# ISOLATION AND INVESTIGATION OF EFFECTIVE-INDIGENOUS RHIZOBIUM BACTERIA FROM GREEN GRAM CULTIVATED SOILS

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# ISOLATION AND INVESTIGATION OF EFFECTIVE-INDIGENOUS RHIZOBIUM BACTERIA FROM GREEN GRAM CULTIVATED SOILS

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**A thesis submitted to the post-graduate committee of the Yezin Agricultural University as a partial fulfillment of the requirements for the degree of Master of Agricultural Science (Plant Pathology)**

**Department of Plant Pathology**
**Yezin Agricultural University**
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The thesis attached hereto, entitled **"Isolation and Investigation of Effective-indigenous Rhizobium Bacteria from Green Gram Cultivated Soils"** was prepared under the direction of the chairperson of the candidate supervisory committee and has been approved by all members of that committee and the board of examiners as a partial fulfillment of the requirements for the degree of **Master of Agricultural Science (Plant Pathology).**

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## DECLARATION OF ORIGINALITY

This thesis represents the original work of the author, except where otherwise stated. It has not been submitted previously for a degree at any other University.

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**DEDICATED TO MY BELOVED PARENTS,**

**U HTAY LWIN AND DAW KYI KYI OO**

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## ABSTRACT

Studies were conducted at Department of Plant Pathology, Yezin Agricultural University from July 2016 to March 2018 to investigate the indigenous rhizobium isolates and to evaluate the response of effective-indigenous rhizobium inoculant on yield and yield components of five green gram varieties. Fifty-two soil samples were collected from green gram fields in Yinmarpin, Palae, Salingyi, Butalin and Monywa Townships in Sagaing Region. In the first experiment, investigation of indigenous rhizobium isolates was carried out by using Completely Randomized Design (CRD) with four replications. Nodulation and plant growth of Yezin-11 green gram variety inoculated with 52 rhizobium isolates were evaluated by most probable number count method (MPN). All tested isolates showed great variation in their capacity to produce nodules and shoot dry matter. Among them, five isolates namely YMP 1, YMP 7, YMP 11, PLE 1 and PLE 5 were found to be effective isolates to enhance the growth of Yezin-11 variety. These isolates were evaluated their effectiveness on five green gram varieties namely MAS-1, Yezin-1, Yezin-9, Yezin-11 and Yezin-14 by using 7 x 5 factorial CRD arrangement (including $N^+$ and $N^-$ control treatments) with three replications. Responses of five rhizobium isolates were varied depending on green gram varieties. YMP 11 isolate gave the highest symbiotic effectiveness percent (SE%) in all tested varieties. The maximum SE% was observed in Yezin-14, followed by Yezin-9, Yezin-1, Yezin-11 and MAS-1. Therefore, the rhizobium inoculant (YMP 11) was selected to evaluate its effectiveness on yield and yield components of five green gram varieties by using 3 x 5 Factorial Randomized Completely Block Design arrangement (including $N^+$ and $N^-$ control treatments) with four replications. YMP 11 inoculant significantly enhanced grain yield 5.21% over un-inoculated ($N^+$) control and 37.10% over un-inoculated ($N^-$) control. Therefore, YMP 11 isolate could be an effective-indigenous rhizobium isolate and may have potential as source of rhizobium inoculant for five green gram varieties.

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# CHAPTER I
# INTRODUCTION

Legumes are very important both ecologically and economically. Among the grain legumes, green gram (*Vigna radiata* L.) is one of the important conventional pulse crops of Myanmar. In 2015-2016, green gram covers nearly 28% of total pulses sown area with an annual production of 1.60 million metric tons (MOALI 2016). It is now becoming popular in the other countries as a cheap, high quality and protein-rich food. Nutritionally green gram seeds contain 28% proteins, 60-65% carbohydrates, 1-1.5% fat and 3.5-4.5% fiber. Sprout is rich in vitamins, minerals and amino acids (especially lizin) which are needed for human body (Delic et al. 2011). Besides, green gram is one of the major export commodities for obtaining foreign revenue during short time period and export value gained 1.19 million metric tons during 2015-2016 (MOALI 2016). In addition to nutritional quality and source of cash, green gram restores and maintains soil fertility through its symbiotic nitrogen-fixation in association with rhizobium (Mansoor 2007). Thus, green gram was grown in rotation with major cereals in traditional low-input agricultural system. As these benefits, nowadays, its cultivated area has been progressively expanded from 0.95 million ha to 1.21 million ha and the average yield increased from 1.05 to 1.32 metric tons $ha^{-1}$ during 2005 to 2015 (MOALI 2016). The green gram potential yield of the world was 1.90 metric tons $ha^{-1}$ (Chauhan and Williams 2018). There are many factors such as application of chemicals, organic and biological fertilizers that can narrow down this yield gap (Elliott and Abbott 2003). One major constraint in green gram yield increase is the absence of specific and effective rhizobia in the soil (Funga et al. 2016). An important feature of green gram is its ability to establish a symbiotic partnership with specific bacteria, setting up the biological nitrogen fixation process in root nodules by rhizobia that may supply the plant's needs of N (Mandal et al. 2009). This ability leads to decrease or absence of N mineral fertilizer application in the field (Abbas et al. 2011).

Application of 30-50 kg mineral N $ha^{-1}$ resulted in significant increase of green gram productivity (Ashraf et al. 2003). One of the principle ways to avoid or decrease environmental pollution associated with really expensive mineral N fertilizer is to insist on maximal use of seed inoculation as microbiological N fertilizer. Green gram in symbiosis with effective *Rhizobium* and *Bradyrhizobium*

spp. can fix 30-60 kg N $ha^{-1}$ depending on agro-ecological conditions (Mansoor 2007). Under unfavorable agro-ecological conditions, although it is capable of fixing atmospheric nitrogen through rhizobia, the yield of green gram is low because of its poor nodulation (Anjum et al. 2006). Selection of suitable green gram-rhizobium is one of the most important means of obtaining higher yield (Uddin et al. 2009). Although rhizobia are present in most soils, variation in number, effectiveness in nodulation and nitrogen fixation were observed (Anjum et al. 2006). Hence, inoculation with efficient rhizobia at planting time is often recommended in environments where compatible rhizobia are absent, soil rhizobial population density has been reduced, or where rhizobia are less effective (Cheminingwa and Vessey 2006). It was found that rhizobium inoculation of green gram increased plant height, leaf area, photosynthetic rate and dry matter production (Thakur and Panwar 1995) and improve nodulation, nitrogen fixation, growth and yield of leguminous crops (Henzell 1988).

One rhizobium strain which is effective on one cultivated legume may not be highly effective on others (Boonkerd and Singleton 2002). With the release of improved varieties of green gram, it is of prime importance to find out the most efficient host-rhizobium combination to attain the maximum yield benefits. Therefore, the evaluation on the effectiveness of indigenous rhizobia isolates on specific host plant should be investigated.

Rhizobial inoculants are now widely used in various parts of the world because they are inexpensive, environment-friendly, and easy to use with no side effects (Tena et al. 2016). Peat is the most commonly used solid carrier in legume inoculants production because rhizobia in a peat carrier persist viable longer both in the package and on the seed (Burton 1984). Usually, the inoculants are not able to occupy a significant proportion of nodules (Vlassak and Vanderleyden 1997). This failure has mostly been contributed to the poor nodulation competitiveness of the introduced rhizobia (Streeter 1994) with native rhizosphere community for nutrients (Bromfeild et al. 1986). For promoting success of inoculants, the native strains that are effective as well as competitive for nodulation can be used as inoculants (Fening and Danso 2002). Rhizobia present in the soil are more adaptable to the soil as well as field conditions (Thies et al. 1991). Therefore, for biofertilizers preparation, selection of effective indigenous rhizobium isolates will obtain increased crop yield through increased nodulation (Agrawal and Choure 2011).

In Myanmar, about 250,000 rhizobial inoculant packets were annually produced for seven legumes crops; groundnut, chickpea, green gram, black gram, soybean, pigeon pea and cowpea by using exotic rhizobium strains from NifTAL (Nitrogen fixation for Tropical Agricultural Legumes) and distributed through Department of Agricultural Research (DAR) (Than et al. 2006). Current inoculant production, however, is sufficient only for 3% of requirement of Myanmar's legumes. Awareness and use of rhizobium inoculants in legume production is limited. Much more inoculants production especially for green gram varieties is still needed in Myanmar and high quality inoculants production is also essential for the farmers' confidence (Than et al. 2003). Most of the rhizobium inoculation studies have concentrated on chickpea and soybean (Aung 2007; Than 2010 and Zaw 2014). Little attention has been paid on other legumes such as green gram, black gram, cowpea and butter bean. Only a few specific rhizobium isolate (DAR 18) for green gram varieties have been investigated (Than et al. 2006). Rhizobium isolates investigated from green gram cultivated soil will be more effective to green gram varieties. Moreover, green gram is mostly grown in Central Dry Zone (CDZ) of the country. Therefore, in this study, the isolate collection site was focused on Sagaing Region, the third largest green gram growing area in Myanmar (MOAI 2014).

According to these, the present study was conducted with the following objectives:

1. to investigate the nodulation efficiency of indigenous rhizobium isolates from different green gram growing areas of Sagaing Region, and
2. to evaluate the response of effective-indigenous rhizobium inoculant on yield and yield components of different green gram varieties.

# CHAPTER II
# LITERATURE REVIEW

## 2.1 Importance of Green Gram in Myanmar

Myanmar is the world's second largest exporter of beans and pulses after Canada and the largest exporter in the ASEAN region. Export value was 1.19 million metric tons during 2015-2016. Twenty one percent of total sown area covered with pulses. Pulses are cultivated throughout Myanmar and mainly in Ayeyawady, Magway, Bago, Mandalay, Sagaing and Yangon Regions (MOALI 2016). Major cultivated pulses are green gram, black gram, pigeon pea, chickpea, soybean, butter bean, kidney bean, cowpea, lab lab bean, sultani and sultapya (FAO 2010). Myanmar has been favored by the India importers in exporting varied pulses because of similarity of the quality as India pulses products, being low freight rates, and being relatively fast delivery. The largest and half of the worldwide green gram production is generated in India, followed by China and Myanmar (Dellaquilla and Tritto 1991; Hussain et al. 2006). In Myanmar, 51% of green gram (154,172 tons), 93% of pigeon pea (275,395 tons) and 97% of chick pea (455,000 tons) produced in the dry zone are exported to India (Srishti and Raghavan 2016).

Green gram is an important and short duration crop of Myanmar. As green gram has high nutritive values, green gram consumption in most of the low income countries has increased from 22% - 66% (Shanmugasundaram 2001). Green gram is used not only primarily as human food, but also it can be used as forage and green manure. Green gram also plays an important role in sustaining soil fertility by improving soil physical properties and fixing atmospheric nitrogen (Reedy et al. 1986). Green gram in symbiosis with effective *Rhizobium* and *Bradyrhizobium* spp. can fix 30-60 kg N $ha^{-1}$ depending on agro ecological conditions (Mansoor 2007). It fits well in many intensive crop rotations and it also helps in preventing soil erosion. It has less water requirement and can be grown twice in a year. Furthermore, it is drought resistant crop that can withstand adverse environmental conditions, and hence successfully be grown in rainfed areas. As a result, green gram production has been improved more than 25% of world production (Shanmugasundaram 2001). In Myanmar, nearly 28% of total pulses sown area covered with green gram and total annual production was about 1.6 million metric tons in 2015-2016. Green gram was mainly grown in Magway Region (24%), Sagaing Region (16.2%), Bago Region

(16.7%), Mandalay Region (11.6%) and Ayeyawady Region (5.8%) as second crop after rice or as pre-monsoon crop in the irrigated areas (MOAI 2014). Myanmar has 50% of improved green gram varieties (Weinberger 2003). Green gram Yezin-6, Yezin-11, Yezin-14 and local varieties are widely grown and a majority of farmers prefer the recommended varieties for their traits to fit with the local areas. Yezin-6, moderate drought tolerant variety, is suitable for dry regions with a considerable yield. Yezin-11 and Yezin-14, highly resistant to yellow mosaic virus disease, could be grown in green gram growing area in which most of the released green gram varieties are affected by yellow mosaic virus disease. Nowadays, green gram sown areas had been increased from 0.95 million ha to 1.21 million ha due to the important attributes such as high market price, benefit cash crop, protein content, drought tolerance and nitrogen fixation (MOALI 2016).

