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THESIS

Symbiotic properties of rhizobium from *Crotalaria ocroleuca* used for intercropping in coffee plantations in South-West Ethiopia

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DECLARATION

I declare that the thesis hereby submitted by me for the degree of masters in Biology to the School of Graduate Studies of Addis Ababa University is my own independent work and has not previously been submitted by me or anybody else at another university. The material obtained from other sources has been duly acknowledged in the thesis.

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Examiner

LIST OF SYMBOLES AND AVERVATION

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
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
Masl	meter above sea level

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ABSTRACT

It has been established that the 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 under Teppi Coffee plantation Enterprise. 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 30^0C .while, 63% and 53% of the isolates were able to tolerate 15^0C and 40^0C 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.

Key words: cultural characters, Gambella, IAR, pH tolerance, symbiotic effectiveness

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 (Sanginga et al, 1995). 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 (Zahran, 1999).

The genus *Crotalaria* L. is one of the most diversified groups of legumes containing moiré than 600 species distributed throughout the tropics and subtropics (Polhill, 1982).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 (Diabate *et al*., 2005).Crotalaria species have a wide range of tolerance to drought and other edaphic conditions, and some are known for their nematicidal properties (Silva *et al*, 1989). .

Crotalaria species are also diverse in their symbiotic effectiveness to fix nitrogen from the atmosphere. Samba et al (2005) 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 *Bradyrhizobium* spp (Giller *et al* 1991). 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* (Moulin et al., 2004; Renier et al.2008), and others isolated from Senegal (Samba *et al*, 1999) and Ethiopia ((Diriba Temesgen and Fassil Assefa, 2010), were nodulated by *Bradyrhizobium* strains only.

Quite recently, new nodulating *Methylobacterium spp*. were isolated from root nodules of three Senegalese species of *Crotalaria glaucoides*, *Crotalaria perrottetii* and *Crotalaria podocarpa* (Sy *et al*., 2001; Samba *et al,*.2002; Jour *et al*., 2004).

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 (Thulin, 1989). 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 rattlebox 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 (Ahmede, *et al*., 2001). 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 (Diriba Temesgen and Fassil Assefa, 2010) 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**

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

2. REVIEW LITERATURE

2.1. Legumes

The legumes are classified into one of the largest groups of flowering plants, the leguminosae. This family comprises of 670 to 750 genera over 20,000 species and it is the third largest family in the plant kingdom (Doyle and Luckow, 2003) and it is classified into three subfamilies including; Caesalpiniodeae, Mimosoideae and Papilionoideae. The legumes that are primarily tropical and subtropical are the subfamilies Caesalpiniodeae, Mimosoideae trees, shrubs, mainly distributed in lowlands, midlands, and arid ecosystems (Dommerugus, 1987). They are multipurpose plants; with a number of significant functions in the use systems (Dommerugus, 1987; Young, 1988).

Legumes are very important both ecologically and agriculturally. This is mainly attributed to their substantial contribution to the global flux of nitrogen from atmosphere N2 to biologically useful forms such as ammonia, and assimilates it into nitrogenous organic compounds (Young and Haukka, 1996). Although the vast majority of the leguminosae nodulate and fix nitrogen; nevertheless some of them do not have this capacity. It is estimated that nodulation could occur in about 23% of the species in Caesalpiniodeae, 90% in Mimosoideae and 97% in Papilionoideae (de Faria *et al*., 1989). Nodulation in legumes may not necessarily lead to effective nitrogen fixation (Vincent, 1980 in Fassil Assefa, 1993), and this could due to a number of factors; such as, the strains of the bacteria, the type of species of the host legume and the different biotic and abiotic environmental factors that affect the process of nitrogen fixation.

2.2.*Crotalaria ocroleuca*

Crotalaria (*C. ochroleuca G. Don*.) has a number of desirable characteristics that suggest it has potential as a green-manure crop for many parts of eastern Africa (Wortmann *et al*., 1994).mainly found in dump grass land, especially in flood plains, depressions and along the edges of swamps and rivers ,but also in deciduous bush land, roadsides and fields. It grows in open localities with adequate sunshine at 300-2000m altitude. Favours warm climate and rear in wild in east Africa. *Crotalaria ocroleuca* and *Crotalaria berviden* commonly known as slender leaf rattle pea or Ethiopian rattlebox or mereja. In

international trade, they referred to as vegetable of small-scale production (Onyango, 2004).

It is closely related to *Crotalaria brevidens*and information cannot always be attributed to either one with certainty, the former is distinguished by Pale yellow or creamy flower colour.it is also erect much branched annual or short lived perennial herbs up to 2.5m tall; branches ascending with short apprised hairs. Leaves alternate, 3-foliolate; stipulate absent; petiole 1-6.5cm long; leaflets linear to lanceolate or elliptical lanceolate. It is promising dual-purpose crop. Beside a high nutritional value, it has great advantage such as its repulsiveness to nematodes and nitrogen fixing ability. Can also as food in some parts of Africa. However, it can result in serious health problem if consumed regularly because of the presence of some toxic chemicals. Its erect growth habit enables efficient inter-cropping with food crops allowing land-scarce farmers to produce a food crop alongside the green manure**.**

(http://database.prota.org/PROTAhtml/crotalaria%20ocroleuca_En.htm).

