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Research Article

Symbiotic Properties of Rhizobium from Crotalaria Ocroleuca Used for Intercropping in CoffeePlantations in South-West Ethiopia

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Abstract

Symbiosis between Rhizobium and legumes are a cheaper and usually more effective agronomic practice for ensuring an adequate supply of nitrogen for legume based crop and pasture production than the application of nitrogen fertilizer. *Crolotalaria ocroleuca* is one of the members of crotalaria grown as cover crop or inter crop in southern and south western parts of Ethiopia. Nineteen isolates were isolated from the root nodules of *Crolotalaria ocroleuca* from coffee growing area in Godere Wereda, Gambella region. Presumptive tests and microscopic features confirmed that the isolates were rod shaped and gram-negative rhizobia. Based on colony and growth characters the isolates were grouped into two groups. Accordingly, 15 isolates were fast growing and acid producing Rhizobium spp; whereas four isolates (AAUCR4. AAUCR9, AAUCR14 and AAUCR18) were slow growing and alkali producing Brady rhizobium spp. Most of the isolates utilized the carbohydrates; sorbitol, xylose, and lactose, whereas only 26% and 15% were able to utilize starch and Na-citrate, respectively. All isolates were resistant to kanamycin and erythromycin at lower concentration of 2.5 and 5 µm/ml. Only 68% and 79% were résistance at (2.5 µm/ml) and 63% and 16% of the isolates were resistant at (5 µm/ml) to neomycin and gentamycin, respectively whereas isolates (AAUCR9 and AAUCR18) were resistant to all the tested antibiotics. The isolates also showed variability in their physiological characteristics. All isolates grew at 30°C. while, 63% and 53% of the isolates were able to tolerate 15°C and 40°C respectively. All isolates were able to grow at lower salt concentration (1-2%), and five isolates (26%) showed tolerance up to 4%. Only isolate (AAUCR9) was considered highly osmo-tolerant since it grew at 5% of salt concentration. Amongst the isolates, 21% were able to form clear zones around their colonies on Pikovaskaya's medium and hence, they were considered as phosphate solubilisers with solubilisation index ranging from (2.2 -3). Relative effectiveness of the isolates was calculated by dry mass of the inoculated plant over the dry mass of positive control has significant difference within and among the treatment and positive and negative control at ($p<0.05$ Tukey's test HSD) and accordingly, 47%, and 32%, of the isolates (79%) were highly effective (80-100%), and effective (50-80%) in nitrogen fixation. Generally, with over all competency analysis measurement two isolates AAUCR9 and AAUCR18 were the best isolates in terms of effective nitrogen fixation and tolerance to various ecological features that could make them competent against prospective candidates under field conditions.

Keywords

Cultural Characters, Gambella, IAR, pH Tolerance, Symbiotic Effectiveness

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1. Introduction

Legumes are important components of low input and traditional agriculture and agro-forestry systems to enhance soil fertility for they fix nitrogen to the tune of 20-300 kg ha/yr [48]. This is due to their symbiotic relationship with diverse groups of root nodule bacteria, generally known as rhizobia. The symbiosis ensures adequate supply of nitrogen for legume based crop and pasture production than the application of nitrogen fertilizer [68].

The genus *Crotalaria* L. is one of the most diversified groups of legumes containing moir é than 600 species distributed throughout the tropics and subtropics [73]. They are versatile and used as forage and food for cattle and humans or can be applied in different agro- forestry systems, such as in intercrop (alley cropping) or fallow crops in low-input agriculture, to restore soil fertility, and rehabilitate degraded farmlands [77]. Crotalaria species have a wide range of tolerance to drought and other edaphic conditions, and some are known for their nematicidal properties [74].

Crotalaria species are also diverse in their symbiotic effectiveness to fix nitrogen from the atmosphere. [45] estimated that *C. ochroleuca*, fix nitrogen equivalent to 83 kg/ha versus 45 kg/ha and 19 kg/ha for *C. retusa* and *C. perrottetii*, respectively. It has been established that *Crotalaria* are promiscuously nodulated by a wide diverse group of rhizobia that include fast growing *Rhizobium* and slow-growing *Brady rhizobium* spp [72]. However, some of them showed narrow range of host specificity and could not be nodulated by *Brady rhizobium* sp. isolated from other species of *Crotalaria* [38, 44], and others isolated from Senegal [47] and Ethiopia [12], were nodulated by *Bradyrhizobium* strains only.

Quite recently, new nodulating*Methylobacteriumspp*. were isolated from root nodules of three Senegalese species of *Crotalaria glaucoides*, *Crotalaria perrottetii* and *Crotalaria podocarpa* [56, 45, 81]. Crotalaria species are widely distributed in Ethiopia within 300-2000 m altitude. It is the most abundant legume diversified into 87 species of which 15 of them are endemic [76].

They are mainly found in damp grassland, especially in floodplains, depressions and along edges of swamps and rivers, but also in deciduous bush land, road sides and fields. The species *Crotalaria ocroleuca* is commonly known as slender leaf or rattle box and widely cultivated in East and Central Africa such as Kenya, Uganda, Tanzania, and Senegal. Although it is not native to Ethiopia, it is imported and widely used as intercrop or cover crop in some government farms alongside crop plants such as maize and coffee in southern parts of Ethiopia [2]. Although Crotalaria spp. is widely distributed in the country, there is limited information about their agronomic importance, except a study on the nodulation pattern of a few *Crotalaria spp.* among woody legumes collected from some parts of Ethiopia [12] that necessitated the search for pattern of nodulation, diversity and symbiotic effectiveness of rhizobia from the legume.

This study therefore, initiated to isolate and characterize rhizobia from *Crotalaria ocroleuca* under laboratory and greenhouse conditions.

1.1. Objectives

1.1.1. General Objectives

The objective of this study was to evaluate the pattern of nodulation and symbiotic effectiveness of root nodule bacteria from *Crotalaria ocroleuca*.

1.1.2. Specific Objectives

1) Isolation and characterization of rhizobia from root nodules using cultural, biochemical and eco-physiological features.
2) To study symbiotic effectiveness of the isolates under the greenhouse condition.

1.2. Significance of the Research

The research is basically aimed to boost agricultural production by providing valuable insight in screening and authentication of the best fixing rhizobia isolates from *Crotalia ocroleuca* and possibly give directions for government to produce inoculates to replace chemical fertilizer.