**2.2 Role of Nitrogen in Green Gram**

Nitrogen (N) is an abundant common element on earth, it forms approximately 78% in the earth's atmosphere. Detectable inorganic nitrogen forms in soil are nitrate, nitrite, exchangeable and fixed ammonium, nitrogen gas, and nitrous oxide. Nitrate and exchangeable ammonium are important in plant nutrition. The other forms are generally not available for plant nutrition (Young and Aldag 1982). In well-aerated soils, ammonium is oxidized rapidly to nitrate by nitrification, so that nitrate is the major source of plant-available nitrogen in soil (Schmidt 1982). Nitrite is oxidized more rapidly than ammonium. Hence, ammonium or nitrite accumulates in most soils and are toxic to most plants (Goyal and Huffaker 1984). N is most useful for legume because it is the main component of amino acids as well as proteins, important in the growth and development of vital plant tissues, the cell membranes and chlorophyll (Tajer 2016). Plants with sufficient N will experience high rates of photosynthesis and typically show vigorous plant growth and development. Nitrogen deficiencies result in decreased crop leaf area, photosynthetic assimilation and seed development (Sinclair and Vadez 2002).

Research revealed that green gram yield could be improved by the use of chemical fertilizer and biofertilizers (Ghosh and Joseph 2007; Achakhai et al. 2012). Anjum et al. (2006) reported that application of 30 kg N $ha^{-1}$ significantly increased seed yield (4.6 g $plant^{-1}$) as compared to control (3.83 g $plant^{-1}$) and maximum seed yield (5.01 g $plant^{-1}$) obtained from seed inoculation with 15 kg N $ha^{-1}$ treatment.

Uddin et al. (2009) also stated that most of the growth and yield components of mungbean significantly influenced by *Bradyrhizobium* inoculation. The maximum seed yield (1.4 t $ha^{-1}$) obtained by fertilizer application 45:80:55 kg NPK $ha^{-1}$+ rhizobium inoculation (Hossain et al. 2011).

### 2.3 Biological Nitrogen Fixation

All organisms require nitrogen for the function of biochemical agents like chlorophyll, enzymes and nucleic acids such as DNA and RNA (Kramer 2000). Although nitrogen is abundant in the atmosphere, it is not readily available to plants (Unkovich et al. 2008). Atmospheric nitrogen can be fixed industrially through biological nitrogen fixation (BNF) (Mulongoy 1992). BNF is becoming more important for not only as potential cheap alternative to mineral N fertilizers for providing N to crops but also in seeking more sustainable agricultural production (Boddey et al. 1997; Giller et al. 1997). Biological fixation of atmospheric nitrogen can be estimated at about 175 million metric tons $year^{-1}$ or about 70% of all nitrogen fixed on the earth per year, the remaining is by some micro-organisms, autotrophs or heterotrophs 'free' fixers (Peter et al. 2002). The transformation or fixation of nitrogen from the unavailable gaseous form in the atmosphere to forms that plants and other organisms can use (either $NH_4^+$ or $NO_3^-$) is mediated by (i) bacteria in symbiotic relationships with vascular plants, (ii) symbiosis between cyanobacteria and fungi (lichens) or plants, (iii) free living heterotrophic or autotrophic bacteria that are typically associated with soil or detritus and (iv) abiotic reactions occur without microbes in the atmosphere associated with lightening (Timoth 1999).

#### 2.3.1 Nitrogen fixing organisms

No plant species is able to reduce atmospheric dinitrogen ($N_2$) into ammonia ($NH_3$) and use it directly for its growth. Only a number of prokaryotic microorganisms including bacteria and cyanobacteria possess the ability to fix dinitrogen (Nghia and Gyurjan 1987). Nitrogen fixing prokaryotes are able to make completely useful associations with plants: from loose associations to intercellular symbioses. There exist associative symbiosis in which nitrogen fixing prokaryotes (eg, *Azospirillum* and *Azotobacter*) have been found in rhizosphere of different plants such as sugarcane, maize, wheat, rice, grasses and others. Both plant and bacteria can live separately but the association is very beneficial for them. It was reported that in

plants, up to 25% of total nitrogen came from nitrogen fixation (Affourtit et al. 2001). Activity of nitrogen fixing microorganisms depends greatly upon excessive amount of carbon compounds that obtained directly from photosynthesis or decay of organic wastes in soils and adequately low level of combined nitrogen (Andrew et al. 2007). Also plant roots release substances into soil, which support colonization and nitrogen fixing activity of bacteria in rhizosphere of plants (Nghia and Gyurjan 1987). Plants that are capable of releasing exudates exhibit higher nitrogen fixation activity in soil (Egamberdieva and Kucharova 2008). Besides, many microorganisms are able to produce hormones and these substances can influence plant growth effectively (Andrew et al. 2007). Nitrogen fixing organisms are generally active in plant root zone. Nitrogen fixing organisms are *Rhizobium*, *Azospirillum*, *Azotobacter*, *Azolla*, *Cyanobacteria* and *Gluconacetobacter diazotrophicus* (Shridhar 2012). The legume-rhizobium symbiosis is one of the most efficient fixing systems which is able to fix approximately from 100 to more than 300 kg N $ha^{-1}$ $year^{-1}$ (Nghia and Gyurjan 1987).

### 2.3.2 Legume-rhizobium symbiosis

The interaction between plants and symbiotic soil microorganisms are important indicators of ecosystem productivity and diversity (Thrall et al. 2011). The fixation of atmospheric $N_2$ by the legume-rhizobium symbiosis is a central element of the N-cycle in agricultural and natural ecosystems (Reichman 2007). In legume-rhizobium symbiosis, macrosymbiont is the legume plant and microsymbiont is the prokaryotic bacteria (rhizobium) (Brahmaprakash and Pramod 2012). Nitrogen fixation involves a complicated interaction between rhizobia and host plants that results in the formation of specialized organs called nodules (Sessitsch et al. 2002). The establishment of nodulation is a complex process involving (1) molecular recognition of the rhizobia by the host plant and the host plant by the rhizobia, (2) formation of an infection thread and invasion by the rhizobia, (3) formation of nodules, (4) conversion of bacteria into bacteroids, and (5) establishment of symbiotic nitrogen fixation (Freiberg et al. 1997).

The specific symbiotic association between rhizobia and leguminous plants results in the formation of specialized structures, called root nodules, where bacteria can convert dinitrogen into ammonia and supply it to the host plant in exchange for carbohydrates (Young 1992). Formation of symbiotically effective root nodules

involves signaling between host and microsymbiont. Flavonoids and/or isoflavonoids released from the root of legume host induce transcription of nodulation genes in compatible rhizobia, leading to the formation of lipochitooligosaccharide (nod factors) that in turn, signal the host plant to begin nodule formation. Nod factors produced by rhizobia determine the host range (Long 1996) and play a pivotal role in the molecular signal exchange, infection and induction of symbiotic developmental responses in legumes (Reddy et al. 1998). A complete and efficient nitrogen fixation in legume-rhizobium symbiosis requires the coordinate interaction of several major classes of genes present in rhizobia: the *nif* genes and *fix* genes (Kaminski et al. 1998) for atmospheric nitrogen fixation, and the *nod*, *nol* and *noe* genes for nodulation (Downie 1998).

In response to nod factors, many of the developmental changes occurred in the host plant. Rhizobia present in the rhizosphere begin to multiply on the surface of young root of an emerging legume plant. The legume roots begin to curl and rhizobia enter the roots hairs through the infection thread. After about two weeks, small bumps appear on the roots. These bumps eventually become larger and mature into fully functional nodules. Within the developing nodule, the rhizobia become swollen. At this stage, they are called bacteroids. Nitrogen gas from the soil atmosphere reaches the bacteroids through pores in the nodule. The bacteroids produce the enzyme nitrogenase. 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). Nitrogenase catalyzes the ATP dependent reduction of atmospheric dinitrogen to ammonia. The ammonia attaches to a compound provided by the plant, forming amino acids. These amino acids move out of the nodule to other parts of the plant where they undergo further changes. They are mainly used to produce proteins. The bacteroids need large amounts of energy to support their nitrogen-fixing activity. The plant provides energy as sugars, produced through photosynthesis. Legume-rhizobium symbiosis requires about 10 kg of carbohydrates (sugars) for each kg of $N_2$ fixed. This process is 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 (Lindemann and Gloves 2003). Various legume crops and pasture species often fix as much as 200 to 300 kg N $ha^{-1}$ (Ghosh et al. 2007) or

about 70 million metric tons N year$^{-1}$ (Sebbane et al. 2006).

**2.4 Rhizobia Diversity and Specificity**

Rhizobia are genetically diverse and physiologically heterogeneous group of bacteria that were originally classified together with their nodulating members of leguminosae. Morphologically, rhizobia are gram-negative, rod-shaped cells, medium-sized, 0.5-0.9 µm in width and 1.2-3.0 µm in length. They are motile by a single polar flagellum or six peritrichous flagella, grow well in the presence of oxygen and utilize relatively simple carbohydrates and amino compounds. Optimal growth of most strains occurs at a temperature range of 25˚C-30˚C and pH of 6.0-7.0. They do not form endospores but increase through cell division. Their life cycle consists of three phases: saprophytic, infective and symbiotic. As saprophytes rhizobia live in the soil without their legume host. These are referred to as native rhizobia (Somasegaran and Hoben 1994).

Differences in rates of growth allowed early separation of rhizobia into two basic groups, fast and slow growers. The main genera *Rhizobium* is characterized by fast growth, have generation times of less than 6 hours and generally forms visible colonies within 2-5 days. They produce an acid growth reaction. Rhizobia isolated from pea, bean, clover, alfalfa, chickpea, and leucaena are all fast growers. The *Bradyrhizobium* are slow growers that have generation times exceeding 6 hours and give detectable growth after more than 5 days under aseptic conditions. They produce an alkaline reaction. The soybean and cowpea rhizobia are slow growers (Jordan 1984).

The occurrence of a wide diversity of strains increases the opportunity for a legume host to find a compactible rhizobium in any particular soil. Although legumes species can be nodulated by several rhizobia species, some are very restrict for nodulation. For example, common bean (*Phaseolus vulgaris*) is known as a promiscuous host, since it can be nodulated by rhizobia belonging to diverse genera such as *Bradyrhizobium*, *Rhizobium* and *Ensifer* while chickpea (*Cicer arietinum* L.) is considered a restrict host, because it is nodulated only by *Mesorhizobium* species. Nevertheless, the host range depends on the legume cultivar used and conditions tested (Romero 2003). Nodulation and nitrogen fixation require not only host and microorganism are compatible but also the soil environment be appropriate for the exchange of signals that precedes infection (Hirsch et al. 2003). The specificity of the

legume-rhizobium symbiotic interaction is largely determined by the recognition of signal molecules produced by both the bacteria and the plant host. Rhizobia can recognize their compatible host when specific flavonoid molecules are released either from the seed or the roots of legumes. This recognition event triggers gene transcription in the bacterium leading to the expression of nodulation genes commonly known as Nod factors. In turn, the nodulation genes encode enzymes that synthesize a very special signal molecule, called a lipo-chitooligosaccharide. The nodulation signal is emitted and then recognized by the plant through specific receptors (Sprent 2009).

**2.5 Assessment of Symbiotic Effectiveness**

Symbiotic effectiveness is the term used to describe the ability of a nodulated plant to fix nitrogen. It can be expressed qualitatively as high, moderate, or low effectiveness or quantitatively based on a comparison of the dry weight or total plant nitrogen of plants receiving adequate combined nitrogen with those of plants inoculated with a standard strain of known performance.

Symbiotic nitrogen fixation can be determined directly by measuring symbiotic nitrogen fixation and fixation ability or indirectly by identification of all characteristics or traits that contribute to symbiotic nitrogen fixation (Htut 1990). Accurate measurement of symbiotic biological nitrogen fixation in legumes is important for improving the efficiency of nitrogen fixation and determining its contribution to an agricultural system (Wani et al. 1995).

Plants should be harvested during flowering period to estimate the amount of nitrogen fixed (Karaca and Uyanoz 2012). Size and color of nodules should be examined for more accurate evaluation (Rebah et al. 2002). Nodules harboring efficient rhizobia are usually large and they contain leghemoglobin and are colored pink to red. Nodules formed by inefficient rhizobia are small and white. Total nodule mass formed by effective rhizobia and the quantity of nitrogen fixed is linearly related (Wadisirisuk and Weaver 1985). Hardarson and Atkins (2003) mentioned that the number and mass of nodules can provide a rough indication of the amount of nitrogen fixed. The shoot dry weight of plants harvested at floral initiation is generally accepted criterion for nitrogen-fixing effectiveness (Prevost and Antoun 2006). As total nitrogen content and nodule dry weight frequently correlate well with shoot dry weight, the latter parameter provides an acceptable distinct factor for strain

comparison (Somasegaran and Hoben 1994).

### 2.6 Factors Affecting Biological Nitrogen Fixation

The biological nitrogen fixation efficiency depends on (i) soil fertility conditions and macro/micronutrient supply, (ii) climatic factors (temperature and photoperiod), (iii) bacterial strain competitiveness, (iv) the amount and the quality of the inoculant, (v) the care in the inoculation process and (vi) the absence of antagonistic agrochemicals on the seed (Campo and Hungria 2004). These factors affect the microsymbiont, the host-plant, or both.