Crotalaria plants have a high dry matter production potential and are able to grow on poor soil with low nitrogen content (Daimon *et al*., 1995). They have also been reported as good intensive fallowing cover crops to regenerate the soil (Müller Sämann and Kotschi, 1994). These annual or perennial plants can be used as green manure or in intercropping farming systems.. In *C. ochroleuca*, the total amount of nitrogen fixed is equivalent to 83 kg/ha versus 45 kg/ha and 19 kg/ha for *C. retusa* and *C. perrottetii*, respectively.

2.3. Biological Nitrogen Fixation

Biological nitrogen fixation (BNF) is a process by which N2 in the atmosphere reduced into a biologically useful, combined form of N-ammonia by living organisms (Giller, 2001). It estimated that endosymbiotic biological nitrogen fixation globally represents approximately 90% of all the fixed nitrogen in the terrestrial environment. Chemically fixed in the form of N-fertilizer contributes an estimated amount of 140 tons of additional fixed nitrogen each year which is mainly used for agricultural fertilizers (Gage, 2004). The greatest proportion of N found on the earth is located in the atmosphere, as N_2. Nevertheless, the majority of organisms cannot utilize this free and abundant, but highly

stable source of N because they can only use N, which combined with other atoms into plant usable forms, such as ammonium, nitrates, and ammonia (Giller, 2001).

The process of making N2 available constitutes a specialized and intricately evolved interaction of soil microbe‟s bacteria (rhizobia) and higher plants and legumes via the formation of nodules (Sessitsch *et al*., 2002).

Biological nitrogen fixation is mediated by the nitrogenase enzyme system that catalyses the ATP dependent reduction of atmospheric dinitrogen to ammonia. Nitrogenase consists of two component metalloproteins, the MoFe-protein with the FeMo-cofactor that provides the active site for substrate reduction, and the Fe-protein that couples ATP hydrolysis to electron transfer (Rees *et al*., 2005).The process that changes inert N_2 to biologically useful NH_3. This process mediated in nature only by bacteria. Other plants benefit from nitrogen-fixing bacteria when the bacteria die and release nitrogen to the environment or when the bacteria live in close association with the plant. Legume BNF involves a symbiosis between legume plants and the rhizobia that live in nodules on their roots.

Rhizobia are agriculturally and environmentally important, because their symbioses with legumes are responsible for conversion of the atmospheric nitrogen into ammonia that can be utilized by plants as a nitrogen source. The process by which N2 converted into ammonia via legume-rhizobia symbiosis is known as Biological Nitrogen Fixation (BNF). A substantial portion of the world‟s supply of organic nitrogen fixed via the symbiosis between rhizobia and leguminous host plants (Postgate, 1986) .Originally, root nodule bacteria were characterized as fast and slow growing rhizobia on the basis of their growth rate and production of acid or base on Yeast extract mannitol agar medium (YEMA) (Jordan, 1984). Later on, fast growing and slow growing root nodule bacteria were classified into the genera of *Rhizobium* and *Bradyhizobium* respectively.

2.4. Biochemistry of Nitrogen Fixation

In all N_2, fixing microorganisms the principle steps of this reaction are the same. The key enzyme complex, referred to as Nitrogenase, is unique to N_2-fixing microorganisms (Thornely, 1992). Nitrogenase consists of two proteins in the ratio of 2:1 azoferredoxin and molydoferredoxin. A common property of all nitrogenase preparation is their sensitivity towards oxygen. The enzyme irreversibly inactivated by oxygen so that nitrogen fixation can be regarded as a strictly anaerobic process (Smith, 1999).

Biological reduction of N_2 to NH_3 is also a highly endergonic process with a minimum energy requirement of Ca.960 KJ mol-1 N- fixed (Sprent and Raven, 1985) sited in Zerihun Belay and Fassil Assefa ,(2011). For the nitrogenase reaction, energy in the form of ATP and reducing equivalents (electrons) are required, supplied by respiration (ATP) and electron carriers, usually ferrodoxin. Nitrogenase catalyses the reduction of several substrates, including H+, N2, and C2H2.

The principal reaction for di-nitrogen reaction is as follows:

N2+8H++8E-+16Mg.ATP 2NH3+H2+16 Mg.ADP+16Pi

All N derived from N2-fixation is obtained in the form of ammonia. The predominant assimilation pathway for ammonia is a two-step process. Glutamine synthetase (GS) adds ammonium to glutamic acid to form glutamine with concomitant hydrolysis of ATP. This glutamine is then used by glutamine-2-oxoglutamate-amino-transfrase (glutamate synthetase or GOGAT) to aminate two molecules of 2-oxaloglutaric acids, with the production of two molecules of glutamate (Lea *et al*., 1990).

2.5. Rhizobia, Taxonomy and Host Specificity

According to (Somasegaran and Hoben, 1994), Rhizobia are genetically diverse and physiologically heterogeneous group of bacteria that were originally classified together with their nodulating members of leguminosae. Morphologically, they are medium-sized, rod-shaped cells, 0.5-0.9 µm in width and 1.2-3.0 µm in length. They are gram-negative, motile by a single polar filagellum or six peritrichous flagella. Rhizobia are predominantly aerobic chemo-organotrophs and are relatively easy to culture. They grow well in the presence of 0_2 and utilize relatively simple carbohydrates and amino

compounds. Optimal growth of most strains occurs at a temperature range of 25^0 C-30^0C and ApH of 6.0-7.0(Somasegaran and Hoben, 1994).