2. Materials and Methods

2.1. Study Site for Sample Collection

Samples were collected from the coffee growing areas under Teppi Green Coffee Estate Share Company in Godere wereda, Mejang Zone (Kabo), Gambella region. The location is between 708^0N latitude and 35020^0 E longitude, altitude of 1200-1900 (masl). annual rainfall about 1737mmHg, temperature with max. 27°C and min.12°C as well as Soil P^H ranging from 4.5-6.0 with HumicNitosol and about 687 km away from Addis Ababa.

Figure 1. *Map of sample site (GodereWereda, Mejang Zone, Gambella).*

2.2. Collection of Plant Material for Identification

The plant samples were randomly collected from coffee plantation field where the legume, *Crotalaria ocroleuca* grown as a green manure. It was pressed within layers of cartoon with passport data that were used for identification at the National Herbarium at Addis Ababa University.

2.3. Nodule and Seed Collection

Fresh nodules were collected from roots of randomly selected and excavated from the plant, and brought to Applied Microbiology Laboratory preserved in vials filled with silcagel (desiccant), and covered with 1cm layer of cotton wool for further study as described by [33]. Similarly, health seed were collected from dried pods, collected in paper bags, and kept in the refrigerator until use.

2.4. Isolation of Rhizobia from Nodules

Isolation of rhizobia was undertaken from nodules according to [76]. The reserved nodules were imbibed in sterile water on petri-dish overnight, and surface sterilized with 95% ethanol for 10 seconds, and transferred to 3% (v/v) solution of sodium hypo-chlorate for 3-4 minutes. The surface sterilized nodules then rinsed in five changes of sterile distilled water to completely remove the chemicals. Then nodules were transferred into sterile Petri-dishes and crushed with alcohol flamed sterile glass rod in a drop of normal saline solution (0.85% NaCl). Loopful nodule suspensions was streaked on plate containing Yeast Extract Mannitol Agar (YEMA) and incubated at 28 ±2°C for 4-10 days.

Yeast Extract Mannitol Agar (YEMA) [84].

YEMA contained;

Mannitol 10 g/l

K2HP04 0.5 g/l

MgS04.7H20 0.2 g/l

NaCl 0.1 g/l

Yeast Extract 0.5 g/l

Agar 15 g/l

Distilled Water 1000 ml

PH 7 ±0.1

They were Autoclaved at 121°C for 15 minutes.

2.5. Purification and Preservation of Isolates

Single colonies were repeatedly streaked on sterile YEMA plates, and incubated at 28±2°C to ensure the purity and uniformity of colony types. Purified colonies were transferred in YEMA slant containing 0.3% (W/V) $CaCO_3$ and preserved at 4°C for further use [76].

2.6. Presumptive Screening of Pure Cultures

Each isolate were examined for presumptive purity using Peptone-Glucose Test (PGT), gram staining and growth response to YEMA-CR medium [54].

2.6.1. Gram Staining Test

Gram staining was carried out to confirm that all isolates were gram negative and did not contain any gram-positive bacteria or contaminants.

2.6.2. Congo Red Absorption Test

Stock solution of Congo red was prepared by dissolving 0.25g of Congo red in 100ml of sterile distilled water. From stock solution, 10ml was added to a liter of YEMA and autoclaved. Loop full of test isolates were streaked on the medium, covered with aluminum foil in a dark condition, and incubated at 28±2°C for 3 to 7 days to detect Congo red absorption by the colonies [76].

2.6.3. Peptone-Glucose Test

Isolates were inoculated on Peptone Glucose medium prepared by dissolving 5g of glucose, 10g of peptone, 15g of agar and 10ml of bromocresol purple (BCP) in a liter of distilled water and the pH was adjusted to 6.8 with 1N NaOH and HCl. They were incubated at 28±2°C for 3 to 7 days. [33].

2.7. Designation of Rhizobial Isolates

All the isolates that were presumptively identified as root nodule bacteria were AAUCR:-Addis Ababa University Crotalaria Rhizobia followed by Arabic numerals (1, 2, 3, 4……19).

2.8. Characterization of the Rhizobia Isolates

All characteristics were assessed by inoculating them with 10^6cells per milliliter, P^H 6.8, and then incubating them for 4-10 days at 28°C±2, unless stated otherwise. The entire test was made in triplicate, including the control plates. Colony morphology was determined based on colony size (diameter), shape, texture, and gum production, according to [2].

2.8.1. Cultural Characteristics

(i). Colony Morphology

The cultural characteristics of the isolates were determined according to [33]. Isolates were inoculated onto YEMA and incubated at 28±2°C for 3-7days. They were characterized as Small dry (SD), Large mucoid (LM) and Large watery (LW) with the production of excess polysaccharides [80].

(ii). Determination of Growth Rate

Each isolate were grown for 24 hours and 1 ml (10^6) inoculated into 100ml YEM broth in 250ml Erlenmeyer flask and kept on orbital shaker at 125 rev. min-1. Turbidity was periodically measured by taking samples every 6 hours using (UV-7804C, Ultraviolet Visible spectrophotometer at optical density (OD540nm) reading. Samples were simultaneously taken serially diluted (10^{-1}-10^{-10}) from which 0.1 ml sample was dispersed on to YEMA plates to determine the colony forming units (CFU) [54]. Mean generation (doubling) time was calculated from the logarithmic phase of either the optical density (OD) reading of spectrophotometer or viable count of colony forming units (C.F.U) [79].

The formula below was used to calculate mean generation time:

$$g = \frac{log2(t)}{logx - logx0}$$

where,

g is generation time

tis time elapsed

xo is the first OD reading in logarithmic phase

xis the second OD reading in logarithmic phase

(iii). Acid-Base Production Test

The ability of isolates to produce either acid or alkali by indicated by color change was determined according to [25]. To this end, the isolates were inoculated into YEMA containing bromothymol blue (BTB) (0.025 w/v) and incubated at 28±2°C for 3-7days.

2.8.2. Utilization of Carbohydrates

Carbon utilization of isolates was determined following the method of [54]. Eight carbohydrates were prepared as 10% (w/v) solution in water and mixed to YEMA medium, modified by reducing the yeast extract to 0.05g/ liter and omitting mannitol. The heat labile carbohydrates were separately sterilized by membrane filtration using Millipore with pore size of 0.22µm and added to the autoclaved carbohydrate free basal medium. The heat-stable carbohydrates were autoclaved together with the medium. YEMA medium without and with mannitol were used as a negative and positive control, respectively.