Among the physical properties of soil, the type, texture and structure affect the nitrogen fixing microbes and thereby the amount of nitrogen fixed. In general, loamy and clay soils favour better nitrogen fixation than sandy soils. This is attributed to the poor microbial activity and lesser water-holding capacity of the latter types of soil (Puiatti and Sodek 1999). Excessive moisture and waterlogging prevent the development of root hairs and sites of nodulation, and interfere with a normal diffusion of $O_2$ in the root system of plants. The lack of $O_2$ is a major problem for root respiration and results in loss of nitrogenase activity (Sprent and Gallacher 1976). The number of rhizobia in soil declines drastically as soil dries. And drought also affects the process of infection and fixation. Prolonged drought will promote nodule decay (Giller 2001). Salinity does not affect rhizobia colonization but does retard the initiation or growth of new nodules (Rao et al. 2002). Soil acidity limits symbiotic nitrogen fixation by reducing rhizobium survival, persistence in soils and nodulation. At or below pH 4.8, aluminium will reduce root growth while manganese disrupts photosynthesis and other functions of growth resulting in the reduction of nitrogen fixation by rhizobia (Duncan 1999). Phosphorous (P) fertilization is the major mineral nutrient yield determinant among legume crops (Chaudhary 2008). Symbiotic plants need higher rates of P fertilization for nodule development, function, signal transduction and membrane biosynthesis than nitrogen fed plants (Ribet and Drevon 1996). Mineral N inhibits the rhizobium infection process from impairment of the recognition mechanisms by nitrates and nitrogen fixation in active nodules that is due to diversion of photosynthates toward assimilation of nitrates (Mulongoy 1992).

The two important climatic determinants are temperature and light. Extreme temperatures affect nitrogen fixation adversely because nitrogen fixation is an

enzymatic process. Rhizobia have a poor growth at temperature below 10˚C or above 37˚C. 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 (Bordeleau and Prevost 1994). In tropical and subtropical areas, as high soil temperatures (>40˚C) decrease rhizobial survival and establishment, repeated inoculation of grain legumes and higher rate of inoculation may frequently be needed (Thies et al. 1991). The availability of light regulates photosynthesis, upon which BNF depends.

Among biotic factors, the absence of the required rhizobium constitutes the major constraint in the nitrogen fixation process. The use of efficient isolates of rhizobium as seed inoculant has proved to be the cheapest and most effective way to increase the yield of pulse crops (Kumar and Shrivastava 1994). Because of the native rhizobia are well adapted to the soil conditions, rhizobial isolates (in inoculants) are more competitive and are able to occupy more nodules in host plant.

Various agronomic practices have a profound influence on microbial activity, rhizosphere aeration and crop performance. These, in turn, influence the rate of nitrogen fixation. Seed inoculation with efficient strains of *Bradyrhizobium*, a starter dose of nitrogen through fertilizers, light irrigations to avoid waterlogging and avoiding the use of plant protection chemicals that harm the microbes, positively influence the BNF and lead to greater amounts of nitrogen fixation. On the other hand, untimely sowing, a poor or uneven plant stand, lack of seed inoculation, heavy doses of nitrogen fertilizers, result in poor nodulation and a lower amount of nitrogen fixation (Siyeni 2016).

## 2.7 Role of Rhizobia in Green Gram Production

### 2.7.1 Inoculation with green gram-rhizobium

Inoculation of legumes with rhizobia is a less expensive and more effective agronomic practice for supplying N to these crops, compared with the application of N fertilizers (Crews and Peoples 2004). Inoculation is necessary for any soil in which a well-nodulated green gram crop has not been grown within the prior three years. In sandy soils if the percentage of sand is greater than 90%, inoculation is also necessary because rhizobial viability and survival from one season to the next is relatively low. Moreover, in many soil, the strain, number, quality, or virulence of indigenous nodulating bacteria is not adequate for nitrogen fixation (FAO 1984).

Therefore, inoculation of seeds or soils with effective strains is necessary because biological nitrogen fixation depends on the occurrence, survival, and efficiency of rhizobia in the soil (Adamovich and Klasens 2001). Selection of rhizobia for inoculants that are well adapted to the edaphic and climatic conditions of the agricultural region is essential to maximize the BNF in legume systems (Hardarson and Atkins 2003) and cannot be a process carried out over a single year of field trials and over few locations.

Among seed-applied inoculation and soil-applied inoculation methods, seed-applied inoculation method is more effective when the inoculants are mixed with water, forming slurry to coat the seeds. Peat-based inoculants are mostly used in this inoculation method (Bashan and Carrillo 1996). The soil-applied inoculation method is more convenient and easier than the seed-applied method, but it is more expensive. Therefore, seed-applied inoculation method is generally chosen by most farmers (Elmore 1984) because inoculation of legume seeds with an appropriate rhizobium strain before sowing is effective (Tittabutr et al. 2005). It was observed that inoculated crop gave 51% higher yield than un-inoculated control (Singh 1977). Tripathi et al. (1994) obtained the seed yield of green gram with rhizobium inoculation similar to that of 20 kg N $ha^{-1}$. Inoculation of green gram with rhizobium increased plant height, leaf area, photosynthetic rate and dry matter production (Thakur and Panwar 1995). Applying seed inoculation with effective rhizobium strains is important agronomic measure which improves intensity of symbiotic nitrogen fixation and satisfies plant need for N (Herridge et al. 2005).

### 2.7.2 Inoculant production in Myanmar

Since 1978, peat based rhizobial inoculant research and commercial production has been conducted in Myanmar by Plant Pathology Section under Department of Agricultural Research, Yezin. Initially, rhizobial inoculant for groundnut, chickpea, green gram, black gram, soybean, pigeonpea and cowpea were produced by using exotic strains from NifTAL project. Investigation of indigenous (local) rhizobium strains has been initiated since 2004. Nowadays, three or four strains (exotic and indigenous) were used to produce inoculant for each legume. Chickpea rhizobial inoculant was produced by using TAL 620, TAL 480 and TAL 1148 strains. TAL 441, TAL 420 and TAL 169 exotic strains were selected to manufacture black gram and green gram inoculants. Local strain (DAR 18) was used

for green gram inoculant. Soybean inoculant was prepared by inoculating exotic strains; TAL 379, TAL 162 and local DAR 1 strain into sterilized peat. Rhizobial inoculant for pigeon pea was manufactured by using exotic and indigenous namely TAL 569, TAL 1127 and DAR 21 strains. TAL 209, TAL 173 and DAR 25 were used to produce cowpea rhizobial inoculant. About 250,000 packets of inoculants are annually produced and sufficient only for 3% of requirement (Than 2010).

# CHAPTER III
# MATERIALS AND METHODS

The experiments were conducted in the laboratory and screen house of Department of Plant Pathology, Yezin Agricultural University (YAU) from 2016 to 2018.

## 3.1 Nodulation Efficiency of Indigenous Rhizobium Isolates on Green Gram Varieties

### 3.1.1 Investigation of indigenous rhizobium isolates

#### 3.1.1.1 Study sites and soil samples collection

Fifty-two soil samples were collected from the green gram growing Townships of Sagaing Region (the third largest green gram growing area of Myanmar) – Yinmarpin (14 soil samples from four villages), Palae (12 soil samples from four villages), Salingyi (10 soil samples from three villages), Butalin (5 soil samples from one village) and Monywa (11 soil samples from three villages) (Appendix 1). Green gram cultivated fields which have no previous history of inoculation with rhizobia produced by Department of Agricultural Research (DAR) were selected from each site and 30 g of soil samples were collected from the upper 15 to 20 cm depth by using cross diagonal pattern. Then these soil samples were pooled together and composite samples (150 g) were put in plastic bags (Jida and Assefa 2011).

#### 3.1.1.2 Rhizobium isolates

Fifty-two indigenous rhizobium isolates were named base on collection sites. Sources of isolates collected from 52 green gram cultivated fields were shown in Table 3.1.

#### 3.1.1.3 Preparation of test plant

Yezin-11 green gram seeds, provided by Food Legumes Section, Department of Agricultural Research (DAR), were surface-sterilized in 5% NaOCl solution for three-four minutes and then thoroughly washed with sterilized water for three times. Sterilized seeds were germinated in Petri dishes. The pots were sterilized using a 10% HCL solution and then washed with distilled water. The pots were filled with 500 g of sterilized sand and 50 ml of N free nutrient solution.

**Table 3.1 Designation of isolates, host varieties and collection sites**

| Sr. No | Isolate | Host variety | Collection site | |
|---|---|---|---|---|
| | | | Village | Township |
| 1 | YMP 1 | Yezin-11 | Lingyauk | Yinmarpin |
| 2 | YMP 2 | Yezin-11 | Lingyauk | Yinmarpin |
| 3 | YMP 3 | Yezin-11 | Lingyauk | Yinmarpin |
| 4 | YMP 4 | Yezin-11 | Ngarmaung | Yinmarpin |
| 5 | YMP 5 | Yezin-11 | Ngarmaung | Yinmarpin |
| 6 | YMP 6 | Yezin-11 | Ngarmaung | Yinmarpin |
| 7 | YMP 7 | Yezin-11 | Nonegyi | Yinmarpin |
| 8 | YMP 8 | Yezin-11 | Nonegyi | Yinmarpin |
| 9 | YMP 9 | Yezin-14 | Nonegyi | Yinmarpin |
| 10 | YMP 10 | Yezin-11 | Nonegyi | Yinmarpin |
| 11 | YMP 11 | Yezin-11 | Nonegyi | Yinmarpin |
| 12 | YMP 12 | Yezin-14 | Ywartarwar | Yinmarpin |
| 13 | YMP 13 | Yezin-11 | Ywartarwar | Yinmarpin |
| 14 | YMP 14 | Yezin-11 | Ywartarwar | Yinmarpin |
| 15 | PLE 1 | Yezin-14 | Latbagan | Palae |
| 16 | PLE 2 | Yezin-14 | Latbagan | Palae |
| 17 | PLE 3 | Yezin-14 | Latbagan | Palae |
| 18 | PLE 4 | Yezin-11 | Kokekokone | Palae |
| 19 | PLE 5 | Yezin-11 | Kokekokone | Palae |
| 20 | PLE 6 | Yezin-11 | Kokekokone | Palae |
| 21 | PLE 7 | Yezin-14 | Ayekone | Palae |
| 22 | PLE 8 | Yezin-14 | Ayekone | Palae |
| 23 | PLE 9 | Yezin-14 | Ayekone | Palae |
| 24 | PLE 10 | Yezin-14 | Chaungu | Palae |
| 25 | PLE 11 | Yezin-14 | Chaungu | Palae |
| 26 | PLE 12 | Yezin-14 | Chaungu | Palae |
| 27 | SLG 1 | Yezin-11 | Ywarthamin | Salingyi |
| 28 | SLG 2 | Yezin-11 | Ywarthamin | Salingyi |
| 29 | SLG 3 | Yezin-11 | Ywarthamin | Salingyi |

**Table 3.1 (continued)**

| Sr. No | Isolate | Host variety | Collection site | |
|---|---|---|---|---|
| | | | Village | Township |
| 30 | SLG 4 | Yezin-11 | Kyartat | Salingyi |
| 31 | SLG 5 | Yezin-14 | Kyartat | Salingyi |
| 32 | SLG 6 | Yezin-14 | Kyartat | Salingyi |
| 33 | SLG 7 | Yezin-14 | Kanni | Salingyi |
| 34 | SLG 8 | Yezin-14 | Kanni | Salingyi |
| 35 | SLG 9 | Yezin-14 | Kanni | Salingyi |
| 36 | SLG 10 | Yezin-14 | Kanni | Salingyi |
| 37 | BTL 1 | Yezin-11 | Kwunchan | Butalin |
| 38 | BTL 2 | Yezin-11 | Kwunchan | Butalin |
| 39 | BTL 3 | Yezin-11 | Kwunchan | Butalin |
| 40 | BTL 4 | Yezin-11 | Kwunchan | Butalin |
| 41 | BTL 5 | Yezin-11 | Kwunchan | Butalin |
| 42 | MWA 1 | Yezin-11 | Kyarpaing | Monywa |
| 43 | MWA 2 | Yezin-11 | Kyarpaing | Monywa |
| 44 | MWA 3 | Yezin-11 | Kyarpaing | Monywa |
| 45 | MWA 4 | Yezin-11 | Tawpu | Monywa |
| 46 | MWA 5 | Yezin-11 | Tawpu | Monywa |
| 47 | MWA 6 | Yezin-11 | Tawpu | Monywa |
| 48 | MWA 7 | Yezin-11 | Tawpu | Monywa |
| 49 | MWA 8 | Yezin-11 | Myaynae | Monywa |
| 50 | MWA 9 | Yezin-11 | Myaynae | Monywa |
| 51 | MWA 10 | Yezin-11 | Myaynae | Monywa |
| 52 | MWA 11 | Yezin-11 | Myaynae | Monywa |

### 3.1.1.4 Inoculum preparation and inoculation

Collected soil samples were air-dried, ground and passed through a 2 mm sieve to remove stones and large pieces of organic matter. Two grams of each composite soil sample was mixed with 98 ml of sterilized yeast mannitol broth (Appendix 2) solution in a 200 ml conical flask. The flask was shaken on a rotary shaker at 120 rpm for one hour to prepare a well-mixed soil suspension. 5 ml of aliquot soil suspension was inoculated beside the pre-germinated seed (Htwe 2016). Then the surface of the substrate was covered with sterilized gravel (about 2 cm) as an anti-contamination layer. N free nutrient solution was provided to inoculated and un-inoculated ($N^-$ control) plants at the rate of 50 ml $plant^{-1}$ at three day interval. For un-inoculated ($N^+$ control) plants, 70 ppm $KNO_3$ in nutrient solution was supplied (Appendix 3).