Taxonomy based on the concept of cross-inoculation groups failed because of the many exceptions to this rule. It was also widely recognized that rhizobium classification should adjust to general bacterial taxonomy and include a panel of genomic, phenotypic and phylogentic features instead of the sole nodulation properties (Zakhia and de Lajudie, 2001).Differences in rates of growth allowed early separation of rhizobia into two basic groups, fast growers and slow growers.

Fast growers have generation times of less than 6 hours and generally forms visible colonies (2-4 mm in diameter) on agar media within 2-5 days; whereas slow growers have generation times exceeding 6 hours and give detectable growth after more than 5 days under standardized conditions. Most of the slow growing rhizobia produce alkali while fast growers produce acid (Jordan, 1984) .

According to current classification rhizobia belong to the alpha subdivision of protobacteria, that were first classified into two genera, the genus Rhizobium including the fast growing strains and the new genus Bradyrhizobia, created for the slow growing ones (Jordan, 1984). Since then, isolation of rhizobia from an increasing number of plant species around the world and their characterization by modern polyphasic taxonomy has necessitated the description of additional new genera and species. Six genera; *Rhizobium*, *Bradoyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium*, *Allorhizobium*, and 28 species have been recognized (Zakhia and de Lajudie, 2001).The species name of the microsymbionts reflects in most cases the corresponding host plant nodulated and suggests that symbiosis is a species-specific process. It is clear that the degree of host specificity varies tremendously among the rhizobia (Young and Johnston, 1989).

2.6. Nodule Formation

Specificity of Rhizobium-Legume symbiosis is also governed by specific genes on the bacterial chromosomes that codes for proteins involved in recognition and uptake of specific signal molecules present in root exudates. These root exudates contain chemical attractants such as flavonoids and betains, secreted by the roots (Krishanan and Pueppke,

1993). Each species of Rhizobium possesses a system for sensing the flavonoids produced by the plants species with which it interacts (Matthysse, 1996).These compounds stimulate the expression of a set of Rhizobium genes; the nod genes (Heidstra and Bisseling, 1996). The nod genes encode approximately 25 proteins required for bacterial synthesis and export of nod factor. Nod factor is a lipo-oligosaccharide signal consisting of a chitin backbone, four to five N-acetyl-glucosamine units in length, with a lipid attached to non-reducing end and host specific modification on the backbone (Gage, 2004).In response to nod factors, many of the developmental changes seen in the host plant early in the nodulation process including root hair deformation to establish a meristem and nodule permordeium. Rhizobia continue to differentiate inside the nodule and synthesize proteins required for nitrogen fixation and for the maintenance of the mutualistic partnership (Gage, 2004).

2.7. Nodulation pattern in Crotalaria

The Crotalaria genus (Fabaceae family, Papilionoideae subfamily, Crotalarieae tribe) is composed of more than 600 species (Allen and Allen, 1981), located in the subtropical and intertropical regions (Polhill and Raven, 1981). In Senegal, Crotalaria species have been found to be nodulated by *Bradyrhizobium* strains (Samba *et al*.,1999). Surprisingly; *Methylobacteriumspp*. strains have also been isolated from root nodules of three Senegalese species of Crotalaria, *Crotalaria glaucoides, Crotalaria perrottetii* and *Crotalaria podocarpa* (Samba *et al*., 1999; Sy *et al*., 2001b).

Over the last 15 years, *Methalobacterium* spp. Strains have been isolated from root nodule legumes belonging to the genera *Crotalaria* and *Lontononis* (*Fabaceae* and *Crotalariae*) (Jaftha *et al*.2002;Samba *et al*,1999;Sy *et al*.2001a and b).Non-pigmented strains isolated from three Crotalaria spp.(i.e.*Crotalaria glaucoides,C.perrottetii*, and *C.podocarpa*) were described as actually being a single novel *Methalobacteriumb spp*.(Sy *et al*.2001a and b). This new species, which has the same methylotrophic function as other *Methalobacterium spp*., was named *M. nodulans* for its ability to nodulate and fix nitrogen specifically during symbiosis with *Crotalaria spp.(*Jourand *et al.*2004;Sy *et al.*2001a and b). Only a few African species have the unusual ability to symbiotically and specifically associated with the newly described *M.nodulans* (Gao *et al*.1994;Samba *et at*.2002).These symbiotic strains belong to a single species, named *M. nodulans*, for its ability to nodulate and fix nitrogen specifically during symbiosis with Crotalaria spp. (Jourand *et al*., 2004). Interestingly, the three species of Crotalaria

associated with *M. nodulans* are not nodulated by Bradyrhizobium sp. isolated from other species of Crotalaria (Samba *et al*., 1999, Sy *et al*., 2001b.; Renier *et al*., 2008. ;Moulin *et al*., 2004).

The specificity of the Methylobacterium–Crotalaria interaction has already been assessed by an evaluation of the role of the methylotrophic properties in the symbiotic process. Inoculation of C. podocarpa species with methylotrophic minus mutants resulted in a reduction of the nodule number and a drastic decrease of plant biomass (Jourand *et al*., 2005).

Host specificity in crotalaria species has for the first time been shown and is different from earlier reports, which indicate that these species was promiscuous (Date, 1976; Giller and Wilson, 1991).

2.8. Ecological factors that affect Biological Nitrogen Fixation

The marked influence of environment on symbiotic nitrogen fixation has been known for a long time. The delicate balance between the host plant and the micro-symbiotic disturbed by adverse environmental conditions, which have otherwise no effect on plant growth. Quite obviously, environmental factors affect the growth of both plants and bacteria. As plants must develop a rhizosphere to support rhizobial growth as well as build a morphologically developed nodule to house the invading rhizobia, we may assume that any factor that adversely affects plant growth will also profoundly affect competition for nodulation (Dowling and Broughton, 1986).