2.8.3. Intrinsic Antibiotic Resistance (IAR)

The intrinsic antibiotic resistance of isolates was determined using four antibiotics (Neomycin, Kanamycin, Erythromycin and Gentamycin) on solid YEM medium containing filter sterilized antibiotics using 0.22 µm size membrane filters, at concentration of 2.5,5 and 10µg/ml according to [82]. Plates without antibiotics were used as control groups. All plates were incubated at 28°C for 10 days to detect presence or absence of growth.

2.8.4. Phosphate Solubilizing Ability of Isolates

The ability of the isolates as phosphate solubiliser was examined by streaking a loop full of 72 hours old YEM broth cultures on Pikovaskaya's medium as indicated by [72]. The plates were incubated at 28°C for 5-7 days.

Based on diameter of clear halo zones, solubilisation index (SI) [61] was calculated using the following formula:

$$SI: \frac{colony\ diameter + diameter\ of\ clear\ zone}{colony\ diameter}$$

Where, SI- solubilisation inde

Components g/l

Glucose10.0

Ca (PO4) 5.0

(NH4)2SO4 0.5

MgSO4.7H2O 0.1

NaCl0.2

KCl0.2

Yeast extract 0.5

$MnSO4.H_2O$ 0.002

FeSO4.7H2O0.002
Agar15.0
Distilled Water 1000 ml

2.9. Ecological Characteristics

2.9.1. Temperature Tolerance

The isolates were inoculated on YEMA medium and incubated at temperatures of 4°C, 10°C, 15°C, 35°C, 40°C and 45°C [33].

2.9.2. Salt Tolerance

The ability of the isolates to grow at different level of salt concentrations was determined by inoculating them on the YEMA media containing 1%, 2%, 3%, 4%, 5% [33].

2.9.3. P^H Tolerance

The capacity of each rhizobial isolate to grow on acidic and alkaline media was determined by inoculating them on YEMA adjusted to pH of 4, 5, 6, 8, 10 using NaOH and HCl as described by [33].

2.10. Authentication and Symbiotic Effectiveness in Sand Culture

In order to test the definitive purity of all rhizobia isolates and their symbiotic effectiveness, nodulation test was carried out on sand culture under greenhouse conditions.

2.10.1. Preparation of Sand and Pots

River sand was socked with sulfuric acid and thoroughly washed with tap water. About 1.5kg of sand and transferred into 2-kg-capacity pots.

2.10.2. Seed Preparation and Sowing

Healthy *crotalaria ocroleuca* seeds were surface sterilized with 70% ethanol for 5 seconds and with 3% (v/v) solution of sodium hypo-chlorate for 3-minutes, and washed thoroughly with five changes of sterile distilled water. They were allowed to germinate on, plated into sterilized water agar (0.75% w/v), and incubated at 28^OC. five germinated seeds were transferred into the pots that were thinned down into three after a week.

2.10.3. Inoculants Preparation and Inoculation

Isolates were grown in 10ml YEM broth on orbital shaker 150 rev/min for 72hrs at room temperature for 3 days from which, One ml of the broth culture (about10^9cells, 0.93 OD 540) was inoculated into the base of the seedlings after one week of germination [75].

2.10.4. Greenhouse Conditions, Nutrient Supply and Watering

The pots were arranged in a randomized complete block design (RCBD) in the green house with the photoperiod of 12 hours day/night with 28°C and 10°C respectively. Three replicates were used for each treatment by including pots treated with 0.05% (w/v) KNO_3 as un-inoculated positive and negative control. All of them were fertilized with 100ml of N-free medium per pot [85]. All the pots were watered every two days in order to prevent salt accumulation.

Table 1. *Nitrogen free nutrient solution.*

Stock solution	Chemical g/litre	g/litre
1	CaCl2.2H2O	294.0
2	KH_2PO4	136.1
3	$FeC_6H_50_7.3H_20$	6.700
	$MgSO_4.7H_2O$	123.3
	$K_2SO_4.H_2O$	87.00
4	$MnSO_4.H_2O$	0.338
	H_3BO_3	0.247
	$ZnSO_4.7H_2O$	0.228
	$CuSO_4.5H_2O$	0.100
	$CoSO_4.7H_2O$	0.056
	$NaMoO_2.2H_2O$	0.048

Source: Adapted from [54].

2.10.5. Harvesting the Plants and Assessing Symbiotic Effectiveness

The plants were harvested 12 weeks after inoculation. Nodules were collected from each plant to count nodules and determine nodule dry weight. The shoots were cut at the level of the sand, collected in paper bags, and dried at 70°C for 48 hours to determine shoot dry weight. The roots and adhering sand dislodged in to a coarse sieve (0.76mm) and were washed with a gentle tap water and observed for nodules.

The relative effectiveness of isolates in accumulating plant shoot dry matter calculated as described in [54]. as follows:

$$SE=\frac{TDM}{NDM}X100$$

where, TDM- treatment dry mass
SE- symbiotic effectiveness
NDM-nitrogen fertilizer control plant dry mass

The rate of nitrogen fixing effectiveness is evaluated as highly effective > 85%, Effective 55-85%, Lowly effective 35-54% and Ineffective <35%. According to [86].

2.10.6. Plant Total Nitrogen Analysis

The total nitrogen content of the shoots was determined by modified "Wet" Kjeldahl method according to [46]. Modified "Wet" Kjeldahl method makes use of apparatus (Digestion block, Digestion tubes and Distillation unit) and reagents: concentrated H_2SO_4, 30% H_2O_2, selenium powder, salicylic acid, sulfuric acid - selenium mixture (made by mixing 3.5g selenium powder with 1 liter of H_2SO4 by heating to 300°C until the color of the solution became light yellow), digestion mixture (made by dissolving 7.2g of salicylic acid in 100ml of sulfuric acid-selenium mixture), mixed indicator (contained mixture of 0.5g bromocresol green and 0.1g methyl red dissolved in 100 ml of 95%ethanol and the pH was adjusted to 4.5 using 1N NaOH and HCl), 40% NaOH (made by dissolving400g of NaOH in 800ml distilled water in one liter volumetric flask and bringing the volume to one liter with distilled water after cooling in tapered flask), 2% boric acid solution (made by dissolving 20g boric acid in 600ml distilled water in a one liter volumetric flask and making to the volume with distilled water), and 0.1N H_2SO4 solution (made by pipetting 2.82 ml of 96%H_2SO4, in to distilled water in a liter of volumetric flask and making to the volume with distilled water).

