate in cytoplasm and change in the composition of lipopolysaccharides (Vriezen et al., 2007). Another common response to acid shock from the bacteria is to produce acid shock proteins which contribute to acid tolerance by conferring acid protection on the bacteria without altering the internal pH of the cell. Exopolysaccharides (mucus produced by rhizobia) are also reported to have a protective role, as rhizobia that produced greater amounts of mucus are able to survive in acidic conditions more successfully than rhizobia that can only produce smaller amounts of mucus (Graham et al., 1994).

4.2.3. Salt tolerance
Cowpea-nodulating rhizobial isolates showed great variations in colony development when cultured on YEMA adjusted to different NaCl concentrations to test their tolerance to salinity. All isolates were tolerant of NaCl concentration levels ranging from 0.1% to 1% (Figure 3). The number of isolates and their growth decreased as the concentration of salt increased. When the salt concentration was increased above 1%, the number of isolates growing and the density of colony
Table 3. Tolerance of rhizobia nodulating cowpea at different pH levels

| Isolate | 5   | 5.5 | 6   | 6.5 | 7   | 7.5 | 8   | 8.5 | 9   | 9.5 | 10  | 10.5 |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| HUCR-1  | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | -    | 5.5–10 |
| HUCR-2  | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | -    | 5.5–10 |
| HUCR-3  | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +    | 5.5–10.5 |
| HUCR-4  | -   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | -    | 6–10 |
| HUCR-5  | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | -    | 5–9 |
| HUCR-6  | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | -    | 5.5–9.5 |
| HUCR-7  | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +    | 5–10.5 |
| HUCR-8  | -   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | -    | 5.5–10 |
| HUCR-9  | -   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | -    | 5.5–10 |
| HUCR-10 | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | -    | 5–9 |
| HUCR-11 | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +    | 5–10.5 |
| HUCR-12 | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +    | 5.5–10 |
| HUCR-13 | -   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +    | 5–10 |
| HUCR-14 | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | -    | 5.5–10 |
| HUCR-15 | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +    | 5–10.5 |
| HUCR-16 | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | -    | 5–9 |
| HUCR-17 | -   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | -    | 5.5–10 |
| HUCR-18 | -   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +    | 5–10.5 |
| HUCR-19 | -   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +    | 5–10.5 |
| HUCR-20 | -   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +    | 6–10 |
| HUCR-21 | -   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +    | 6–10.5 |
| HUCR-22 | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | -    | 5–9 |
| HUCR-23 | -   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | -    | 5.5–10 |
| HUCR-24 | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | -    | 5.5–10 |
| HUCR-25 | -   | -   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +    | 6–10.5 |

(Continued)
Table 3. (Continued)

| Isolate   | 5   | 6   | 6.5 | 7   | 7.5 | 8   | 8.5 | 9   | 9.5 | 10  | Tolerance range |
|-----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----------------|
| HLR-26    | -   | +   | +   | +   | +   | +   | +   | +   | +   | -   | 6-10            |
| HLR-27    | -   | +   | +   | +   | +   | +   | +   | +   | +   | -   | 5.5-8.5         |
| HLR-28    | -   | +   | +   | +   | +   | +   | +   | +   | +   | -   | 5.5-9           |
| HLR-30    | -   | +   | +   | +   | +   | +   | +   | +   | +   | -   | 5.5-9           |
| Total     | 8   | 20  | 28  | 28  | 28  | 28  | 28  | 28  | 28  | 20  | 5-10            |
| % tolerated | 28.57 | 71.43 | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 96.43 | 71.43 |

*Presence of growth; absence of growth.*
development progressively reduced and no colony development was observed at NaCl concentration of more than 6% (Figure 4). More than 85% of the tested isolates were able to develop colonies on YEMA with 3% NaCl, while 57.14%, 21.43%, and 7.14% were able to grow at a salt concentration of 4%, 5% and 6% NaCl, respectively. Zahran (1999) reported that increasing salt concentrations have a negative effect on the growth of rhizobia due to its direct toxicity and indirectly by osmotic stress caused by salinity.