2.8.1. Extreme Temperature

Rhizobia are mesophylls and most have a poor growth at temperature below 100C or above 37^0C (Graham, 1992). Although response to temperature is strains dependent, rhizobia found to tolerate between 4-42^0C. However, growth at 40C is rare, and only *R. meliloti* can grow at 42.5^0C. *R. leguminosarum* isolates from lentil plants in Southern Nile Valley of Egypt were tolerant to 35 to 400C inducing less effective symbiosis with their legume host. For most rhizobia, the optimum temperature range for growth in culture is 28 to 31^0C. (Bordeleau and Prevost, 1994).

Changes in temperature strongly affect bacterial infection and N2 fixation in several legume species (Arayankoon et al., 1990; Kishinevsky *et al*., 1992). Nodulation and symbiotic nitrogen fixation depend on the nodulating strain in addition to plant cultivars (Arayankoon *et al*., 1990).

Elevated temperature may delay nodule initiation and development, and interfere with nodule structure and functioning in temperate legumes, whereas nitrogen fixation efficiency mainly affected in tropical legumes. Furthermore, temperature changes affect the competitive ability of Rhizobium strains (Roughley, 1970; Bordeleau and Prevost, 1994).

High soil temperature in tropical and subtropical areas is a major problem for biological nitrogen fixation of legume crops (Michiels *et al*., 1994). Maximum soil temperatures in the tropics regularly exceed 40^0C at 5 cm and 50^0C at 1cm depth (Eaglesham and Ayanaba, 1984). Because high temperatures decrease rhizobial survival and establishment in tropical soils, repeated inoculation of grain legumes and higher rate of inoculation may frequently be needed (Thies *et al*., 1991).

2.8.2. Salt stress

Salt stress is one of the major environmental stresses adversely affecting legume production in arid and semi-arid regions (Bernstein and Ogata, 1966). High soil salinity can deleteriously affect symbiotic association between legume and Rhizobium by osmotic stress and ionic toxicity and imbalance (Aparicio-Tejo, 1982). Increased salt concentration affect the soil micro-biota by causing osmotic stress on them. The legume-Rhizobium symbiosis and nodule formation on legumes are more sensitive to salt or osmotic stress than are rhizobia (Graham, 1992). In contrast to their host legumes, some rhizobia can survive in the presence of extremely high levels of salt both in culture and in soil (Bordeleau and Prevost, 1994). Thus, organisms such as *S. meliloti* tolerate 200-300mM (milimole) NaCl, while nodulation and nitrogen fixation in their host can be inhibited at 50-100mM salt concentration (Serraj and Sinclair, 1998). The depressive effect of salt stress on N_2-fixation by legumes is directly related to the salt induced decline in dry weight and N_2 content in the shoot (Cordovilla *et al*., 1995).

Many species of bacteria adapted to saline conditions by intracellular accumulation of low molecular weight organic solutes; the osmolytes (Csonka and Hanson, 1991). The accumulation of osmolytes thought to counteract the dehydration effect of low water

activity in the medium but not to interfere with macromolecular structure or function. Rhizobia utilize this mechanism of osmotic stress adaptation (Smith *et al*., 1994). Generally, salinity is a serious threat to agriculture in arid and semi-arid regions. Most of these areas confined to the tropics and Mediterranean regions. Successful Rhizobium-legume symbiosis under salt stress requires the isolation and development of salt tolerant rhizobial inoculant (Zahran, 1991). The selection of salt tolerant plant gene also plays an important role in confronting the salt stress when it matched with the salt tolerant gene of Rhizobium (Cordovilla *et al*., 1995).

2.8.3. Water deficiency Stress

Shortage of water compromises plant and rhizobial growth, and is a major cause of nodulation Failure and low N2-fixation. The modification of rhizobial cells by water stress will eventually leads to a reduction in infection and nodulation of legumes (Zahran, 1999). Symbiotic nitrogen Fixation of legumes is also highly sensitive to soil water deficiency. A number of temperate and tropical legumes and shrub legumes exhibited a reduction in nitrogen fixation when subjected to soil moisture deficit. This is because water stress affects the formation and longevity of nodules, synthesis of leghaemoglobin and nodule function. In general, the wide range of moisture level characteristic of ecosystems where legumes have been shown to fix nitrogen suggested that rhizobial strains with different sensitivity to soil moisture could be selected. Studies have shown that sensitivity to moisture stress varies for a variety of rhizobial strains (Fuhrmann *et al*., 1986). Thus, it can assume that rhizobial strains selected with moisture stress tolerance within the range of their legume host. Optimization of soil moisture for growth of the host plant, which is generally more sensitive to moisture stress than bacteria, results in maximal development of fixed-nitrogen inputs into the soil system by the Rhizobium-legume symbiosis (Tate, 1995).

2.8.4. Soil Acidity

Legume and their rhizobia exhibit varied responses to acidity. Some rhizobial species can tolerate acidity better than others can, and tolerance may vary among strains within species (Brockwell *et al*., 1995). The optimum pH for rhizobial growth is considered to be between 6.0 and 7.0 (Jordan 1984), and relatively few rhizobia grow well at pH Less than 5.0. The fast growing strains of rhizobia have generally been considered less tolerant to acid pH than have slowly growing strains of Bradyrhizobium (Graham *et al*., 1994).