Ground shoot sample (0.3g) was transferred into digestion tube; 2.5ml of the digestion mixture was added to each digestion tube, swirled carefully to moisten the ground shoot samples and let stand for 2 hours. Then, the tubes were placed on heating block and heated at 100°C for 2 hours. After two hours, the tubes were removed from the block and allowed to cool and three 1ml of 30% $H_2O_2$was added successively in to each digestion tube and mixed thoroughly. The digestion tubes were again placed on the preheated block and heated at 300°C until the digest turned to colorless or light yellow. Then the tubes were removed from the block, cooled to room temperature and 48.3ml of distilled water was added to each tube, mixed and then let stand overnight. On the next day, the content of each digestion tube was mixed again by shaking, filtered on a 100ml volumetric flask, brought to the volume with distilled water. Each 100 ml of the acid digest was transferred into a macro-Kjeldahl tube and 20ml of boric acid solution was measured from a dispenser flask into 250ml Erlenmeyer flask corresponding to the number of samples and 2 drops of mixed indicator solution were added to each 20ml boric acid solution, mixed thoroughly and placed under the condenser. After adding 75ml of 40% NaOH solution to each digestion tube containing the digest, it was fitted to the corresponding holder and distillation was started.

When the distillation was completed, that is, when about 80ml of the distillate had been collected to boric acid, the flask was removed and distillation process of another sample was continued. Titration was then performed by using 0.1N H_2SO_4 until the color of the distillate turned from green to pink at the end and the utilized H_2SO_4 for titration was recorded volumetrically.

Finally the per cent of N_2 content of the samples were calculated after correcting for the blank as described by [46]

$$\%N = \frac{(a-b)*N*0.014*100*mcf}{S}$$

Where, a = ml of H2S04 required for titration of sample
b = ml of H_2S0_4 required for titration of blank
S = Sample weight in mg
N = Normality of H2So4
0.014= meq weight of nitrogen in g
Mcf=moisture correction factor

2.11. Comparative Analysis on the Basis of Competency of Isolates

Based on eco-physiological, biochemical and symbiotic performance isolates were given grades 1 up to 5, 1 being poor for having the lowest performance and 5 being excellent for having the highest performance based on the total number of physiological factors each isolates tolerated divided by number of each factors tested multiplied by 100.

Therefore, grade (5) for Greater than 80%, (4) between 60-80%, (3) between 50-60, (2) between 35-50%and (1) below 35% were given.

2.12. Data Analysis

Comparison between treatments was analyzed by one-way ANOVA (Tukey's HSD tests) (SPSS.21). One-way analysis of variance (ANOVA) and the Student t test (post-hoc analysis) were employed to assess the difference between treated and control plants in biomass of shoot, nodule and nodule numbers and total nitrogen.

3. Result and Discussion

3.1. Isolation of Rhizobia

In this study, 19 root nodule bacteria were isolated from nodules of *Crotalaria ocroleuca* grown in Godere Woreda, MejangZone, Gambella region. All isolates were Gram-negative rod shaped bacteria and did not grow on peptone glucose agar. Most of them did not absorb Congo red on YAMA media except isolatesAAUCR4 andAAUCR12, which turned Congo red to pink. [54], had reported the absorption of Congo red by some rhizobial spp. The isolates displayed three types of colony texture of which equal number of isolates showing large mucoid (LM) and large watery (LW) colonies whereas four isolates, AAUCR4, AAUCR9, AAUCR16 and AAUCR 18 showed small dry (SD) colony types. In the LW colonies, growth was accompanied by exopolysaccharide production (Table 2).

Isolates were characterized by mean generation time be-

tween (2.0and 4hrs), except isolates AAUCR4, AAUCR9, AAUCR16, and AAUCR18 that showed generation time above 4hrs. They also showed variations in that 79% of the isolates turned YEMA-BTBin to yellow color indicating acid production; whereas isolates AAUCR4, AAUCR9, AAUCR16and AAUCR18 changed the medium from green to blue color showing alkaline production.

Based on fast generation time 2-4h, large colony size and acid production, all but AAUCR4, AAUCR9, AAUCR16 and AAUCR18 were fast growers that could be tentatively classified into the genus *Rhizobium*-like and the latter could be classified into the genus *Bradyrhizobium* according to [25] (Table 1). This also complement with the early works of [66] who characterized slow growers and fast growers based on acid and alkaline production on YEMA medium. Several studies also showed that *Crotalaria* nodulates with both fast growing rhizobia including the recently identified non-nodule *Methylobacterium*spp, and the slow growing *Bradyrhizobiumjaponicum* [47, 56, 12].

Table 2. *Colony and growth characteristics of rhizobia isolates on YEMA medium containing CR, BTB, andMean generation time.*

Isolates	Colony characters	ColonyDiameter (mm)	Growth on YEMA-CR	Growth on YAMA- BTB	Growth on PGA	MGT (hrs)	PSB (SI)	Type of rhizobia
AAUCR1	LM	5	Colourless	Yellow	-	2.49	-	Rhizobia
AAUCR2	LW	6	Colourless	Yellow	-	2.36	-	‘’
AAUCR3	LM	2	Colourless	Yellow	-	2.7	-	‘’
AAUCR4	SD	2	Pink	Blue	-	5.85	-	Bradyrhizobia
AAUCR5	LM	2	Colourless	Yellow	-	3.68	-	Rhizobia
AAUCR6	LW	5	Colourless	Yellow	-	3.0	-	‘’
AAUCR7	LM	2	Colourless	Yellow	-	4.02	2.5	‘’
AAUCR8	LW	5	Colourless	Yellow	-	2.54	2.4	‘’
AAUCR9	SD	1.	Colourless	Blue	-	5.3	2.2	Bradyrizobia
AAUCR10	LM	3	Colourless	Yellow	-	3.72	-	Rhizobia
AAUCR11	LW	4	Colourless	Yellow	-	2.0	-	“
AAUCR12	LM	2	Pink	Yellow	-	3.07	-	‘’
AAUCR13	LW	8	Colourless	Yellow	-	3.99	-	‘’
AAUCR14	LW	5	Colourless	Yellow	-	3.38	-	‘’
AAUCR15	LM	3	Colourless	Yellow	-	3.42	-	‘’
AAUCR16	SD	1	Colourless	Yellow		4.83	-	Bradyrhizobia
AAUCR17	LW	6	Colourless	Yellow	-	2.35	-	Rhizobia
AAUCR18	SD	1	Colouless	Blue	-	6.38	3.0	Bradyrizobia
AAUCR19	LW	8	Colourless	Yellow	-	2.0	-	Rhizobia

LW-large watery, LM-large mucoid, SD-sm-all dry; PSB=Phosphate solubilization; SI= solubilisation index.