### 3.1.1.5 Experimental design and data recording

The experiment was laid out in Completely Randomized Design (CRD) with four replications. Plants were harvested at 35 days after sowing (DAS) (Karaca and Uyanoz 2012). The plants were uprooted and gently washed with water not to remove the root hairs and nodules. Nodules were carefully separated from the roots by hand. The plants were cut at the root crown to separate the shoot and root portions. The nodules and shoots were oven dried at 60˚C for 48 hours and 72 hours respectively (Cheminingwa et al. 2007). Nodule dry weight (g), shoot fresh weight (g) and shoot dry weight (g) were recorded. Symbiotic effectiveness percent (SE%) of the isolates was also calculated by using the following formula (Elkan 1987).

$$SE\ \% = \frac{\text{Shoot dry weight of inoculated plants}}{\text{Shoot dry weight of un-inoculated } (N^+) \text{ control plants}} \times 100\ \%$$

Finally, the symbiotic effectiveness (SE) values of the isolates were rated as highly effective (>80%), effective (51-80%), lowly effective (35-50%) and ineffective (<35%) (Beck et al. 1993).

Percent differences in shoot dry weights (SDW) between inoculated and $N^+$ control plants were calculated as the following formula described by Zerihun and Fassil (2010).

$$\text{Increased SDW (\%)} = \frac{\text{SDW of inoculated plants} - \text{SDW of } N^{+} \text{ control plants}}{\text{SDW of } N^{+} \text{ control plants}} \times 100\ \%$$

#### 3.1.1.6 Statistical analysis

Analysis of variance (ANOVA) was performed on the data using Statistix Version 8.0 program and means were separated by Least Significant Difference (LSD) test at 5% probability level.

### 3.1.2 Authentication of symbiotic effectiveness of selected rhizobium isolates on different green gram varieties

#### 3.1.2.1 Test varieties

Five commercial green gram varieties; MAS-1, Yezin-1, Yezin-9, Yezin-11 and Yezin-14 supplied by Food Legumes Section, Department of Agricultural Research (DAR), were used as test varieties.

#### 3.1.2.2 Rhizobium isolates

Five indigenous rhizobium isolates; YMP 1, YMP 7, YMP 11, PLE 1 and PLE 5 were selected based on the results of the previous experiment.

#### 3.1.2.3 Isolation of effective-indigenous rhizobium isolates

Isolation was conducted at Department of Plant Pathology, YAU. Root nodules from 35-day-old plant were initially washed in sterilized distilled water to remove the soil. Then, the nodules were surface-sterilized in 5% sodium hypochloride solution (NaOCl) for three minutes and rinsing three times in sterilized distilled water. The nodules were crushed in small bottles containing 1 ml of sterilized distilled water. A loopful of the bacterial suspension was isolated on plated yeast mannitol agar media (YMA) containing congo-red (25 µg $ml^{-1}$) (Appendix 2) by streaked plate method and single colonies were selected after incubation at room temperature for five-seven days (Plate 3.1) (Bala et al. 2011). Single colonies were sub-cultured on YMA slants to obtain pure cultures. The isolates were examined by using gram staining method and checked under the microscope.

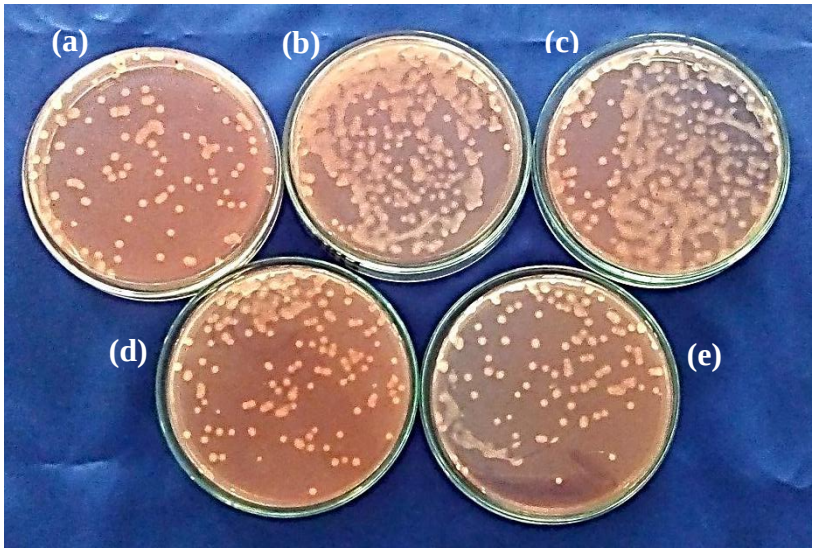



**Plate 3.1 Colonies of five isolates (a) YMP 1, (b) YMP 7, (c) YMP 11, (d) PLE 1 and (e) PLE 5 on Yeast Mannitol Agar (YMA) medium**

### 3.1.2.4 Inoculum preparation and inoculation

YMP 1, YMP 7, YMP 11, PLE 1 and PLE 5 isolates were used as the inocula. Inocula were prepared by suspending the bacterial mass in sterilized water. Bacterial population in broth culture was measured by using Spectrophotometer and was estimated to be $10^8$ cells $ml^{-1}$. Then, 1 ml of bacterial suspension was inoculated beside the pre-germinated seed by using sterilized syringe (Somasegaran and Hoben 1985). Then, the surface of the substrate in the plastic pot was covered with sterilized gravel (about 2 cm thick layer) as an anti-contamination layer. Un-inoculated $N^+$ control and $N^-$ control treatments were also provided. N free nutrient solution was poured at the rate of 50 ml $plant^{-1}$ at three day interval.

### 3.1.2.5 Experimental design and data recording

Factorial experiment was laid out in a Completely Randomized Design (CRD) with three replications. There were two factors involving five rhizobium isolates and two un-inoculated controls as the first factor, and five green gram varieties as the second factor. Data recording was done as in the section 3.1.1.5.

### 3.1.2.6 Statistical analysis

Analysis of variance (ANOVA) was performed on by using Statistix Version 8.0 program and means were separated by Least Significant Difference (LSD) test at 5% probability level.

## 3.2 Effect of Indigenous Rhizobium Inoculant on Yield and Yield Components of Different Green Gram Varieties

### 3.2.1 Inoculum preparation

YMP 11 rhizobium isolate was cultured on yeast mannitol agar media at 28˚C for five days (Plate 3.2). Bacterial suspension was prepared by suspending the bacterial mass in sterilized water. One ml of rhizobial suspension was transferred to 250 ml of yeast mannitol liquid media. Bacterial suspension was incubated in the shaker for 72 hours at 28˚C (Weaver and Frederick 1982). Bacterial population in broth culture was estimated to be $10^8$ cells $ml^{-1}$ by using Spectrophotometer. The carrier (peat) was first ground, sieved and packed in polyethylene bags. The pH of the peat was adjusted to 6.7 adding 17.5 g $Na_2CO_3$ for 50 g peat packet (Valdiviezo

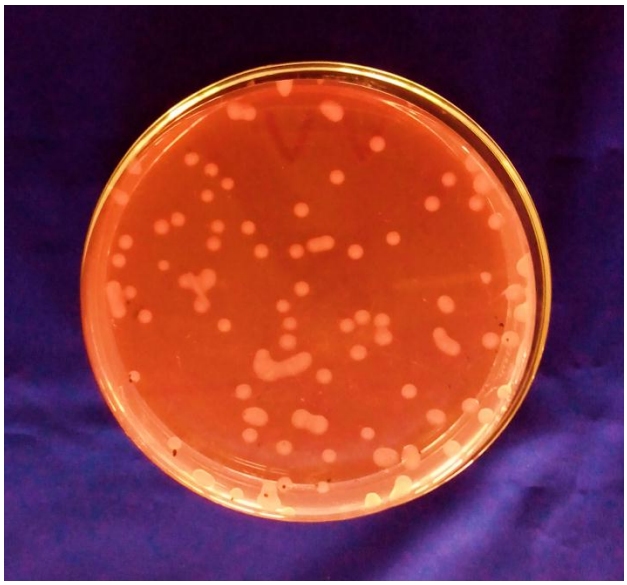


**Plate 3.2 Colonies of YMP 11 isolate on Yeast Mannitol Agar (YMA) medium**

**Plate 3.3 Pot experiment for evaluation of effective-indigenous rhizobium inoculant (YMP 11) on yield and yield components of five green gram varieties**

et al. 2015). The peat was sterilized at 121˚C for 20 min. Then 25 ml of bacterial suspension ($10^8$ cells $ml^{-1}$) was injected into the 50 g sterile ground peat with sterile plastic disposable syringe fitted with a sterile needle gauge. The punctured area was wiped with ethyl alcohol and the punctured hole was immediately covered with cellophane tape. Each bag was kneaded thoroughly to ensure even distribution and proper missing of the broth culture with the substrates. All operations were carried out aseptically in a laminar flow chamber. After injection, the packets were incubated for two weeks to contain $10^8$ cells $g^{-1}$ of peat (Somasegaran and Hoben 2004).

### 3.2.2 Preparation of test plants and inoculation

Seeds of test varieties; MAS-1, Yezin-1, Yezin-9, Yezin-11 and Yezin-14 were surface-sterilized as in the previous experiment. Accordingly, using peat based inoculation method at the recommended rate of 10 g per kilogram of seed, 6g of green gram seed was soaked in 1 ml of 5% sugar solution (Funga et al. 2016). The contents were stirred gently. Sugar solution improves the adhesion of inoculant to the seeds. Then 0.06 g of inoculant was added on the wetted seed and mixed in a plastic cup until the seeds were fairly evenly coated. To avoid direct exposure of coated seeds to sunlight that damages the bacteria, the whole inoculation procedure was performed in shade. The seeds were sown immediately at three seeds per a sterilized plastic pot (15 cm in diameter) which were filled with 1.5 kg of sterilized soil. Plastic discs were placed under the pots to prevent the percolation of nutrient solution from the pots. Thinning was done 3 days after sowing and only one plant was left in a pot. The un-inoculated seeds were sown before the inoculated ones to avoid contamination of the former. Un-inoculated nitrogen-fertilized $N^+$ control plants and un-inoculated $N^-$ control plants were included. The plants were irrigated with distilled water every three days and they were supplied once a week with a nitrogen-free nutrient solution. Although positive control received $KNO_3$ as nitrogen source weekly, all other plants received this solution initially as starter nitrogen only one (Somasegaran and Hoben 1994).

### 3.2.3 Experimental design and data recording

Factorial experiment was laid out in a Randomized Completely Block Design (RCB) with four replications (3 ×5 × 4) (Plate 3.3). There were two factors involving

rhizobium inoculant and un-inoculated controls as the first factor, and five green gram varieties as the second factor. Plant height (cm) was recorded weekly from 14 DAS until flowering time. At harvest, number of pods, pod length and number of grains $pod^{-1}$ were recorded. And then, harvested shoots and grains were oven-dried at 60˚C for 48 hours. Dry matter weights and grain weight were determined. After that, yield components such as grain yield (g $plant^{-1}$), hundred grains weight (g) and total dry matter (g $plant^{-1}$) were also recorded (Han 2012).

### 3.2.4 Statistical analysis

Statistix program Version 8.0 was used for the collected and analyzed data. All means were separated by Least Significant Differences (LSD) at 5%.probability level.

# CHAPTER IV
# RESULTS AND DISCUSSION

## 4.1 Nodulation Efficiency of Different Indigenous Rhizobium Isolates on Green Gram Varieties

### 4.1.1 Investigation of indigenous rhizobium isolates

All tested 52 indigenous rhizobium isolates collected from Yinmarpin, Palae, Salingyi, Batalin and Monywa soils were able to nodulate Yezin-11 green gram. Nodule formations were observed on the root of Yezin-11 variety when inoculated with each isolate. However, there was no nodule formation in the plants treated with $N^+$ and $N^-$ controls (Plate 4.1, 4.2, 4.3, 4.4 and 4.5). Nodule dry weight and shoot dry weight of green gram inoculated with 52 rhizobium isolates were significantly different from each other (Table 4.1 and 4.2). Than (2010) and Zaw (2014) found that the effectiveness of different indigenous rhizobium isolates differed significantly with each other in terms of nodule number, nodule dry weight and shoot dry weight, respectively in chickpea. Vijila and Jebaraj (2008) also reported that all tested strains nodulated their host very well with different level of infectivity in green gram. In this study, these differences pointed out rhizobium isolates performed differently in their efficiency in nodulation and plant growth of Yezin 11 green gram. YMP and PLE isolates gave better performance in nodulation and plant growth than SLG, BTL and MWA isolates (Figure 4.1 and 4.2). Rhizobial strains within a species vary in their ability in nodulation and nitrogen fixation (Kucey et al. 1988).