Although the basis for differences in pH tolerance among strains of Rhizobium and Bradyrhizobium is not clear (Correa and Barneix, 1997), differences in lipopolysacchardes composition, proton exclusion and extrusion accumulation of cellular polyamines, and synthesis of acid shock proteins (Zarhan, 1999), and composition and structure of outer membrane (Graham *et al*.,1994), have been implicated with pH tolerance of endo-symbioants.(Vlassak and Vanderleyden,1997) reported that, nodulation of legumes is reduced in acid soil, mainly because of sensitivity of early nodulation events, such as attachment, root hair curling and initiation of infection thread formation.

In addition, low pH can affect the production and excretion of nodulation factors in some strains of rhizobia (Mckay and Diordjevic, 1993).Soil acidity is a significant problem facing agricultural production in many areas of the world and limits legume productivity (Bordeleau and Prevost, 1994; Correa and Barnex, 1997). Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depended on symbiotic N2 fixation (Bordeleau and Prevost, 1994).

Two strategies have been adopted to solve the problem of soil acidity. First selecting the optimal combination of rhizobial inoculums and the legume genotype for acidic soils where high H+, Al, or Mn may limit the effectiveness of some rhizobia-host combination (Wood *et al*., 1984; Rai, 1992) and, liming the acidic soil to improve the effects of acidic conditions (Rai, 1992). Liming has been considered the most efficient practice in overcoming soil acidity, with some of the benefits to legume crops not only due to increased soil pH, but also to increased availability of Ca to plant, bacteria and the symbiosis.

In general, low soil pH is often associated with increased Al and Mn toxicity, reduced calcium (Ca) supply phosphorus (P) and molybdenum (Mo) deficiencies. These additional stresses affect the growth of rhizobia, of the host legume and symbiosis. The effect on symbiosis is evident from the fact that, nodulated legumes are more sensitive to Al and Mn toxicity than plants receiving mineral N.

3. MATERIALS AND METHODS

3.1. Study site for sample collection

Figure 1. Map of sample site(Godere Wereda, Mejang Zone, Gambella)

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.27^0C and min.12^0C as well as Soil P^H ranging from 4.5-6.0 with Humic Nitosol and about 657 km away from Addis Ababa.

3.2. 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.

3.3. 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 silca- gel (desiccant), and covered with 1cm layer of cotton wool for further study as described by Lupwayi and Haque (1994). Similarly, health seed were collected from dried pods, collected in paper bags, and kept in the refrigerator until use.

3.4. Isolation of rhizobia from Nodules

Isolation of rhizobia was undertaken from nodules according to Vincent (1970). 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 ± 2^0C for 4-10 days.

Yeast Extract Mannitol Agar (YEMA) (Vincent, 1974)

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 121^0C for 15 minutes.

3.5. Purification and preservation of isolates

Single colonies were repeatedly streaked on sterile YEMA plates, and incubated at 28±2^{0}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^{0}C for further use (Vincent, 1970).

3.6. 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 (Somasegaren and Hoben, 1994).

3.6.1. 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.

3.6.2. 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 litre of YEMA and autoclaved. Loop full of test isolates were streaked on the medium, covered with aluminium foil in a dark condition, and incubated at 28±2^{0}C for 3 to 7 days to detect Congo red absorption by the colonies (Vincent, 1970).

3.6.3. 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 litre of distilled water and the pH was adjusted to 6.8 with 1N NaOH and HCl. They were incubated at 28±2^{0}C for 3 to 7 days.(Lupwayi and Haque, 1994)

3.7. 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).

3.8. Characterization of the rhizobia isolates

All characteristics were assessed by inoculating them with 10^6cells per millilitre, P^H 6.8, and then incubating them for 4-10 days at 28^0C±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 Ahmed *et al*.(1984).

3.8.1. Cultural characteristics

3.8.1.1. Colony morphology

The cultural characteristics of the isolates were determined according to Lupwayi and Haque (1994). Isolates were inoculated onto YEMA and incubated at 28±2^0C for 3-7days. They were characterized as Small dry (SD), Large mucoid (LM) and Large watery (LW) with the production of excess polysaccharides. (Martinez-Romero *et al*., 1991).

3.8.1.2. 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) (Somasegaren and Hoben, 1994). 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) (White, 1995).

The formula below was used to calculate mean generation time:

$$g = \frac{log2(t)}{logx - logx0}$$ where, g is generation time

t is time elapsed

xo is the first OD reading in logarithmic phase

x is the second OD reading in logarithmic phase

3.8.1.3. Acid-Base production Test

The ability of isolates to produce either acid or alkali by indicated by colour change was determined according to Jordan (1984). To this end, the isolates were inoculated into YEMA containing bromothymol blue (BTB) (0.025 w/v) and incubated at 28±2^{0}C for 3-7days.

3.8.2. Utilization of Carbohydrates

Carbon utilization of isolates was determined following the method of Somasegaran and Hoben (1994). 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/ litre 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.

3.8.3**.** 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 Beynon and Josey, (1980). Plates without antibiotics were used as control groups. All plates were incubated at 28^{0}c for 10 days to detect presence or absence of growth.