3.2. Phosphate Solubilising Property of Isolates

The data showed that fewer (21%) of the isolates; i.e. isolates, AAUCR7 AAUCR8, AAUCR9, and AAUCR18formed clear zone around their colonies on Pikovskaya’s medium with Solubilisation indices of 2.5, 2.4, 2.2, and 3.0, respectively (Table 1). The number of rhizobia with capacity of phosphate solubilisation (PSB) in crotalaria in this study was similar to the number of PSB (20%) isolated from the same group [12], but more than the 14% recorded from woody legumes such as acacia species [51].

3.3. Biochemical and Physiological Characterization

3.3.1. Biochemical Test

(i). CarbohydrateUtilization

The isolates showed variability in terms of carbon utilization as shown in (Table 2). All isolates were able to grow on rhamnose, xylose, and lactose; whereas more than 80% of the isolates were able to utilize trehalose and glycerol as carbon sources, respectively. However, some isolate (26%) (AAUCR2, AAUCR9, AAUCR12, AAUCR13 and AAUCR18) and 15% (AAUCR14, AAUCR17 and AAUCR19) were able to utilize starch and Na-citrate, respectively (Table 2). It is not uncommon to find limitation in the utilization of citrate in that only 20% of rhizobial isolates were capable of growing on citrate ([12]. Similarly, [83] and [51], showed fewer rhizobial isolates from woody legume species utilized Na-citrate, However, none of the isolates utilized starch in contrast to the present study. Generally, the was no difference in terms of patterns of carbon utilization in between fast and slow growing rhizobia isolates.

(ii). Inherent Antibiotic Resistance (IAR)

Isolates acquired inherent antibiotic resistance (IAR) to one or more the tested antibiotics at different concentrations (Table. 2). All isolates were resistant to Kanamycin and Erythromycin at lower concentrations (2.5-5ug/ml) and with few exception were able to grow on YEMA containing the same antibiotics at higher concentration (10 ug/ml). However, they showed different pattern of antibiotic resistance to Neomycin and Gentamycin. Accordingly, majority of the isolates 68% and 79% were tolerance at (2.5ug/ml) concentration while, 63% and 58% showed tolerance at (5ug/ml), respectively. However, fewer 16% and 26% of the isolates were tolerant to the same antibiotics at higher concentration (10ug/ml). The most resistant isolates were; AAUCR9 and AAUCR18, AAUCR4, AAUCR7 andAAUCR14; while the most sensitive isolate was AAUCR10. Similarly, [51], indicated that most isolates from Acacia species were sensitive to neomycin sulphate at higher concentration. However, [12] from Ethiopia and [40] from Kenya, reported that some isolates from woody legumes were sensitive to Kanamycin mono-sulphate contrary to the present study. Over all, slow growing (Bradyrhizobia) were highly resistant to the tested antibiotics as compared to fast growing isolates.

Table 3. *Carbon utilization and antibiotic resistance (IAR) by isolates from Crotalaria ocroleuca.*

Isolates	Group	Rhamnose	xylose	lactose	Trehalose	Starch	Na-Citrate	Glycerol	Total	Neomycin (ug/ml)			(Kanamycin (ug/m)			Erythromycin (ug/m)			Gentamicin (ug/ml)			Total
										2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	
AAUCR1	R	+	+	+	-	-	-	-	3	+	-	-	+	+	+	+	+	+	-	-	-	7
AAUCR2	R	+	+	+	+	+	-	+	6	+	+	-	+	+	+	+	+	+	+	-	-	9
AAUCR3	R	+	+	+	+	-	-	+	5	-	-	-	+	+	+	+	+	+	-	-	-	6
AAUCR4	B	+	+	+	-	-	-	+	4	+	+	+	+	+	+	+	+	+	+	+	-	11
AAUCR5	R	+	+	+	+	-	-	+	5	-	-	-	+	+	+	+	+	+	+	+	-	8
AAUCR6	R	+	+	+	+	-	-	+	5	-	-	-	+	+	+	+	+	+	-	-	-	6
AAUCR7	R	+	+	+	+	-	-	+	5	+	+	-	+	+	+	+	+	+	+	+	+	11
AAUCR8	R	+	+	+	+	-	-	+	5	+	+	-	+	+	+	+	+	+	+	+	-	10
AAUCR9	B	+	+	+	+	+	-	+	6	+	+	+	+	+	+	+	+	+	+	+	+	12
AAUCR10	R	+	+	+	-	-	-	-	3	-	-	-	+	+	-	+	+	-	-	-	-	4
AAUCR11	R	+	+	+	+	-	-	+	5	-	-	-	+	+	-	+	+	+	+	+	-	7
AAUCR12	R	+	+	+	+	+	-	+	6	+	+	-	+	+	+	+	+	+	+	+	-	10
AAUCR13	R	+	+	+	+	+	-	+	6	+	+	-	+	+	-	+	+	+	+	-	-	8
AAUCR1	R	+	+	+	+	-	+	+	6	+	+	-	+	+	+	+	+	+	+	+	+	11

Isolates	Group	Rhamnose	xylose	lactose	Trehalose	Starch	Na-Citrate	Glycerol	Total	Neomycin (ug/ml)			(Kanamycin (ug/m)			Erythromycin (ug/m)			Gentamicin (ug/ml)			Total
										2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	
AAUCR15	R	+	+	+	+	-	-	+	5	+	+	-	+	+	-	+	+	+	+	-	-	8
AAUCR16	B	+	+	+	+	-	-	+	5	-	-	-	+	+	+	+	+	+	+	+	+	9
AAUCR17	R	+	+	+	+	-	+	+	6	+	+	-	+	+	+	+	+	+	+	-	-	9
AAUCR18	B	+	+	+	+	+	-	+	6	+	+	+	+	+	+	+	+	+	+	+	+	12
AAUCR19	R	+	+	+	+	-	+	+	6	+	+	-	+	+	+	+	+	+	+	+	-	10
Total (%)		100	100	100	84	26	16	89		68	63	16	100	100	79	100	100	95	79	58	26	

+ growth- no growth; R= fast growing rhizobia; B=slow growing bradyrhizobia.