Isolates HUCR-3 (South Ari) and HUCR-7 (Humbo, Wolaita) showed the highest tolerance to salt concentration up to 6% NaCl (Table 4). A report by Degefu et al. (2018) also indicated that rhizobia isolates collected from southern Ethiopia were able to tolerate the highest salt concentration of 5.5%. This ability can make them highly competitive in colonizing the rhizosphere and fixing nitrogen in salinity affected soils. Mpepereki et al. (1997) reported that the existence of tolerant isolates in salt-affected conditions may be an indication of their adaptation to osmotic stress in response to increasing the ion concentration and the difference in soil moisture during dry seasons.
### Table 4. Tolerance of rhizobia nodulating cowpea at a different salt concentration

| Isolate | Salt concentration (%) | Tolerance range |
|---------|------------------------|-----------------|
|         | 0.1 | 0.4 | 0.8 | 1   | 2   | 3   | 4   | 5   | 6   | 7   |
| HUCR-1  | +   | +   | +   | +   | -   | -   | -   | -   | -   | -  | 0.1–1 |
| HUCR-2  | +   | +   | +   | +   | +   | +   | -   | -   | -   | -   | 0.1–3 |
| HUCR-3  | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.1–6 |
| HUCR-4  | +   | +   | +   | +   | +   | +   | -   | -   | -   | -   | 0.1–3 |
| HUCR-5  | +   | +   | +   | +   | +   | +   | -   | -   | -   | -   | 0.1–3 |
| HUCR-6  | +   | +   | +   | +   | +   | +   | +   | +   | +   | -   | 0.1–6 |
| HUCR-7  | +   | +   | +   | +   | +   | +   | +   | +   | +   | -   | 0.1–6 |
| HUCR-8  | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.1–4 |
| HUCR-9  | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.1–4 |
| HUCR-10 | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.1–5 |
| HUCR-11 | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.1–4 |
| HUCR-12 | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.1–4 |
| HUCR-13 | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.1–4 |
| HUCR-14 | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.1–3 |
| HUCR-15 | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.1–5 |
| HUCR-16 | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.1–3 |
| HUCR-17 | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.1–4 |
| HUCR-18 | +   | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.1–4 |
| HUCR-19 | +   | +   | +   | +   | +   | +   | -   | -   | -   | -   | 0.1–2 |
| HUCR-20 | +   | +   | +   | +   | +   | +   | -   | -   | -   | -   | 0.1–4 |
| HUCR-22 | +   | +   | +   | +   | +   | +   | -   | -   | -   | -   | 0.1–2 |
| HUCR-23 | +   | +   | +   | +   | +   | +   | -   | -   | -   | -   | 0.1–5 |
| HUCR-24 | +   | +   | +   | +   | +   | +   | -   | -   | -   | -   | 0.1–3 |
| HUCR-25 | +   | +   | +   | +   | +   | +   | +   | -   | -   | -   | 0.1–5 |

(Continued)
| Isolate   | Salt concentration (%) | Tolerance range |
|-----------|-------------------------|-----------------|
|           | 0.1 | 0.4 | 0.8 | 1   | 2   | 3   | 4   | 5   | 6   | 7   |       |
| HUCR-26   | +   | +   | +   | +   | +   | -   | -   | -   | -   | -   | 0.1–3 |
| HUCR-27   | +   | +   | +   | +   | +   | +   | -   | -   | -   | -   | 0.1–4 |
| HUCR-28   | +   | +   | +   | +   | -   | -   | -   | -   | -   | -   | 0.1–2 |
| HUCR-30   | +   | +   | +   | +   | +   | -   | -   | -   | -   | -   | 0.1–3 |
| Total     | 28  | 28  | 28  | 28  | 26  | 16  | 6   | 2   | -   | -   | 0.1–3 |
| % tolerated | 100 | 100 | 100 | 100 | 92.86 | 85.71 | 57.14 | 21.43 | 7.14 | 0   |       |

*presence of growth; absence of growth*

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The numbers of isolates decreased from 28 to 26, 24, 16, 6, 2 and 0 as the concentration of NaCl increased from 1 to 2%, 3%, 4%, 5%, 6% and 7%, respectively (Table 4). Bernard et al. (1986) found a strong hampering of growth on 15 different rhizobial strains as the salt concentration increased in the growth medium. Bouzerao et al. (2015) also showed that the growth of isolates decreased when the NaCl concentration increases and the adverse effect of higher salt concentration appears beyond 1% NaCl. In their study on native rhizobia strain isolates from cultivated cowpea in Bangladesh, Nushair et al. (2017) reported that rhizobia were able to grow at 1% NaCl but incapable to tolerate a higher concentration of NaCl, further showing their sensitivity to the higher NaCl concentration.