3.8.4. Phosphate solubilizing ability of isolates

The ability of the isolates as phosphate solubilizes was examined by streaking a loop full of 72 hours old YEM broth cultures on Pikovaskaya‟s medium as indicated by Tawari *et al*. (2004). The plates were incubated at 28^0C for 5-7 days. Based on diameter of clear halo zones, solubilisation index (SI) (Vazquez *et al*., 2000) was calculated using the following formula:

SI:

$$\frac{colony\ diameter + diameter\ of\ clear\ zone}{colony\ diameter}$$ Where, SI − solubilisation inde

Components	g/l
Glucose	10.0
Ca (PO4)	5.0
(NH4)2SO4	0.5
MgSO4.7H2O	0.1
NaCl	0.2
KCl	0.2
Yeast extract	0.5
MnSO4.H2O	0.002
FeSO4.7H2O	0.002
Agar	15.0
Distilled Water	1000 ml

3.9. Ecological characteristics

3.9.1. Temperature tolerance

The isolates were inoculated on YEMA medium and incubated at temperatures of 4^0C, 10^0C, 15^0C, 35^0C, 40^0C and 45^0C (Lupwayi and Haque, 1994).

3.9.2. 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% (Lupwayi and Haque, 1994).

3.9.3. PH 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 (Lupwayi and Haque, 1994).

3.10. 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.

3.10.1. Preparation of sand and pots

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

3.10.2. 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^{O}C. five germinated seeds were transferred into the pots that were thinned down into three after a week.

3.10.4. Inoculant 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^{9}cells, 0.93OD 540) was inoculated into the base of the seedlings after one week of germination (Vincent, 1970).

3.10.5. 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 28^0C and 10^0C 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 (Broughton and Dilworth, 1970).All the pots were watered every two days in order to prevent salt accumulation.

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
	$MnSO_4.H_2O$	0.338
4	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 Somasegaren and Hoben (1994)

3.10.6. 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 70^0C 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 Somasegaren and Hoben (1984) as follows:

$SE=\frac{TDM}{NDM}X100$ where, TDM- treatment dry mass

SE- symbiotic effectiveness

NDM-nitrogen fertilized 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 Date *et al*., (1979).

3.10.7. Plant total nitrogen analysis

The total nitrogen content of the shoots was determined by modified "Wet" Kjeldahl method according to Sahlemedhin Sertsu andTaye Bekele (2000).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, sulphuric acid - selenium mixture (made by mixing 3.5g selenium powder with 1 litre of H_2SO4 by heating to 300^0C until the colour of the solution became light yellow), digestion mixture (made by dissolving 7.2g of salicylic acid in 100ml of sulphuric 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 dissolving 400g of NaOH in 800ml distilled water in one litre volumetric flask and bringing the volume to one litre with distilled water after cooling in stoppered flask), 2% boric acid solution (made by dissolving 20g boric acid in 600ml distilled water in a one litre 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 litre 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 100^0C 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 300^0C until the digest turned to colourless 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 H2SO4 until the colour of the distillate turned from green to pink at the end and the utilized H2SO4 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 Sahlemedhin Sertsu and, Taye Bekele,(2000).

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

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

3.11. 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.

3.12. Data analysis

Comparison between treatments was analysed 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 .

4. RESULT and DISCUSSION

4.1. Isolation of rhizobia

In this study, 19 root nodule bacteria were isolated from nodules of *Crotalaria ocroleuca* grown in Godere Woreda, Mejang Zone,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 isolates AAUCR4 and AAUCR12, which turned Congo red to pink. Somasegran and Hoben, (1994), 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 ex-polysaccharide production (Table 1).

Isolates were characterized by mean generation time between (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-BTB in to yellow colour indicating acid production; whereas isolates AAUCR4, AAUCR9, AAUCR16 and AAUCR18 changed the medium from green to blue colour 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 Jordan (1984) (Table 1). This also complement with the early works of (Young,1996) 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 *Bradyrhizobium japonicum*(Samba et al., 1999; Sy et al., 2001; Diriba Temesgen and Fassil Assefa, 2010).

Table 1.Colony and growth characteristics of rhizobia isolates on YEMA medium containing CR, BTB, and Mean generation time

Isolates	**Colony characte**	**Colony Diamete**	**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

4.2. Phosphate solubilizing property of isolates

The data showed that fewer (21%) of the isolates; i.e. isolates, AAUCR7 AAUCR8, AAUCR9, and AAUCR18 formed 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 **(**Deriba Temesgen and Fassil Assefa, 2010), but more than the 14% recorded from woody legumes such as acacia species (Shishay, 2008).

4.3**.** Biochemical and Physiological characterization

4.3.1. Biochemical test

4.3.1.1. Carbohydrate utilization

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 (Lindstrom and Lehtomaki, 1988). Similarly, Diriba Temesgen and Fassil Assefa, (2010) and Shishay,(2008), 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.

4.3.1.2. 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 and AAUCR14; while the most sensitive isolate was AAUCR10. Similarly, Shishay Mesfin,(2008), indicated that most isolates from Acacia species were sensitive to neomycin sulphate at higher concentration. However, Diriba Temesgen and Fassil Assefa ,(2010) from Ethiopia and Odee *et al* (1997) 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 2.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)**			**Genta micin (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
AAUCR14	R	+	+	+	+	-	+	+	6	+	+	-	+	+	+	+	+	+	+	+	+	11
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

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4.3.2. Physiological characterization of the isolates

4.3.2.1. Temperature tolerance

The study also showed isolates had different pattern of grow at different incubation temperatures15-40^{0}C. All of the isolates were able to grow at 30^{0}C and none of the isolates was able to grow at 4^{0}C and 45^{0}C (Table 3). It appeared that more than half of the isolates 63% were able to grow at 15^{0}C and 40^{0}C. Zehari *et al*. (2000) and Fassil Assefa , (1993) also isolated some rhizobial strains from some woody legumes that could tolerate the highest temperature ranges between 40 and 43^{0}C. Similar report by Shishay Mesfin, (2008) from Acacia showed some rhizobia were the tolerant to grow at 15^{0}C and 40^{0}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 sub-tropical areas (Michiel *et al*., 1994).