3.3.2. Physiological Characterization of the Isolates

(i). Temperature Tolerance

The study also showed isolates had different pattern of grow at different incubation temperatures 15-40°C. All of the isolates were able to grow at 30°C and none of the isolates was able to grow at 4°C and 45°C (Table 3). It appeared that more than half of the isolates 63% were able to grow at 15°C and 40°C. [69, 16] also isolated some rhizobial strains from some woody legumes that could tolerate the highest temperature ranges between 40 and 43°C. Similar report by [51] from Acacia showed some rhizobia were the tolerant to grow at 15°C and 40°C indicating that most of the isolates can overcome high soil temperature, which is one of the major problems for biological nitrogen fixation in tropical and subtropical areas [79].

(ii). pH Tolerance

Isolates showed variability in tolerance to different pH range (4-10). All of the isolates were able to grow at pH 6, while majority of the isolates were able to grow in between pH ranges 5-10 (Table 3). Seven isolates; AAUCR2, AAUCR8, AAUCR10, AAUCR12, AAAUCR14, AAUCR16 and AAUCR19) (37%) were highly tolerant to lower pH 4 of which most of them were fast growers except, AAUCR16. Similarly, the study by [51] revealed that isolates from acacia species were tolerant to pH 4. Only one isolate (AAUCR16) seems to be more tolerant to both alkaline and acidic ranges. Generally, slow- growing alkali producing isolates grew better at pH level (5-10) than at low pH level. Similarly, [12], reported the high acid susceptibility of slow-growing strains of rhizobia isolated from woody legume trees. However, the slow-growing bradyrhizobia isolates of *Acacia saligna* were found to be alkali sensitive and acid-tolerant [34]. Isolates that grew over a wider pH ranges imply that they can be good candidates as inoculants over an indicated P^H.

(iii). Salt Tolerance

All of the tested isolates were tolerant (100%) to 1 and 2% of salt concentration. However, 47% of the isolates were failed to tolerate a concentration of NaCl above 3%. One isolate (AAUCR9) was able to grow at all concentrations (5%) followed by the isolates AAUCR1, AAUCR5, AAUCR7, AAUCR12 and AAUCR18 (26%) grow in between concentration range 1-4% and failed to grow at 5% of NaCl concentration (Table 3). Only isolate AAUCR9 was able to tolerate the higher salt concentration of 5%, hence it can be considered as the most osmo-tolerant isolate.

Different studies showed that many rhizobial isolates from some woody legumes such as *Acacia, Prosopis* and *Leucaena* species tolerated a concentration of NaCl up to 5% [31, 55, 51]. Similarly, [51] reported that fast growing isolates from some acacia species were tolerant to high concentration of salt than slow growing ones. However, [16] reported that some isolates (slow growing rhizobia) from woody legumes were more salt tolerant than fast growers.

Table 4. *Temperature, pH and salt tolerance of rhizobial isolates collected from Crotalaria ocroleuca.*

Isolate	Temperature (°C)						pH tolerance						Na Cl tolerance					
	4	15	30	40	45	total	4	5	6	8	10	Total	1	2	3	4	5	Total
AAUCR1	-	+	+	-	-	2	-	+	+	-	-	2	+	+	+	+	-	4
AAUCR2	-	-	+	+	-	2	+	+	+	+	-	4	+	+	-	-	-	2
AAUCR3	-	+	+	-	-	2	-	+	+	+	-	3	+	+	+	-	-	3
AAUCR4	-	+	+	-	-	2	-	+	+	+	+	4	+	+	-	-	-	2
AAUCR5	-	+	+	-	-	2	-	+	+	+	-	3	+	+	+	+	-	4
AAUCR6	-	+	+	-	-	2	-	+	+	+	-	3	+	+	-	-	-	2
AAUCR7	-	-	+	+	-	2	-	+	+	+	-	3	+	+	+	+	-	4
AAUCR8	-	-	+	+	-	2	+	+	+	-	-	3	+	+	-	-	-	2
AAUCR9	-	-	+	+	-	2	-	+	+	+	+	4	+	+	+	+	+	5
AAUCR10	-	+	+		-	2	+	+	+	-	-	3	+	+	+	-	-	3
AAUCR11	-	+	+	+	-	3	-	+	+	+	+	4	+	+	+	-	-	3
AAUCR12	-	+	+	+	-	3	+	+	+	+	-	4	+	+	+	+	-	4
AAUCR13	-	+	+	-	-	2	-	+	+	+	+	4	+	+	-	-	-	2
AAUCR14	-	+	+	+		3	+	+	+	-	-	3	+	+	-	-	-	2
AAUCR15	-	+	+	-	-	2	-	+	+	+	-	3	+	+	-	-	-	2
AAUCR16	-	+	+	+	-	3	+	+	+	+	+	5	+	+	-	-	-	2
AAUCR17	-	-	+	-	-	1	-	-	+	+	+	3	+	+	-	-	-	2
AAUCR18	-	-	+	+	-	2	-	+	+	+	+	4	+	+	+	+	-	4
AAUCR19	-	-	+	+	-	2	+	+	+	-	-	3	+	+	+	-	-	3
Total (%)		63	100	53			37	95	100	74	37		100	100	53	32	5	

3.4. Symbiotic Effectiveness of the Isolates on Sand Culture

Isolates show significant variation at P<0.001 (using Tukey's test HSD) on nodule number (NN), nodule dry weight (NDW), shoot dry weight (SDW), shoot length (SL). While, total nitrogen (TN) and symbiotic effectiveness (SE) showed significant difference at P<0.05 within their group and with respect to +N and -N control. Accordingly, the inoculated plants showed significant variations at p< 0.001 in mean nodule number ranging from the lowest (AAUCR2) to the highest (AAUCR18), with 10-102mean nodule number respectively**.** Only six plants inoculated with AAUCR9, AAUCR12, AAUCR8, AAUCR13, AAUCR18, and AAUCR19) were able to induce more than 50 nodules per plant (Table 4). Similarly, [51] recorded the highest nodule number from some Acacia species with 107 nodules per plant, but, none of them were below 25 nodule per plant.