The nodule dry weight and shoot dry weight of the plants inoculated with 14 isolates from Yinmarpin Township were ranged from 36.75 to 114.50 mg $plant^{-1}$ and 1061.8 to 1584.5 mg $plant^{-1}$, respectively. Among 14 isolates, the highest nodule dry weight was recorded in plants inoculated with YMP 11, which was significantly higher than those of the plants inoculated with other tested isolates. The nodule dry weights of the plants inoculated with YMP 1 and YMP 7 were significantly different from those of YMP 3, YMP 4, YMP 6, YMP 8, YMP 9, YMP 10, YMP 12 and YMP 14. The maximum shoot dry weight was observed in plants inoculated with YMP 11 and was significantly higher than those of the plants treated with YMP 5, YMP 8, YMP 9, YMP 10, YMP 12 and YMP 13 isolates (Table 4.2).

Nodule dry weight and shoot dry weight of plants inoculated with 12 tested isolates from Palae Township were recorded between 38.50 and 69.50 mg $plant^{-1}$,

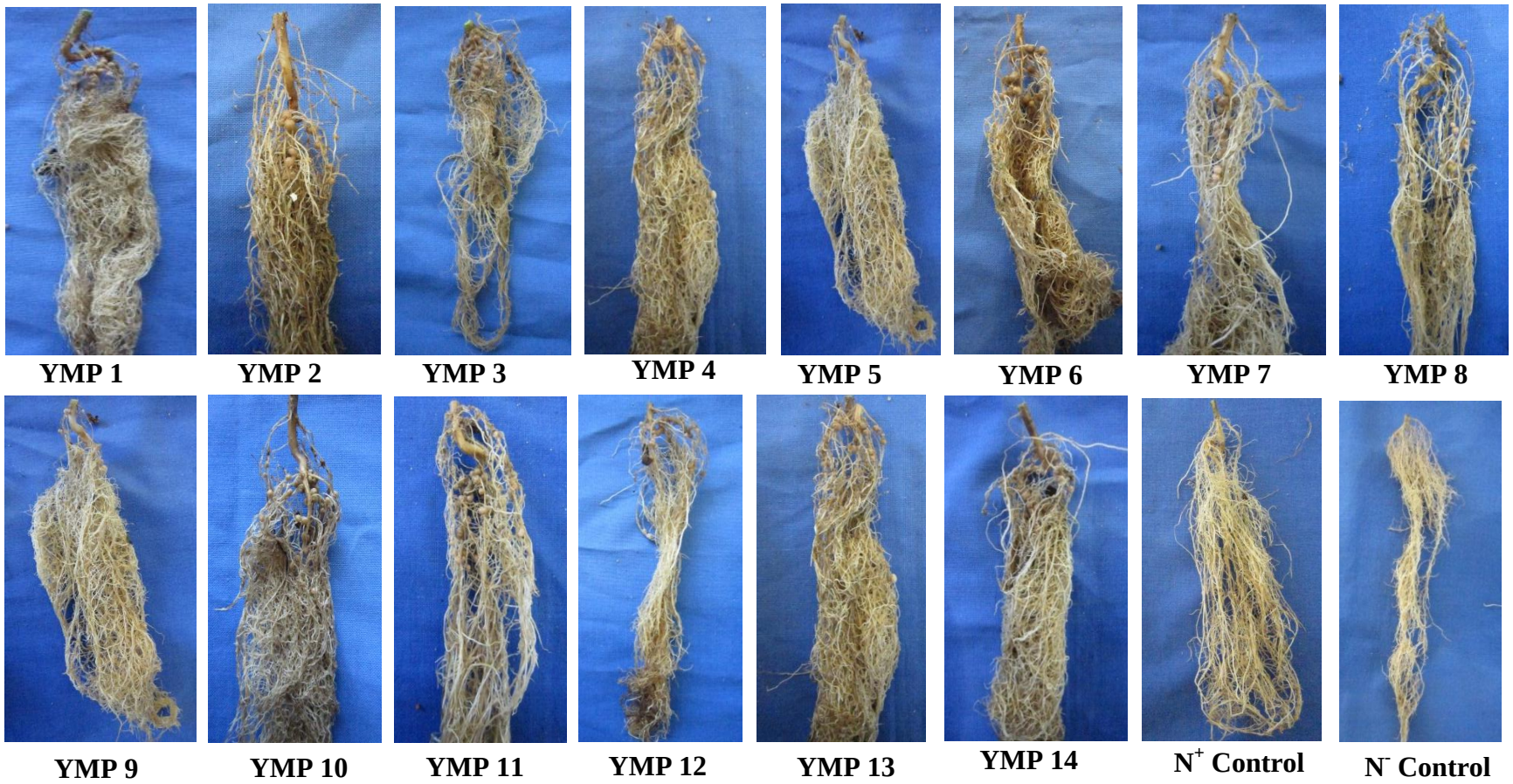



**Plate 4.1 Nodule formation of Yezin-11 green gram, inoculated with 14 rhizobium isolates collected from Yinmarpin Township and un-inoculated controls ($N^+$ and $N^-$) at 35 DAS**

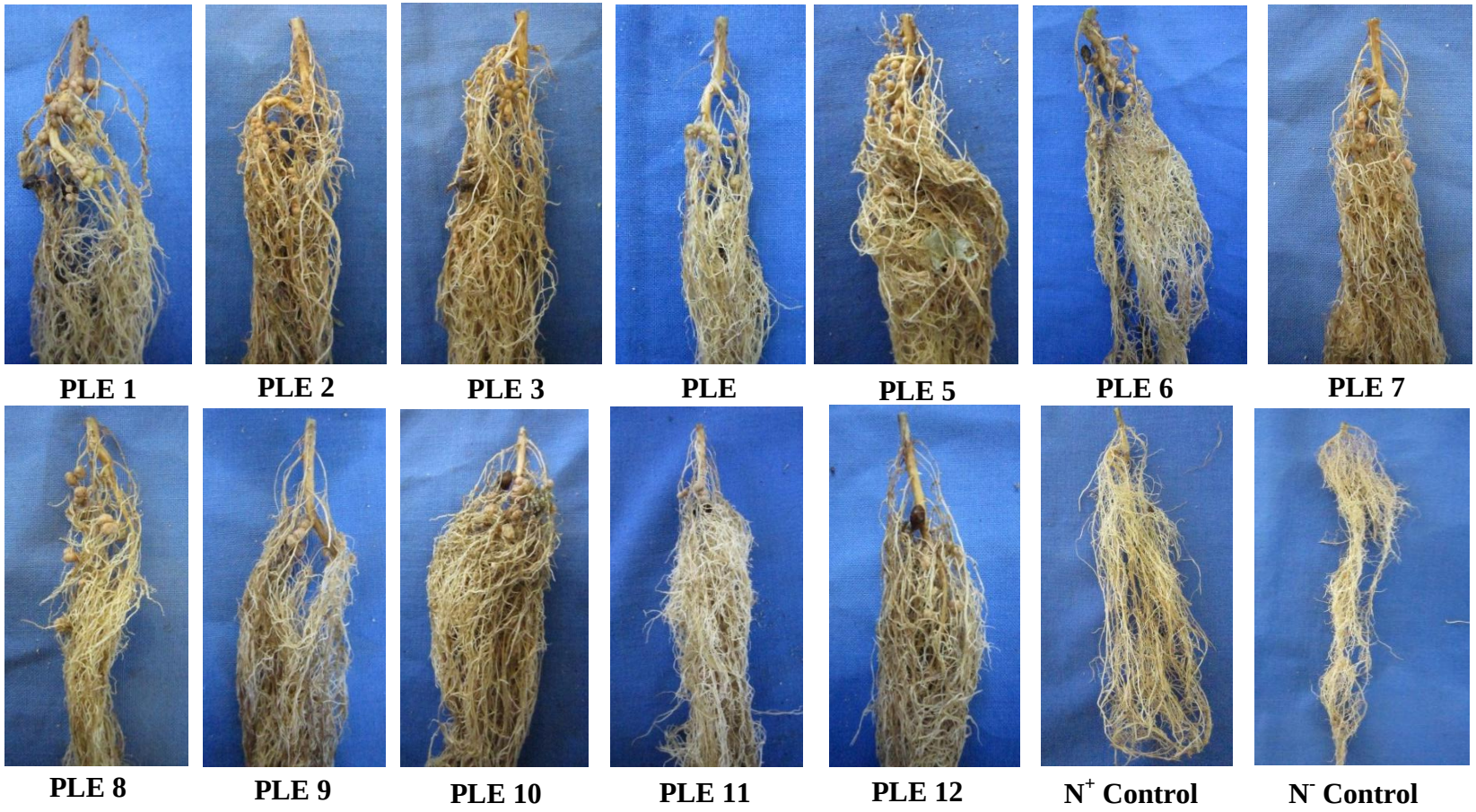



**Plate 4.2 Nodule formation of Yezin-11 green gram, inoculated with 12 rhizobium isolates collected from Palae Township and un-inoculated controls ($N^+$ and $N^-$) at 35 DAS**

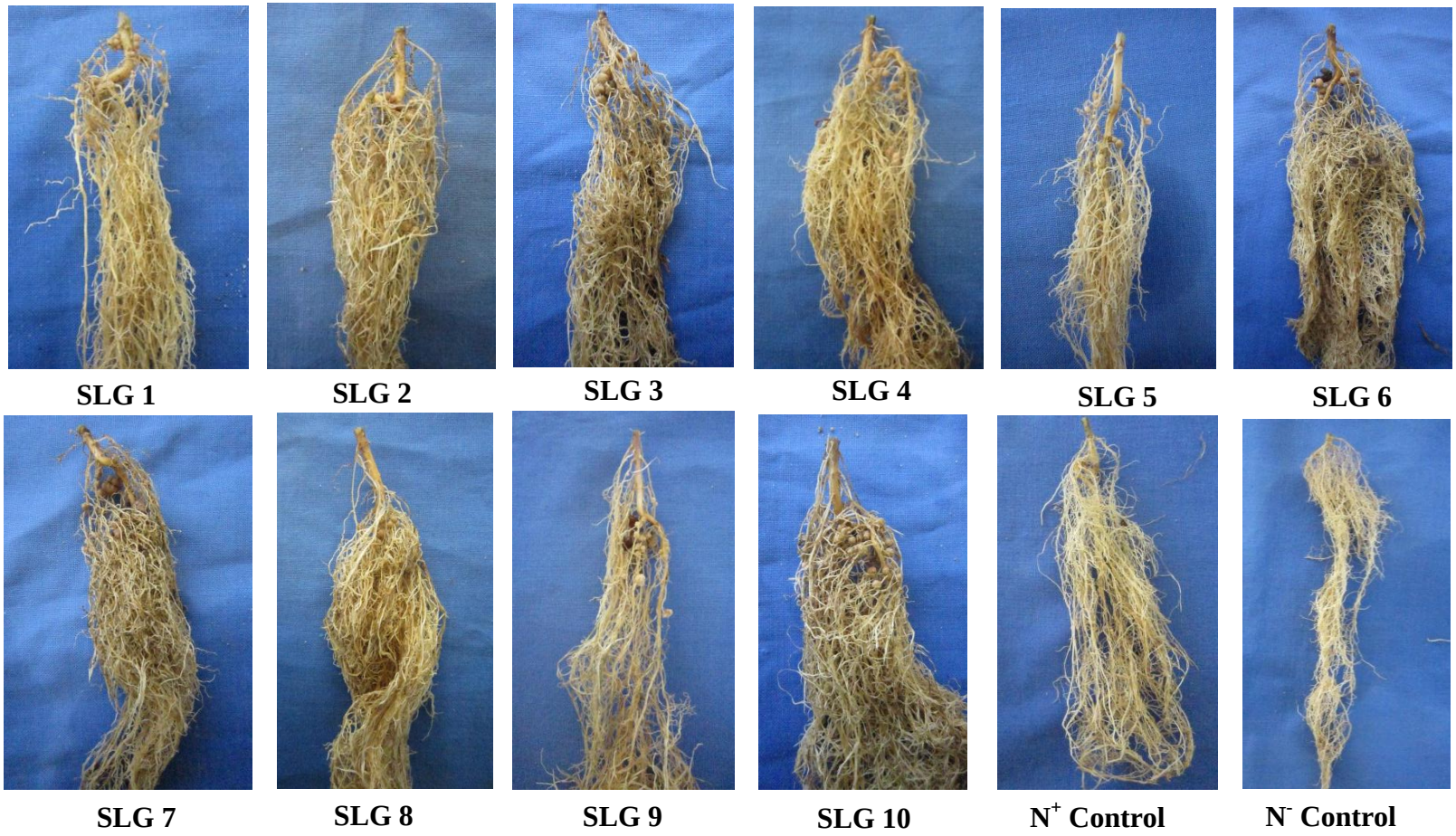



**Plate 4.3 Nodule formation of Yezin-11 green gram, inoculated with 10 rhizobium isolates collected from Salingyi Township and un-inoculated controls ($N^+$ and $N^-$) at 35 DAS**