4.3.2.2. 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 Shishay Mesfin,(2008) 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, Deriba Temesgen and Fassil Assefa, (2010), 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 (Marsudi *et al*.,1999). Isolates that grew over a wider pH ranges imply that they can be good candidates as inoculants over an indicated P^H.

4.3.2.3. 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 %(Lal and Khanna, 1995; Surange *et al*., 1997; Shishay Mesfin, 2008). Similarly, Shishay Mesfin, (2008) reported that fast growing isolates from some acacia species were tolerant to high concentration of salt than slow growing ones. However, Fassil Assefa ,(1993) reported that some isolates (slow growing rhizobia) from woody legumes were more salt tolerant than fast growers.

Table: 3. Temperature, pH and salt tolerance of rhizobial isolates collected from *Crotalaria ocroleuca.*

Isolate	Temperature(^{0}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	

4.4. 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 10-102 mean 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, Shishay Mesfin, (2008) 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/plant to 90.9 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 Samba, (2002) showed that, the highest nodule dry weight of *Crotalaria ocroleuca* with 154mg per plant and Shishay Mesfin, (2008) 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 AAUCR10 with 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. Shishay Mesfin, (2008) 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 4. 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).

4.5. 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 5). 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 5).

Table 5. Comparative analysis of the eco-physiological and biochemical characteristics of isolates with symbiotic effectiveness.

Isolates	SE (%)	P^H tolerance	Temp(^{0}C)	Salt tolerance	Carbon Utilization	IAR	PSB	Total Nitrogen	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

5. 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 sources in 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) of 1.2 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:-

- ✓ Field trial on different soil need to be carried out for further approval of competitiveness among the isolates against a reference control.
- ✓ Checking whether the isolate were host specific or promiscuous group or not.
- ✓ 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.

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7. **APPENDICE**

APPENDEX 1.Image of Mature Crotalaria *ocroleuca* intercropped between Coffee seedlings as cover crop in the field**.**

Appendix 2.Turbidity measure every six hours at optical density (OD540nm) using spectrometer (Jenway, 640UV/vis spectrometer) for isolates to determine their growth rate(MGT)

Isolates	6hrs	12hrs	18hrs	24hrs	30hrs	36hrs	42hrs	48hrs	MGT
AAUCR1	0.180	0.301	0.374	0.465	0.480	0.495	0.518	0.541	5.49
AAUCR2	0.222	0.294	0.460	0.512	0.556	0.600	0.644	0.688	5.36
AAUCR3	0.075	0.129	0.246	0.314	0.372	0.430	0.583	0.735	2.7
AAUCR4	0.200	0.311	0.414	0.501	0.515	0.528	0.547	0.565	5.85
AAUCR5	0.121	0.273	0.472	0.557	0.574	0.591	0.608	0.628	3.68
AAUCR6	0.013	0.007	0.003	0.045	0.323	0.600	0.667	0.734	1.5
AAUCR7	0.162	0.347	0.504	0.580	0.583	0.585	0.658	0.731	4.02
AAUCR8	0.058	0.258	0.396	0.477	0.545	0.613	0.622	0.630	2.54
AAUCR9	0.187	0.43	0.565	0.697	0.729	0.761	0.792	0.822	6.38
AAUCR10	0.145	0.310	0.471	0.522	0.585	0.647	0.695	0.743	3.72
AAUCR11	0.003	0.107	0.257	0.427	0.486	0.544	0.575	0.605	1.14
AAUCR12	0.190	0.338	0.502	0.572	0.585	0.597	0.633	0.668	3.07
AAUCR13	.069	.071	.119	.175	0.215	0.256	0.286	.316	3.99
AAUCR14	0.119	0.348	0.441	0.523	0.543	0.563	0.641	0.718	3.38
AAUCR15	0.079	0.126	0.196	0.248	0.244	0.240	0.534	0.828	4.42
AAUCR16	0.190	0.338	0.502	0.572	0.585	0.597	0.633	0.668	4.83
AAUCR17	0.081	0.224	0.439	0.593	0.622	0.651	0.734	0.817	2.35
AAUCR18	0.160	0.283	0.367	0.414	0.441	0.467	0.494	0.520	5.5
AAUCR19	.033	.084	.246	.373	.504	.634	.654	.675	2.0

$g = \log_2 (t) / (\log x - \log x_0)$ where, g is generation time

t is time elapsed

Xo is the first OD reading in logarithmic phase

x is the second OD reading in logarithmic phase

Appendix 3. ANOVA result within and between the treatments and the control in terms