The mean nodule dry weight (NDW) also showed significant difference at P<0.001 (using Tukey's test HSD) ranging from 1.2 mg/plantto90.9 mg/plant from plants inoculated with AAUCR17 and AAUCR13, respectively. There was correlation between nodule number and nodule dry weight at (Pearson's correlation r=0.9 and 0.01 significant level (two-tailed). However, there was no association between the highest nodule number and the higher shoot dry weight. However, previous work by [45] showed that, the highest nodule dry weight of *Crotalaria ocroleuca* with 154mg per plant and [51] also recorded the highest nodule dry weight of 890mg per plant from Acacia species.

The plants inoculated with the isolated (AAUCR18) displayed the maximum mean shoot dry weight (SDW) of 0.465g/plant with SE of 118%; whereas the minimum shoot dry matter was recorded from isolate AAUCR10with mean shoot dry weight of 0.140g/plant and SE of 34% (Table 4). With respect to mean shoot length, the inoculated plants showed significant difference within each other and with respect to the +N and -N control at p<0.001 (Tukey's test HSD), except AAUCR9. The minimum mean shoot length of

-N control and the maximum (AAUCR9) having the value 9cm to 36.8cm respectively. Shoot dry weight and shoot length showed correlation (Pearson's correlation r=0.6) at significant level of 0.01 (two-tailed).

The maximum and the minimum total nitrogen accumulated by the plant inoculated with AAUCR18 and AAUCR17 with the mean value (2.19%) and (0.85%), respectively.

There was no significant difference between and within the inoculated and un-inoculated control at P<0.05 (Turkey's test HSD) in terms of the mean total nitrogen.

The shoot dry weight was found to be positively correlated with total nitrogen (Pearson's correlation, r=0.6) at a significance level of 0.01 (two-tailed). However, higher shoot dry weight was not necessarily associated with total nitrogen percentage in some woody species as well**.**

The lowest total nitrogen (%) accumulated by the un-inoculated -N control with (0.6%) and the highest value (2.19%) accumulated by inoculated plant with the isolate (AAUCR18) with effectiveness (14%) and (118%), respectively. In this case, the lowest and the highest values of total nitrogen (percentage) and the lowest and the highest effectiveness were perfectly associated. [51], also showed isolates from acacia species scored the highest total nitrogen (2.2%).

The relative effectiveness expressed as percentage of shoots dry mass of inoculated plants over positive control. Accordingly, 9 isolates (47%) were highly effective, 6 isolates (32%) were effective, 3 isolates (16%) were least effective and 1 isolate (5%) was ineffective. indicating that the soil harboured 79% of rhizobia with good symbiotic properties. The isolate AAUCR9 accumulated the highest value i.e. (116%), and showed good performance in terms of mean nodule number, nodule dry weight, shoot dry weight; shoot length, total nitrogen and symbiotic effectiveness of *Crotalaria ocroleuca* under pot experiment.

Table 5. *Mean nodule number, nodule dry weight, shoot dry weight, and shoot length, total nitrogen and symbiotic effectiveness of Crotalaria ocroleuca under pot experiment.*

Treatment	NN/plant	NDW (mg/plant)	SDW (g/plant)	SL (cm/plant)	TN (%)	SE (%)	E
+N control	.0^{a}	.00a	0.395±.080^{abc}	19.3±1.3^{abc}	1.15±.231^{ab}	100	HE
-N control	.0^{a}	.00^{a}	0.0567±.033^{a}	9±.577^{a}	.59±.087^{a}	14	I
AAUCR2	10.0±1.15^{ab}	1.5±.2^{a}	0.227±.005^{ab}	14.8±.722^{ab}	.98±.191^{ab}	57	E
AAUCR3	11.0±.58^{ab}	4±.2^{a}	0.145±.013^{a}	11.8±1.00^{ab}	1.07±.208^{ab}	37	LE
AAUCR17	11.0±.58^{ab}	1.2±.06^{a}	0.144±.013^{a}	13.3±.90^{ab}	.85±.161^{ab}	36	LE
AAUCR1	15.0±.58^{ab}	1.8±.07^{a}	0.172±.025^{ab}	11.8±.441^{ab}	1.24±.266^{ab}	44	LE
AAUCR10	20.0±2.65^{ab}	2.6±.4^{a}	0.140±.050^{a}	13.3±1.20^{ab}	1.03±.196^{ab}	34	I
AAUCR11	25.0±5^{ab}	30.2±6^{abcde}	0.325±.096^{abc}	15.3±.90^{ab}	.90±.173^{ab}	82	HE
AAUCR6	30.0±2.31^{ab}	18.0±1.4^{abc}	0.266±.066^{ab}	11.0±2.60^{a}	1.14±.220^{ab}	67	E
AAUCR15	30.0±3.5^{ab}	48.2±6^{bcdef}	0.256±.102^{ab}	18.3±3.80^{ab}	1.09±.231^{ab}	65	E
AAUCR5	33.3±5.7^{ab}	19.7±3.4^{abcd}	0.292±.070^{abc}	17.8±.73^{ab}	1.43±.277^{ab}	74	E
AAUCR7	35.0±7.64^{ab}	18.6±4.1^{abc}	0.232±.086^{ab}	20.5±1.80^{abc}	.895±.424^{ab}	59	E
AAUCR16	37.3±1.8^{ab}	15.4±.7^{ab}	0.440±.091^{bc}	25.7±4.30^{abc}	1.83±.352^{ab}	111	HE
AAUCR4	40.0±10.6^{ab}	46.1±12^{bcdef}	0.346±.102^{abc}	11.5±1.04^{abc}	1.47±.283^{ab}	88	HE
AAUCR14	45.0±7.8^{b}	35.0±5.7^{abcdef}	0.436±0.142^{bc}	25.3±3.80^{abc}	1.49±.289^{ab}	110	HE
AAUCR9	50.0±5.8^{bc}	55.7±6.4^{defg}	0.459±.150^{bc}	36.8±5.67^{c}	1.34±.289^{ab}	116	HE
AAUCR12	50.0±8.7^{bc}	56.5±.9.8^{efg}	0.427±.173^{bc}	17.7±4.33^{ab}	1.42±.27ab	108	HE
AAUCR19	50.0±7.2^{bc}	69.0±9.9^{fg}	0.449±.112^{bc}	26.0±0.60^{abc}	1.63±.31^{ab}	114	HE
AAUCR8	51.7±10.9^{bc}	52.8±8.7^{cdef}	0.290±.046^{abc}	26.2±3.66^{abc}	1.38±.265^{ab}	73	E
AAUCR13	90.0±12.6^{cd}	90.9±13^{g}	0.445±.111^{bc}	24.0±10.60^{abc}	1.87±.313^{ab}	113	HE
AAUCR18	101.7±26^{d}	90.2±14^{g}	0.465±.09^{bc}	29.7±.90^{bc}	2.19±.421^{b}	118	HE