Successful legume-rhizobia association under salinity stress condition requires isolates that could tolerate and/or resist higher NaCl concentration. Therefore, the presence of high NaCl concentration tolerant isolates (HUCR-3 and HUCR-7) in this study could potentially offer a possibility to enhance legume growth and yield in areas where salinity is problematic. Correspondingly, Keneni et al. (2010) suggested that high NaCl concentrations tolerant isolates are very competitive in colonizing the rhizosphere and nodulating the host plants in severe environmental conditions such as salt-affected soils.

4.3. Physiological characteristics test of the isolates

4.3.1. Carbohydrate utilization
Rhizobia isolates nodulating cowpea were able to utilize a large variety of carbon sources even though there is a variation among the isolates. The tested isolates were able to grow on glucose (100%), fructose (100%), glycerol (100%), mannose (96.43%), lactose (96.43%), galactose (85.71%), arabinose (60.71%) and maltose (46.43%) (Figure 4). This indicates that the majority of isolates were able to use a broad range of carbon sources. Zahran et al. (2012) tested the ability of rhizobial isolates to utilize different carbon sources and revealed that all isolates were able to utilize and grow well on most carbon sources.

All isolates showed colony development on glucose, fructose, and glycerol showing that these carbon sources can provide the carbon needed for the growth of cowpea rhizobia. The lowest utilization percentage (46.43%) was found from maltose. This result agrees with the report of Stowers and Elkan (1984) who reported that all 25 isolates from cowpea showed growth on different carbon sources such as glucose, fructose, galactose and arabinose using them as good energy source and showed limited growth response on lactose and maltose. However, Argaw (2007) and Hassan et al. (2015) reported that rhizobia can also utilize lactose as carbon sources.

According to Degefu et al. (2018), rhizobia selection as inoculant and for biofertilizer production requires isolates with higher tolerance to numerous ecological stresses and their utmost ability to compete for nutrient utilization. In the present study, isolates HUCR-3, HUCR-7, HUCR-11, HUCR-15, and HUCR-25 showed growth on all the provided carbon sources and these isolates have known to use a broader range of carbon sources (Table 5). These isolates have selection advantage due to broader nutrient utilization and, thus, useful in exploiting nitrogen fixation for sustainable production. Therefore, these isolates have a better advantage over those having limited utilization of carbon sources. Degefu et al. (2018) further delineated that isolates that use a broader range of carbon sources are of paramount importance for the production of inoculum that can be used in soils with different carbon sources.

4.3.2. Amino acid utilization
Amino acids utilization test is one of the necessary physiological characteristics for the selection of isolates for different ecological conditions (Jida & Asefa, 2011). This study revealed that there is a difference among cowpea nodulating isolates in utilizing different nitrogen sources. The tested isolates were able to develop colony on media supplemented with L-arginine (92.86%), L-lysine (89.29%), L-tryptophan (89.29%), glutamate (85.71%), tyrosine (82.14%), L-asparagine (78.57%) and Methionine (75%) (Figure 5). This indicates that more than 75% of the isolates were able to
Table 5. Utilization of different carbon sources by rhizobia nodulating cowpea