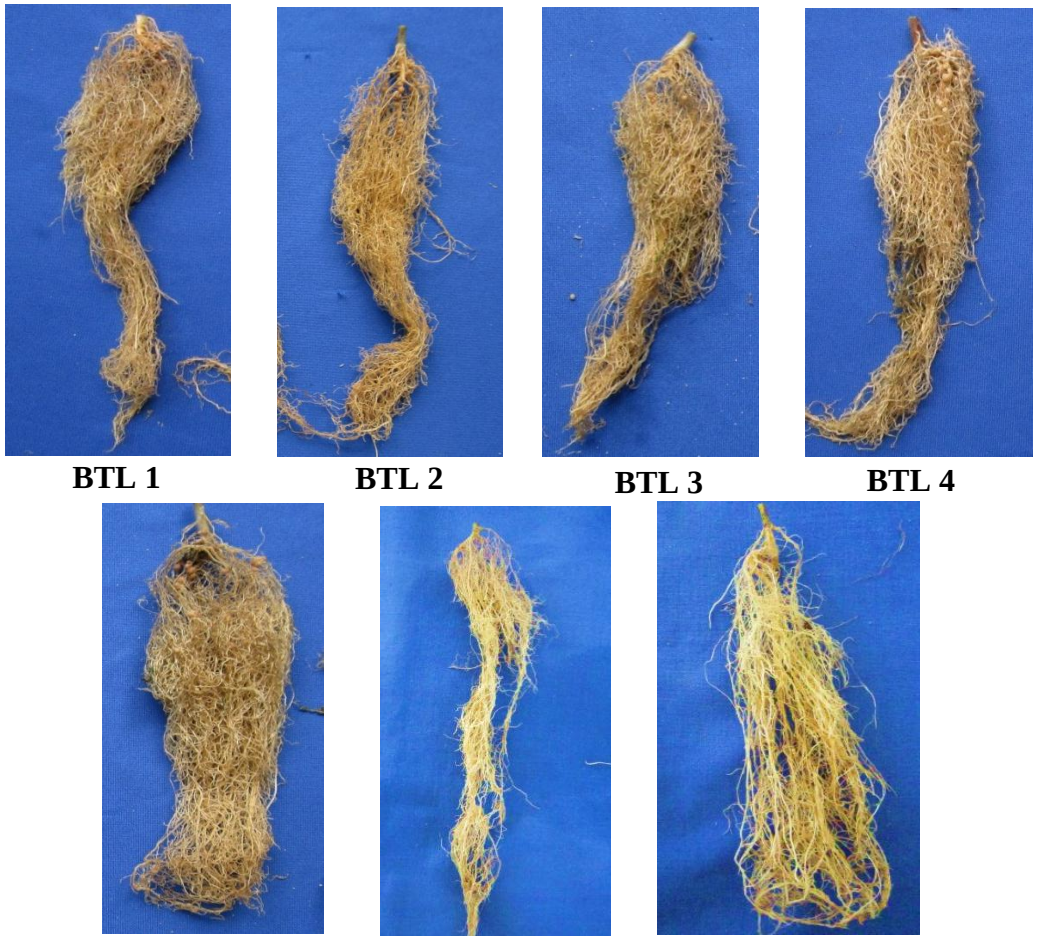



**Plate 4.4 Nodule formation of Yezin-11 green gram, inoculated with 5 rhizobium isolates collected from Butalin Township and un-inoculated control ($N^+$ and $N^-$) at 35 DAS**


**Plate 4.5 Nodule formation of Yezin-11 green gram, inoculated with 11 rhizobium isolates collected from Monywa Township and un-inoculated controls ($N^+$ and $N^-$) at 35 DAS**

**Table 4.1 Analysis of variance for nodule dry weight and shoot dry weight of Yezin-11 green gram inoculated with 52 rhizobium isolates at 35 DAS**

| Source of variation | Degree of freedom | Mean square | |
|---|---|---|---|
| | | Nodule dry weight (mg plant$^{-1}$) | Shoot dry weight (mg plant$^{-1}$) |
| **Treatments** | 53 | 1310.05** | 435250** |
| **Error** | 162 | 229.02 | 71903 |
| **Total** | 215 | | |

** Significant at 1% level

**Table 4.2 Nodule dry weight, shoot dry weight, symbiotic effectiveness (SE%) and percent shoot dry weight increase over $N^+$ control of Yezin-11 green gram inoculated with 52 rhizobium isolates**

| Sr. No. | Isolate | Nodule dry weight (mg plant$^{-1}$)$^x$ | Shoot dry weight (mg plant$^{-1}$)$^x$ | SE% | % increase in SDW over $N^+$ control |
|---|---|---|---|---|---|
| 1 | **YMP 1** | 67.75 bcd$^y$ | 1448.3 abc$^y$ | 283.00 | 183.00 |
| 2 | **YMP 2** | 55.25 b-i | 1280.8 a-h | 250.27 | 150.27 |
| 3 | **YMP 3** | 45.25 f-q | 1356.3 a-g | 265.02 | 165.02 |
| 4 | **YMP 4** | 38.75 h-r | 1249.0 a-h | 244.06 | 144.06 |
| 5 | **YMP 5** | 64.00 b-f | 1145.3 b-j | 223.79 | 123.79 |
| 6 | **YMP 6** | 45.00 f-q | 1349.3 a-g | 263.65 | 163.65 |
| 7 | **YMP 7** | 66.75 b-e | 1403.0 a-e | 274.16 | 174.16 |
| 8 | **YMP 8** | 37.50 h-r | 1201.0 b-i | 234.68 | 134.68 |
| 9 | **YMP 9** | 36.75 i-r | 1061.8 d-m | 207.47 | 107.47 |
| 10 | **YMP 10** | 40.25 g-r | 1142.8 b-j | 223.30 | 123.30 |
| 11 | **YMP 11** | 114.5 a | 1584.5 a | 309.62 | 209.62 |
| 12 | **YMP 12** | 43.00 f-q | 1149.5 b-j | 224.62 | 124.62 |
| 13 | **YMP 13** | 53.75 b-i | 1196.8 b-i | 233.85 | 133.85 |
| 14 | **YMP 14** | 44.75 f-q | 1339.5 a-g | 261.75 | 161.75 |
| 15 | **PLE 1** | 68.25 bc | 1427.8 a-d | 278.99 | 178.99 |
| 16 | **PLE 2** | 52.00 b-k | 1215.8 a-i | 237.57 | 137.57 |
| 17 | **PLE 3** | 54.75 b-i | 1107.5 b-k | 216.41 | 116.41 |
| 18 | **PLE 4** | 44.00 f-q | 1083.0 c-l | 211.63 | 111.63 |
| 19 | **PLE 5** | 69.50 b | 1461.8 ab | 285.64 | 185.64 |
| 20 | **PLE 6** | 38.50 h-r | 924.75 h-p | 180.70 | 80.70 |
| 21 | **PLE 7** | 63.50 b-f | 1369.5 a-f | 267.61 | 167.61 |
| 22 | **PLE 8** | 52.75 b-j | 1149.8 b-j | 224.67 | 124.67 |

**Table 4.2 (continued)**

| Sr. No. | Isolate | Nodule dry weight (mg plant$^{-1}$)$^{x}$ | Shoot dry weight (mg plant$^{-1}$)$^{x}$ | SE% | % increase in SDW over $N^{+}$ control |
|---|---|---|---|---|---|
| 23 | **PLE 9** | 54.00 b-i$^{y}$ | 1247.8 a-h$^{y}$ | 243.82 | 143.82 |
| 24 | **PLE 10** | 60.75 b-g | 1261.3 a-h | 246.46 | 146.46 |
| 25 | **PLE 11** | 46.75 d-o | 1017.5 f-n | 198.83 | 98.83 |
| 26 | **PLE 12** | 40.50 g-r | 990.5 g-o | 193.55 | 93.55 |
| 27 | **SLG 1** | 43.25 f-q | 684.75 n-t | 133.81 | 33.81 |
| 28 | **SLG 2** | 52.50 b-k | 868.25 i-q | 169.66 | 69.66 |
| 29 | **SLG 3** | 48.00 c-n | 912.75 h-p | 178.36 | 78.36 |
| 30 | **SLG 4** | 49.25 b-m | 807.75 j-r | 157.84 | 57.84 |
| 31 | **SLG 5** | 39.75 g-r | 495.00 q-t | 96.73 | -3.27 |
| 32 | **SLG 6** | 58.00 b-h | 1004.5 f-o | 196.29 | 96.29 |
| 33 | **SLG 7** | 46.50 e-p | 706.75 m-t | 138.10 | 38.10 |
| 34 | **SLG 8** | 49.25 b-m | 1047.0 e-n | 204.59 | 104.59 |
| 35 | **SLG 9** | 51.00 b-l | 852.25 i-q | 166.54 | 66.54 |
| 36 | **SLG 10** | 49.75 b-m | 1065.3 d-m | 208.16 | 108.16 |
| 37 | **BTL 1** | 31.75 j-r | 564.50 p-t | 110.31 | 10.31 |
| 38 | **BTL 2** | 26.50 o-r | 686.75 n-t | 134.20 | 34.20 |
| 39 | **BTL 3** | 25.50 pqr | 427.25 st | 83.49 | -16.51 |
| 40 | **BTL 4** | 32.50 j-r | 698.50 m-t | 136.49 | 36.49 |
| 41 | **BTL 5** | 32.00 j-r | 612.25 p-t | 119.64 | 19.64 |
| 42 | **MWA 1** | 24.50 qr | 498.50 q-t | 97.41 | -2.59 |
| 43 | **MWA 2** | 21.50 r | 442.00 rst | 86.37 | -13.63 |
| 44 | **MWA 3** | 27.25 n-r | 591.50 p-t | 115.58 | 15.58 |

**Table 4.2 (continued)**

| Sr. No. | Isolate | Nodule dry weight (mg plant$^{-1}$)$^{x}$ | Shoot dry weight (mg plant$^{-1}$)$^{x}$ | SE% | % increase in SDW over $N^+$ control |
|---|---|---|---|---|---|
| 45 | **MWA 4** | 36.75 i-r$^{y}$ | 764.00 k-s$^{y}$ | 149.29 | 49.29 |
| 46 | **MWA 5** | 29.50 m-r | 635.25 o-t | 124.13 | 24.13 |
| 47 | **MWA 6** | 26.50 o-r | 567.50 p-t | 110.89 | 10.89 |
| 48 | **MWA 7** | 31.25 k-r | 705.75 m-t | 137.91 | 37.91 |
| 49 | **MWA 8** | 30.25 l-r | 556.50 p-t | 108.74 | 8.74 |
| 50 | **MWA 9** | 25.75 o-r | 611.75 p-t | 119.54 | 19.54 |
| 51 | **MWA 10** | 26.75 o-r | 603.75 p-t | 117.98 | 17.98 |
| 52 | **MWA 11** | 32.00 j-r | 721.50 l-t | 140.99 | 40.99 |
| | **$N^+$ control** | 0.00 s | 511.75 q-t | 100 | - |
| | **$N^-$ control** | 0.00 s | 365.75 t | - | - |
| | **Pr>F** | **** | **** | **-** | **-** |
| | **$LSD_{0.05}$** | **21.13** | **374.42** | **-** | **-** |
| | **CV(%)** | **34.83** | **28.30** | **-** | **-** |

$^{x}$ = Means of 4 replications

$^{y}$ = Means followed by the same letter in the same column are not significantly different at 5% level