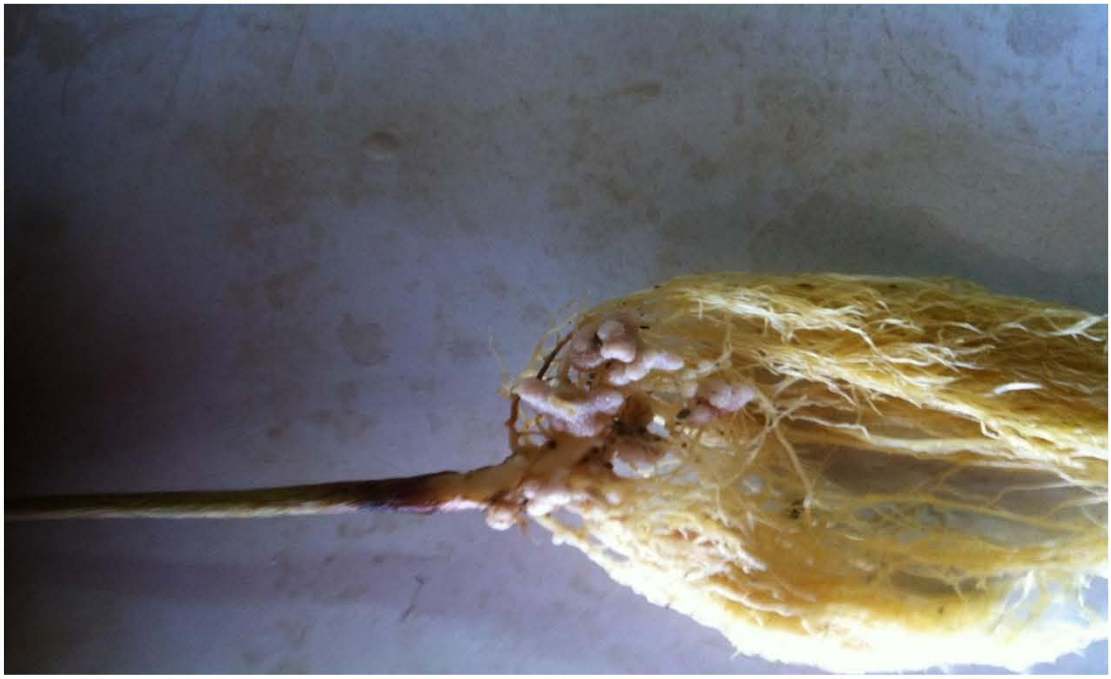
ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
nodule number per plant	Between Groups	40672.190	20	2033.610	9.871	.001
	Within Groups	8652.667	42	206.016		
	Total	49324.857	62			
nodule dry weight per plant	Between Groups	.053	20	.003	19.409	.001
	Within Groups	.006	42	.000		
	Total	.058	62			
shoot dry weight per plant	Between Groups	4.348	20	.217	3.740	.001
	Within Groups	2.441	42	.058		
	Total	6.788	62			
shoot length per plant	Between Groups	3202.704	20	160.135	4.596	.001
	Within Groups	1463.417	42	34.843		
	Total	4666.121	62			
total nitrogen in percent	Between Groups	8.876	20	.444	2.003	.029
	Within Groups	9.308	42	.222		
	Total	18.185	62			

of nodule number, nodule dry weight, shoot dry weight; shoot length, and total nitrogen.

df- degree of freedom Statically, significant at P<0.05 (using Tukey‟s test HSD)

Appendix 4. The arrangement of plant sample (*Crotalaria ocroleuca*) in triplet for infection test in randomized complete block at Addis Ababa University Green House.



Appendix 5.the highest proliferating nodule for plant inoculated with AAUCR18 isolate

Appendix 6. Pearson‟s correlation coefficient between shoot dry weight and symbiotic effectiveness.

		shoot dry weight per plant	symbiotic effectiveness in percent
shoot dry weight per plant	Pearson Correlation	1	.935**
	Sig. (2-tailed)		.000
	Sum of Squares and Cross-products	6.624	1925.385
	Covariance	.107	31.055
	N	63	63
symbiotic effectiveness in percent	Pearson Correlation	.935**	1
	Sig. (2-tailed)	.000	
	Sum of Squares and Cross-products	1925.385	640512.317
	Covariance	31.055	10330.844
	N	63	63

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix 7. Pearson‟s correlation coefficient between shoot dry weight and total nitrogen

		shoot dry weight per plant(g)	total nitrogen
shoot dry weight per plant(g)	Pearson Correlation	1	.584**
	Sig. (2-tailed)		.000
	Sum of Squares and Cross-products	20.632	11.641
	Covariance	.333	.188
	N	63	63
total nitrogen	Pearson Correlation	.584**	1
	Sig. (2-tailed)	.000	
	Sum of Squares and Cross-products	11.641	19.276
	Covariance	.188	.311
	N	63	63

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix 8. Pearson"s correlation coefficient between SDW and shoot length

		shoot dry weight per plant(g)	shoot length(cm)
shoot dry weight per plant(g)	Pearson Correlation	1	.627**
	Sig. (2-tailed)		.000
	Sum of Squares and Cross-products	20.632	190.473
	Covariance	.333	3.072
	N	63	63
shoot length(cm)	Pearson Correlation	.627**	1
	Sig. (2-tailed)	.000	
	Sum of Squares and Cross-products	190.473	4470.232
	Covariance	3.072	72.101
	N	63	63

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix 9. Pearson correlation coefficient between NN and NDW

		nodule number per plant	nodule dry weight per plant(mg)
nodule number per plant	Pearson Correlation	1	.917**
	Sig. (2-tailed)		.000
	Sum of Squares and Cross-products	49324.857	49.260
	Covariance	795.562	.795
	N	63	63
nodule dry weight per plant(mg)	Pearson Correlation	.917**	1
	Sig. (2-tailed)	.000	
	Sum of Squares and Cross-products	49.260	.058
	Covariance	.795	.001
	N	63	63

**. Correlation is significant at the 0.01 level (2-tailed).