NN = nodule number, NDW = nodule dry weight, SDW = shoot dry weight, TN = total nitrogen. Numbers in the same column followed by the letters do not differ significantly at p<0.05 level (Turkey HSD)

3.5. Evaluation of the Pattern of Symbiosis of Highly Effective and Effective Isolates

Based on the eco-physiological, biochemical characters and symbiotic effectiveness of isolates, grades were assigned for each of the above-mentioned tests and comparisons were made among isolates (Table 6). Accordingly, isolate AAUCR9 was found to be highly competent among all the isolates with highest total grade (29) and high symbiotic effectiveness followed by AAUCR18, AAUCR2 with the second highest score of (25) and the highest symbiotic performance. On the contrary, three isolate AAUCR4, AAUCR6 and AAUCR11 were found to be low competent among all the isolates with lowest total grade of (18), even though it was effective. The two isolates (AAUCR9 and AAUCR18) with the highest performance score 29 and 25 with the highest effectiveness range from slow growing categories respectively. However, there are also isolates with optimum performance score ranging from (above 20) from fast and slow-growing category (Table 6).

Table 6. *Comparative analysis of the eco-physiological and biochemical characteristics of isolates with symbiotic effectiveness.*

Isolates	SE (%)	P^Htolerance	Temp (°C)	Salt tolerance	CarbonUtilization	IAR	PSB	TotalNitrogen	Total
AAUCR2	E	4	2	2	5	4	5	2	24
AAUCR4	HE	4	2	2	3	5	0	2	18
AAUCR5	E	3	2	4	4	4	0	2	19
AAUCR6	E	3	2	2	4	3	0	4	18
AAUCR7	E	3	2	4	4	5	5	1	24
AAUCR8	E	3	2	2	4	5	5	2	23
AAUCR9	HE	4	2	5	5	5	5	3	29
AAUCR11	HE	4	3	3	4	3	0	1	18
AAUCR12	HE	4	3	4	5	5	0	2	23
AAUCR13	HE	4	2	2	5	4	0	4	21
AAUCR14	HE	3	3	2	5	5	0	2	20
AAUCR15	E	3	2	2	4	4	0	2	17
AAUCR16	HE	5	3	2	4	4	0	4	22
AAUCR18	HE	4	2	4	5	5	0	5	25
AAUCR19	HE	3	2	3	5	5	0	3	21

4. Conclusions and Recommendation

The present study shows that the newly introduced *Crotalaria ocroleuca* used as intercrop in coffee plantation is nodulated by indigenous rhizobia in the area. Nineteen root nodule rhizobia were isolated and characterized. Accordingly, they were diverse in colony texture, colony size, and growth reaction on different growth media, and 15 isolates (80%) and 4 isolates (20%) were tentatively grouped into fast growing (*Rhizobium*), and slow growing *Bradyrhizobium*sppp. A few isolates (21%) were also phosphate solubilizers with solubilizing indices of 2.2-3.0).

They were also diverse in their ability to utilize different carbon sourcesin that they were versatile in utilizing rhamnose, xylose, and lactose, but limited in their capacity to utilize starch and Na-citrate. The isolates showed significant difference in their IAR where they were more tolerant to Kanamycin and Erythromycin than they were to Neomycin and Gentamycin. The isolates also showed variations in their eco-physiological tolerance to temperatures of 15-40^{o}C, pH 4-10, and NaCl of 2-5%.

The isolates also showed significant difference in their effectiveness in nitrogen fixation based on their symbiotic features. Accordingly, they induced nodules on the host plant with mean nodule number ranging from the lowest 10NN/plant (AAUCR2) to the highest 107NN/plant (AAUCR18), with mean Nodule dry weight (NDW) of1.2

mg/plant (AAUCR17) and 90.9 mg/plant (AAUCR13).

The plants inoculated with the isolated (AAUCR18) displayed the maximum mean shoot dry weight (SDW) of 0.465g/plant; whereas the minimum shoot dry matter was recorded from isolate AAUCR10 with mean shoot dry weight of 0.140g/plant. In general**,** 9 isolates (47%) were highly effective, 6 isolates (32%) were effective, indicating the soil in the area harboured 79% of rhizobia with good symbiotic properties.

Based on the cumulative performance of isolates on their symbiotic effectiveness; eco-physiological features of salt, temperature, pH tolerance and IAR, and versatility in utilizing different carbon sources, and ability to solubilize inorganic phosphate, AAUCR9, and AAUCR18 were the best of all the isolates collected from *Crotalaria ocroleuca.*

Based on the above study the researchers recommend that:-

1) Field trial on different soil need to be carried out for further approval of competitiveness among the isolates against a reference control.
2) Checking whether the isolate were host specific or promiscuous group or not.
3) Further work needed to be carried out on molecular screening of the isolate to determine the strain type for the production of commercially and ecologically productive inoculants.

Abbreviations

ATP	Adenosine Tri-Phosphate
ANOVA	Analysis of Variance
BNF	Biological Nitrogen Fixation
BCP	Bromocreesol Purple
BCP-YEMA	Bromocreesol Purple-Yeast Extract Mannitol Agar
BTB	Bromothymol Blue
BTB-YEMA	Bromothymol Blue- Yeast Extract Mannitol Agar
cm	Centi Meter
CR	Congo Red
CR-YEMA	Congo Red- Yeast Extract Mannitol Agar
log	Logarithm
mg	Mili Gram
HSD	Highest Significant Difference
µg	Micro Gram
Mo-Fe protein	Molybodenum Iron Protein
N	Normality
N_2	Dinitrogen
OD	Optical Density
P^H	Potential of Hydrogen
Rev/min	Revolution/Minute
TN	Total Nitrogen
V/v	Volume/Volume
YEMA	Yeast Extract Mannitol Agar Medium
MGT	Mean Generation Time
GPA	Glucose Peptone Agar
UV	Ultra Violet
RCBD	Randomized Complete Block Design

Author Contributions

Animaw Jarra is the sole author. The author read and approved the final manuscript.

Conflicts of Interest

The author declares no conflicts of interest.

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