**** Significant at 1 % level

YMP = isolate collected from Yinmarpin township

PLE = isolate collected from Palae township

SLG = isolate collected from Salingyi township

BTL = isolate collected from Butalin township

MWA = isolate collected from Monywa township

SE% = Symbiotic Effectiveness Percent

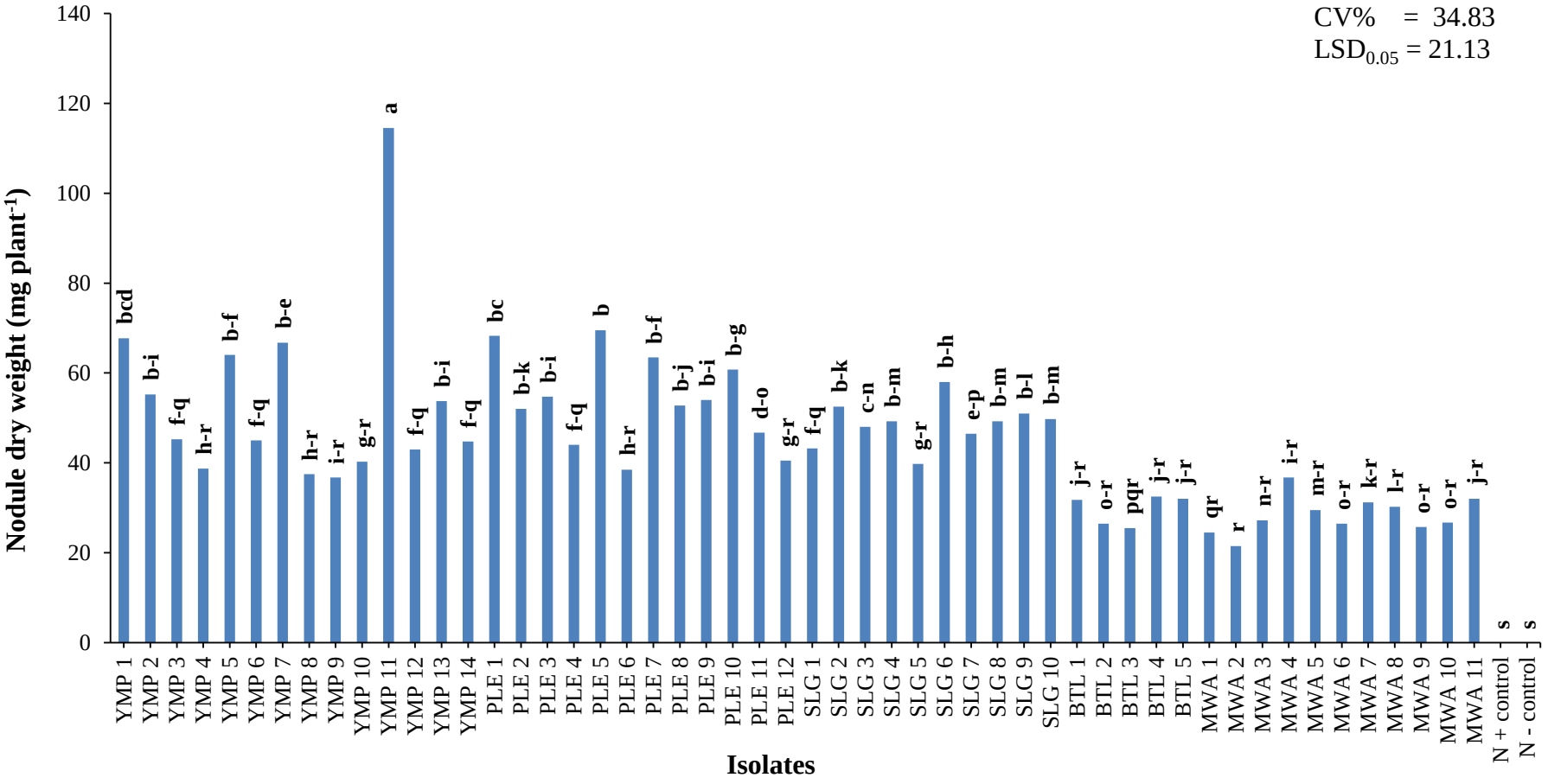



**Figure 4.1 Effect of different indigenous rhizobium isolates on nodule dry weight of Yezin-11 green gram**

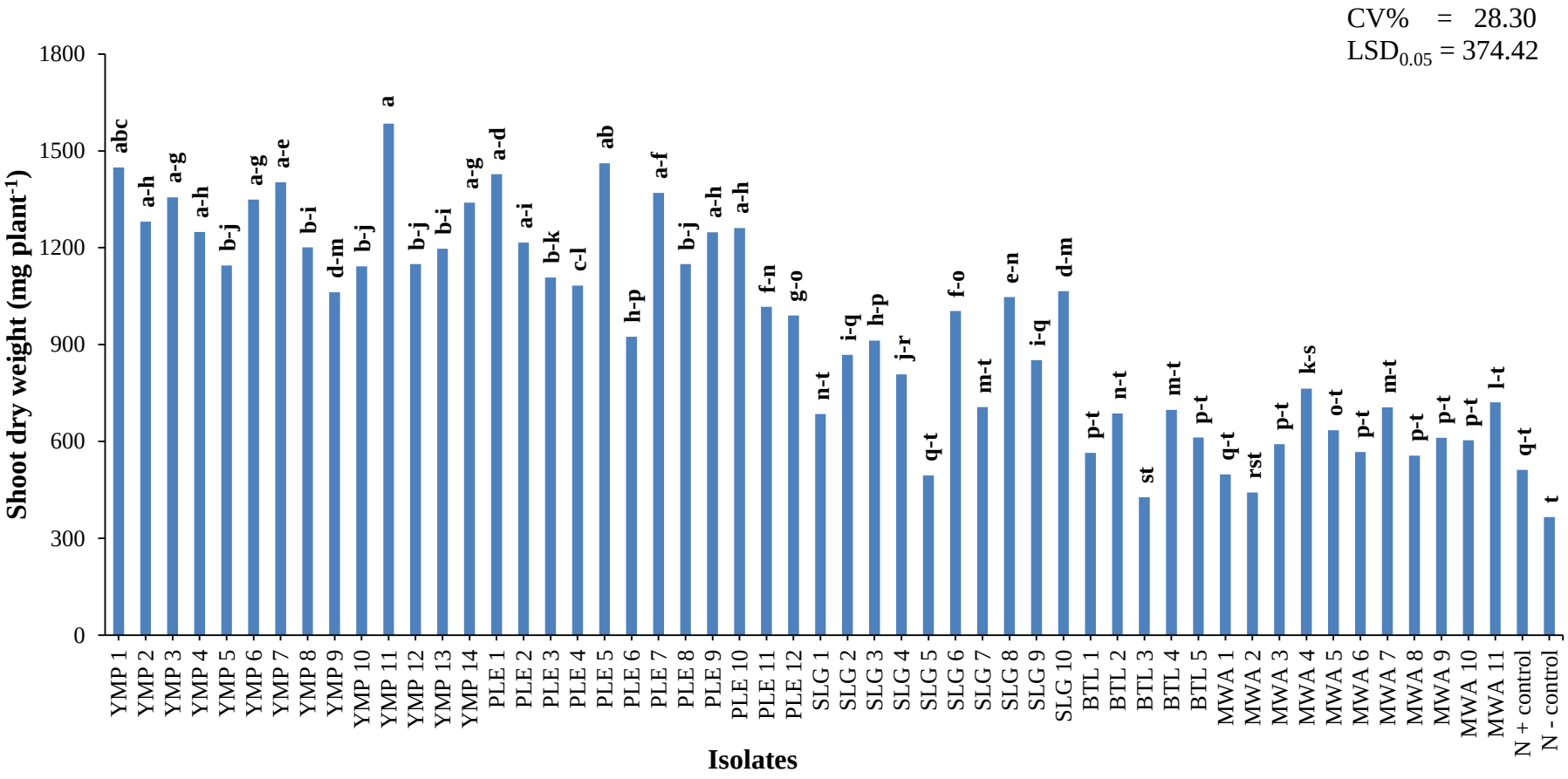



**Figure 4.2 Effect of different indigenous rhizobium isolates on shoot dry weight of Yezin-11 green gram**

and 924.75 and 1461.8 mg plant$^{-1}$, respectively. Out of 12 tested isolates, the nodule dry weight of the plants inoculated with PLE 1 (68.25 mg plant$^{-1}$) and PLE 5 (69.50 mg plant$^{-1}$) were significantly higher than those of the plants inoculated with PLE 4, PLE 6, PLE 11 and PLE 12. The lowest shoot dry weight (924.75 mg plant$^{-1}$) was observed from the plants inoculated with PLE 6, which was not significantly different from PLE 2, PLE 3, PLE 4, PLE 8, PLE 9, PLE 10, PLE 11 and PLE 12 (Table 4.2).

The nodule dry weights of the plants inoculated with 10 rhizobium isolates from Salingyi Township were not significantly different from each other. Out of 10 tested isolates, 4 isolate; SLG 3, SLG 6, SLG 8 and SLG 10 varied significantly from $N^+$ control in shoot dry weight (511.75 mg plant$^{-1}$). The shoot dry weight of the plants inoculated with SLG 1, SLG 5 and SLG 7 were not significantly higher than that of un-inoculated $N^-$ control (Table 4.2).

There were no significant differences among nodule dry weight and shoot dry weight of the plants inoculated with 5 rhizobium isolates from Butalin Township and control plants. The shoot dry weight (427.25 mg plant$^{-1}$) of BTL 3 isolate was lower than that of $N^+$ control, but not significantly different from each other (Table 4.2).

Among 11 rhizobium isolates collected from Monywa Township, MWA 4 gave the highest value, 764.00 mg plant$^{-1}$, in shoot dry weight but its shoot dry weight was statistically similar to $N^+$ control. However, the nodule dry weight of the plants inoculated with MWA 4 was not significantly different from those of the plants inoculated with the rest isolates (Table 4.2).

The correlation analysis result showed that nodule dry weight was highly significant and positively correlated (r = 0.81**) with shoot dry weight (Figure 4.3). Similar relationship (r = 0.49**) was reported by Manalku et al. (2009) between nodule dry weight and shoot dry weight in faba bean. Than (2010) found that shoot dry weight and nodule dry weight, and shoot dry weight and nodule number in chickpea were positively correlated (r = 0.79** and r = 0.80**, respectively). Zaw (2014) also reported that positive and significant correlation (r = 0.81**, r = 0.39**, r = 0.56**) between nodule number, nodule dry weight and shoot dry weight of chickpea.

In the present study, the differences in symbiotic potential of the isolates were examined. When symbiotic effectiveness percent (SE%) was calculated, all tested

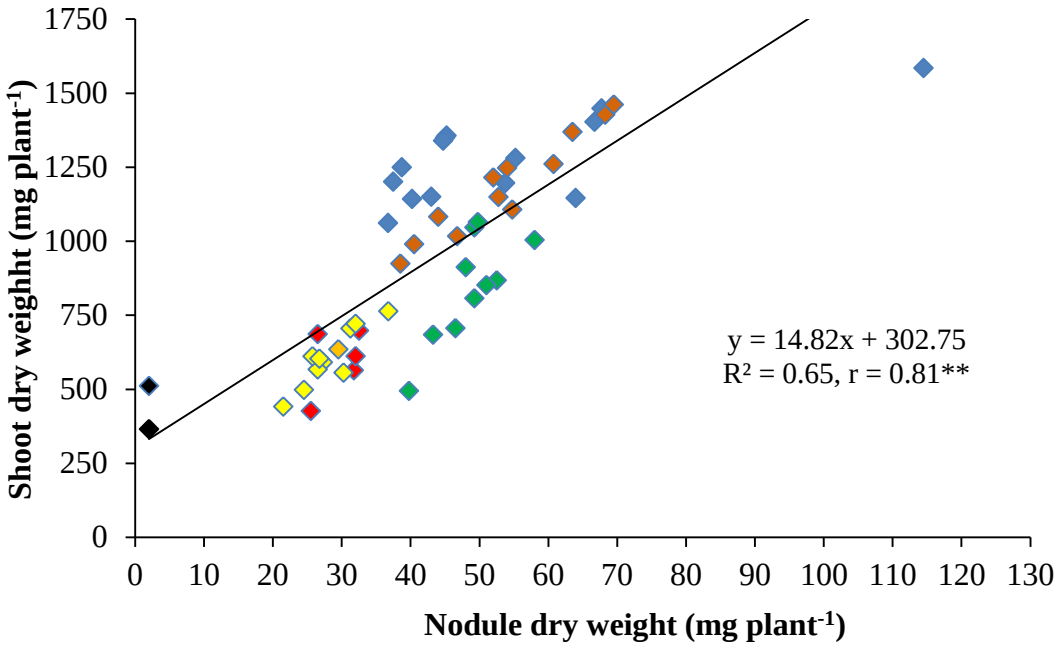



**Figure 4.3 Correlation between nodule dry weight and shoot dry weight of Yezin-11 green gram inoculated with 52 rhizobium isolates**

isolates gave higher SE% (>80%) ranging from 83.49% to 309.62%. It was observed that YMP 11 gave the highest SE% (309.62%) followed by PLE 5 (285.64%), YMP 1 (283.00%), PLE 1 (278.99%) and YMP 7 (274.16%). BTL 3 produced the lowest value (83.49%) (Table 4.2). The results of symbiotic efficiency indicated that rhizobium strains isolated from green gram cultivated soils can be in harmony with Yezin-11 green gram. Karaca and Uyanoz (2012) found that SE% of all tested rhizobium strains isolated from dry bean cultivated soils were higher than that of positive control in dry bean.