| Isolate | Glucose | Fructose | Galactose | Lactose | Maltose | Arabinose | Mannose | Glycerol | %age |
|---------|---------|----------|-----------|---------|---------|-----------|---------|----------|-------|
| HUCR-1  | +       | +        | +         | +       | -       | +         | +       | +        | 87.5  |
| HUCR-2  | +       | +        | +         | +       | -       | -         | +       | +        | 75    |
| HUCR-3  | +       | +        | +         | +       | +       | +         | +       | +        | 100   |
| HUCR-4  | +       | +        | +         | -       | +       | +         | +       | +        | 87.5  |
| HUCR-5  | +       | +        | +         | +       | -       | +         | +       | +        | 87.5  |
| HUCR-6  | +       | +        | +         | -       | -       | +         | +       | +        | 75    |
| HUCR-7  | +       | +        | +         | +       | +       | +         | +       | +        | 100   |
| HUCR-8  | +       | +        | -         | +       | +       | +         | +       | +        | 87.5  |
| HUCR-9  | +       | +        | +         | +       | -       | +         | +       | +        | 87.5  |
| HUCR-10 | +       | +        | +         | +       | +       | -         | +       | +        | 87.5  |
| HUCR-11 | +       | +        | +         | +       | +       | +         | +       | +        | 100   |
| HUCR-12 | +       | +        | +         | -       | -       | +         | +       | +        | 75    |
| HUCR-13 | +       | +        | +         | +       | -       | +         | +       | +        | 87.5  |
| HUCR-14 | +       | +        | +         | +       | -       | +         | +       | +        | 87.5  |
| HUCR-15 | +       | +        | +         | +       | +       | +         | +       | +        | 100   |
| HUCR-16 | +       | +        | +         | +       | -       | +         | +       | +        | 87.5  |
| HUCR-17 | +       | +        | +         | +       | -       | +         | +       | +        | 87.5  |
| HUCR-18 | +       | +        | +         | +       | -       | -         | +       | +        | 75    |
| HUCR-19 | +       | +        | -         | +       | -       | +         | +       | +        | 75    |
| HUCR-20 | +       | +        | +         | +       | -       | +         | +       | +        | 87.5  |
| HUCR-22 | +       | +        | +         | +       | -       | +         | +       | +        | 87.5  |
| HUCR-23 | +       | +        | +         | -       | -       | -         | +       | +        | 75    |
| HUCR-24 | +       | +        | -         | +       | -       | -         | +       | +        | 75    |
| HUCR-25 | +       | +        | +         | +       | +       | +         | +       | +        | 100   |

(Continued)
## Table 5. (Continued)

| Isolate | Sources of C | %age utilized |
|---------|--------------|---------------|
|         | Glucose      | Fructose      | Galactose | Lactose | Maltose | Arabinose | Mannose | Glycerol |
| HUCR-26 | +            | +             | +         | +       | -       | +         | +       | +        | 87.5     |
| HUCR-27 | +            | +             | +         | +       | +       | -         | +       | +        | 87.5     |
| HUCR-28 | +            | +             | +         | +       | -       | -         | +       | +        | 75       |
| HUCR-30 | +            | +             | -         | +       | +       | -         | +       | +        | 87.5     |
| Total   | 28           | 28            | 24        | 27      | 13      | 17        | 27      | 28       | 87.5     |
| % utilized | 100   | 100            | 85.71     | 96.43   | 46.43   | 60.71     | 96.43   | 100      |

*presence of growth; absence of growth
utilize each of the nitrogen sources tested indicating the majority of cowpea nodulating rhizobia were able to utilize a large variety of amino acids.

Twelve isolates (42.86%), namely, HUCR-3, HUCR-5, HUCR-6, HUCR-7, HUCR-10, HUCR-11, HUCR-15, HUCR-16, HUCR-20, HUCR-22, HUCR-25, and HUCR-28 utilized all nitrogen sources provided to them (Table 6). These isolates have been known to utilize a broader range of nitrogen sources making them predominantly significant for the production of inoculum for soils with different nitrogen sources. Jida and Asefa (2011) indicated that the capability of isolates to use a broader range of nitrogen sources gave them survival and competitive advantage in the soil.

Isolate HUCR-4, HUCR-8, HUCR-18, and HUCR-27 have utilized only four among the seven sources of nitrogen provided with the growth media showing that these isolates have selective utilization capability (Table 6). Stowers and Elkan (1984) and Zhang et al. (1991) also showed that some rhizobial isolates have limited utilization of nitrogen sources.

5. Conclusions
Eco-physiological and physiological characteristics of rhizobia are studied to evaluate the eco-physiological and physiological adaptation of the isolates and ensure the selection of superior inoculants. In the present study, rhizobial isolates nodulating cowpea exhibited a wide diversity for their tolerance to different eco-physiological conditions (temperature, pH and salt concentration). Besides, the tested isolates have better and broader utilization of carbon and nitrogen sources to satisfy their nutritional requirements for their growth. This offers them an ecological advantage in occupying the rhizosphere. The ability of the isolates to tolerate different eco-physiological conditions and utilize a diversity of nutrients make known that the isolates can adapt and establish symbiosis in an environment where nutrients are a limiting factor for agricultural production. The difference and diversity of tolerance to different ecological conditions and utilization of various nutrients are also an indication of the possible diversity and enhanced survival of the isolates. The result of this study is limited to cultural characteristics, i.e. eco-physiological and physiological characteristics of rhizobia nodulating cowpea and, thus, a test to confirm these findings using molecular
## Table 6. Utilization of different nitrogen sources by rhizobia nodulating cowpea

| Isolate | L-lysine | L-arginine | Tyrosine | L-tryptophan | L-asparagine | Glutamate | Methionine | %age |
|---------|----------|------------|----------|--------------|--------------|-----------|------------|------|
| HUCR-1  | +        | +          | +        | +            | +            | +         | -          | 85.71|
| HUCR-2  | +        | +          | +        | +            | +            | -         | +          | 85.71|
| HUCR-3  | +        | +          | +        | +            | +            | +         | +          | 100  |
| HUCR-4  | +        | +          | -        | -            | +            | +         | +          | 57.14|
| HUCR-5  | +        | +          | +        | +            | +            | +         | +          | 100  |
| HUCR-6  | +        | +          | +        | +            | +            | +         | +          | 100  |
| HUCR-7  | +        | +          | +        | +            | +            | +         | +          | 100  |
| HUCR-8  | -        | +          | +        | +            | -            | -         | +          | 57.14|
| HUCR-9  | +        | +          | +        | +            | -            | -         | +          | 71.43|
| HUCR-10 | +        | +          | +        | +            | +            | +         | +          | 100  |
| HUCR-11 | +        | +          | +        | +            | +            | +         | +          | 100  |
| HUCR-12 | +        | +          | +        | +            | -            | +         | -          | 71.43|
| HUCR-13 | +        | +          | +        | +            | -            | +         | +          | 85.71|
| HUCR-14 | +        | +          | +        | +            | +            | -         | -          | 71.43|
| HUCR-15 | +        | +          | +        | +            | +            | -         | +          | 100  |
| HUCR-16 | +        | +          | +        | +            | +            | +         | +          | 100  |
| HUCR-17 | +        | +          | +        | +            | -            | +         | +          | 85.71|
| HUCR-18 | +        | +          | -        | +            | +            | -         | -          | 57.14|
| HUCR-19 | +        | -          | +        | +            | +            | +         | +          | 85.71|
| HUCR-20 | +        | +          | +        | +            | +            | +         | +          | 100  |
| HUCR-21 | +        | +          | +        | +            | +            | +         | +          | 100  |
| HUCR-22 | +        | +          | +        | -            | +            | +         | +          | 85.71|
| HUCR-23 | +        | +          | +        | +            | +            | +         | -          | 71.43|
| HUCR-24 | +        | +          | -        | +            | +            | +         | -          | 100  |
| HUCR-25 | +        | +          | +        | +            | +            | +         | +          | 100  |

(Continued)
Table 6. (Continued)

| Isolate  | L-lysine | L-arginine | Tyrosine | L-tryptophan | L-asparagine | Glutamate | Methionine | %age |
|----------|----------|------------|----------|--------------|--------------|-----------|------------|-------|
| HUCR-26  | -        | +          | +        | +            | +            | +         | +          | 85.71 |
| HUCR-27  | +        | -          | +        | +            | -            | -         | +          | 57.14 |
| HUCR-28  | +        | +          | +        | +            | +            | +         | +          | 100   |
| HUCR-30  | +        | +          | +        | -            | +            | +         | -          | 71.43 |
| Total    | 25       | 26         | 23       | 25           | 22           | 24        | 21         | 71.43 |
| % utilized | 89.29    | 92.86      | 82.14    | 89.29        | 78.57        | 85.71     | 75         |       |

*presence of growth; absence of growth*
characterization is needed. Besides, the proper taxonomical determination of the rhizobial isolates was not tested in this study.

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