According to the percentage differences in shoot dry weight of rhizobium inoculated and $N^+$ control plants, SLG 5, BTL 3, MWA 1 and MWA 2 isolates were inferior to $N^+$ control but superior to $N^-$ control in terms of shoot dry weight. The rest isolates performed symbiotically well on Yezin-11 variety and their contributions to shoot dry weight were higher than that of un-inoculated $N^+$ control. And thus, 92% of tested isolates performed better but 8% of them were lower than un-inoculated $N^+$ control (Table 4.2). Kawaka et al. (2014) mentioned that 42% of the native rhizobia isolates nodulating common bean were effective nitrogen fixers and 16% performed as good as the positive controls.

Nodule dry weight may be considered as a usual character to select effective rhizobium isolates (Saleh 2013). Somasegaran and Hoben (1994) and Peoples et al. (2002) explained that shoot dry matter is a good indicator of relative isolate effectiveness. In this experiment, it was observed that the highest nodule dry weight was recorded in plants inoculated with YMP 11, followed by those with PLE 5, PLE 1, YMP 1 and YMP 7 inoculated plants while MWA 1 and MWA 2 were the lowest. Ali (2010) reported that the comparison of inoculated and un-inoculated plants in respective to nodulation was highly significant and the average oven dry weight of effective nodules increased by rhizobium inoculation. Likewise, the highest shoot dry weight was observed from plants inoculated with YMP 11 followed by plants treated with PLE 5, YMP 1, PLE 1 and YMP 7 isolates. The lowest shoot dry weights were shown by the hosts inoculated with MWA 1, SLG 5, MWA 2 and BTL 3 isolates. As five indigenous rhizobium isolates; YMP 1, YMP 7, YMP 11, PLE 1 and PLE 5 had the above indicators, they could be effective-indigenous rhizobium isolates. These isolates were selected to authenticate their symbiotic effectiveness on five green gram varieties namely MAS-1, Yezin-1, Yezin-9, Yezin-11 and Yezin-14.

### 4.1.2 Authentication of symbiotic effectiveness of selected rhizobium isolates on different green gram varieties

Nodule dry weights and shoot dry weights of tested green gram varieties inoculated with five selected indigenous rhizobium isolates were significantly different among the isolates and among the varieties at 5 % level of significance (Table 4.3). Nodules formed on green gram in all inoculated treatments. Absence of nodules on plant roots of un-inoculated control treatments indicated that there were no indigenous rhizobia in the tested sand of present study. Khurana et al. (1984) reported that nodule dry weight compared to nodule number was more closely related to seed yield. In the present study, significant difference in nodule dry weights was observed among the isolates at 1 % level. The nodule dry weights of the isolates ranged from 18.73 to 33.27 mg plant$^{-1}$. Among the isolates, the average nodule dry weight (33.27 mg plant$^{-1}$) of the plants treated with YMP 11 was found to be the highest and significantly different from other tested isolates. The lowest nodule dry weight (18.73 mg plant$^{-1}$) was found in plants inoculated with PLE 1 but statistically similar to that of YMP 7 (Figure 4.4). The results indicated that nitrogen fixing ability of five indigenous rhizobium isolates differed from each other. Their differential abilities in nodule dry weight might be due to their genotypic differences. YMP 11 inoculated plants gave the highest nodule dry weight among the five selected indigenous rhizobium isolates. Some strains of rhizobia are more effective than others to a particular host plant. This property should be attributed to the genetic variation of strains used in chickpea (Mandhare et al. 2005).

Green gram varieties exhibited a significant effect on the nodule dry weight at 1 % level. The nodule dry weights of green gram varieties ranged from 13.86 to 22.29 mg plant$^{-1}$ (Table 4.3). The highest nodule dry weight (22.29 mg plant$^{-1}$) was found in Yezin-11, which significantly varied from those of other tested varieties. The lowest nodule dry weight (13.86 mg plant$^{-1}$) was observed in Yezin-1, but not significantly different from that of Yezin-14 (Figure 4.5). In the present study, nodule dry weight varied with green gram varieties. Among green gram varieties, Yezin-11 was the most nodulated while Yezin-1 was the least nodulated. This could be attributed to highly specific interactions between green gram varieties and rhizobium isolates. Scheffer (2007) reported that leguminous species were very specific in their rhizobial requirement and could nodulate and then increase nodule

**Table 4.3 Effect of indigenous rhizobium isolates and effect of green gram varieties on nodule dry weight and shoot dry weight at 35 DAS**

| Treatments | Nodule dry weight (mg plant$^{-1}$) [x] | Shoot dry weight (mg plant$^{-1}$) [x] |
|---|---|---|
| **Isolate** | | |
| YMP 1 | 26.40 b[y] | 448.53 b[y] |
| YMP 7 | 20.27 cd | 427.07 bc |
| YMP 11 | 33.27 a | 547.93 a |
| PLE 1 | 18.73 d | 390.73 c |
| PLE 5 | 23.00 c | 428.60 bc |
| $N^+$ control | 0.00 e | 393.47 c |
| $N^-$ control | 0.00 e | 288.60 d |
| **$LSD_{0.05}$** | **3.30** | **44.09** |
| **Variety** | | |
| MAS-1 | 18.48 b | 457.29 ab |
| Yezin-1 | 13.86 c | 324.71 d |
| Yezin-9 | 17.67 b | 434.52 b |
| Yezin-11 | 22.29 a | 492.76 a |
| Yezin-14 | 14.62 c | 379.95 c |
| **$LSD_{0.05}$** | **2.79** | **37.26** |
| **Pr>F** | | |
| Isolate | ** | ** |
| Variety | ** | ** |
| Isolate × Variety | ** | * |
| **CV%** | **26.10** | **14.48** |

[x] = Means of 3 replications

[y] = Means in the same column followed by the same letters are not significantly different at 5 % level

** Significant at 1 % level, * Significant at 5 % level

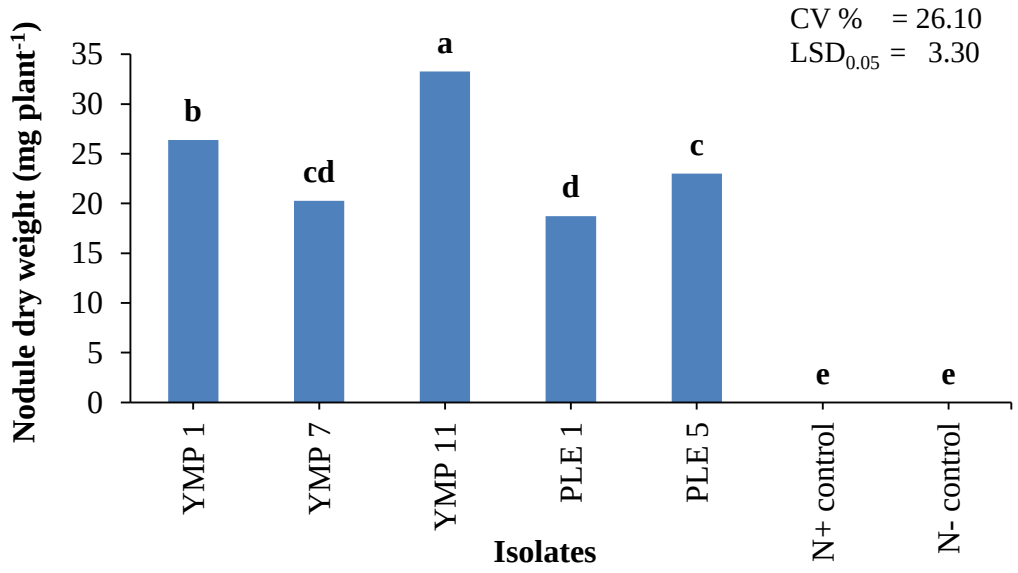



**Figure 4.4 Effect of different indigenous rhizobium isolates on nodule dry weight of green gram at 35 DAS**

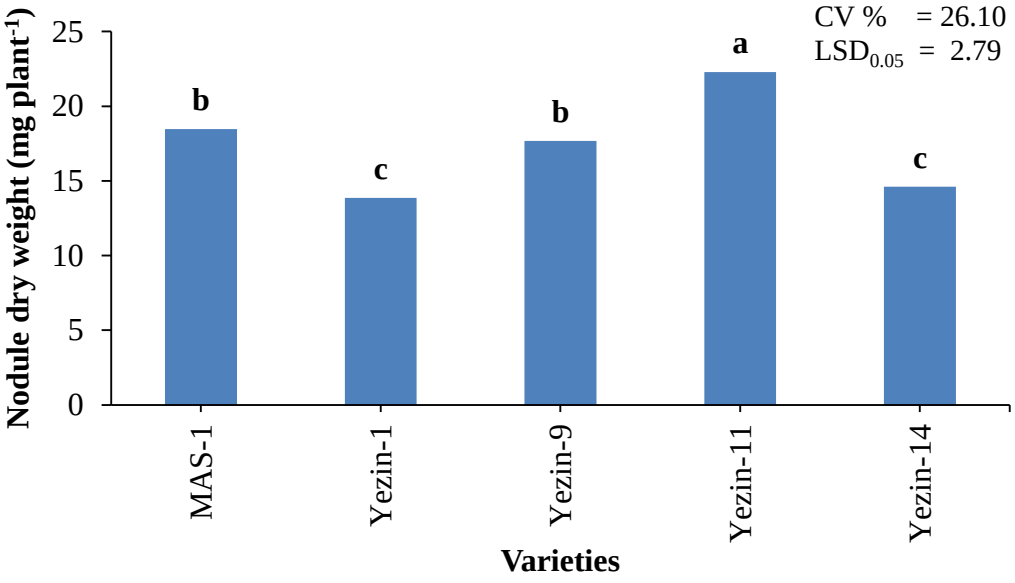



**Figure 4.5 Response of different green gram varieties to nodule dry weight at 35 DAS**

dry weight only by their own rhizobia.

There was an interaction between isolates and green gram varieties for nodule dry weight at 1 % level (Table 4.3). Than (2010) explained that the interaction between chickpea varieties and isolates was significant in nodule dry weight. Saleh et al. (2014) reported that black gram varieties, strains, adhesives as well as interaction between varieties and strains showed significant effect in nodule dry weight. It was obvious that different combination of rhizobium isolates and green gram varieties affected the nodule dry weight (Table 4.4). The highest nodule dry weight (40.00 mg plant$^{-1}$) was found in YMP 11 inoculated Yezin-11 variety while the lowest nodule dry weight (12.67 mg plant$^{-1}$) observed in PLE 1 treated Yezin-14 variety. In all tested green gram varieties, YMP 11 inoculated plant gave the maximum nodule dry weight. In MAS, the nodule dry weight of YMP 11 inoculated plant was not significantly higher than those of YMP 1 and YMP 7 inoculated plants. The nodule dry weights of Yezin-1 variety inoculated with YMP 1 and YMP 11 were not significantly different from one another. In Yezin-9, nodule dry weight of YMP 11 infected plant was significantly higher than those of the plants inoculated with other isolates. In Yezin-11 variety, the nodule dry weight of YMP 11 inoculated plant was statistically similar to that of YMP 1 inoculated plant. In Yezin-14, YMP 11 treated plant gave the maximum nodule dry weight which was not significantly higher than that of PLE 5 treated plant (Figure 4.6). These results indicated that the response to nodulation is variety-specific. And thus, effective rhizobia must have highly effective nitrogen fixing ability with the intended host species. This may be associated with the ability of rhizobial isolate to induce signals for nodulation with many types of soybean germplasm (Romero 2003). In the present study, YMP 11 was compatible with all tested green gram varieties.

Variation in shoot dry weight of the plants was occurred when all tested green gram varieties inoculated with five rhizobia isolates. Nodule dry weight and shoot dry weight were routinely used as indicators of relative strain effectiveness (Somasegaran and Hoben 1994). There was a significant difference in shoot dry weights among the isolates at 1 % level. The shoot dry weights of the isolates ranged from 288.60 to 547.93 mg plant$^{-1}$ (Table 4.3). The maximum shoot dry weight (547.93 mg plant$^{-1}$) was obtained in plants inoculated with YMP 11 isolate that was significantly different from plants inoculated with the rest isolates and un-inoculated control plants. The minimum shoot dry weight (288.60 mg plant$^{-1}$) was found in N$^{-}$

**Table 4.4 Interaction effect of isolate and variety on nodule dry weight and shoot dry weight of green gram at 35 DAS**

| Isolate | Variety | | | | |
|---|---|---|---|---|---|
| | **MAS-1** | **Yezin-1** | **Yezin-9** | **Yezin-11** | **Yezin-14** |
| **Nodule dry weight (mg plant$^{-1}$)$^{x}$** | | | | | |
| **YMP 1** | 28.67 c-f$^{y}$ | 24.33 d-g | 23.00 e-h | 34.00 abc | 22.00 f-i |
| **YMP 7** | 29.00 c-f | 14.33 jkl | 14.00 kl | 28.67 c-f | 15.33 i-l |
| **YMP 11** | 33.00 abc | 27.00 c-g | 36.67 ab | 40.00 a | 29.67 b-e |
| **PLE 1** | 23.00 e-h | 15.33 i-l | 21.00 g-k | 21.67 f-j | 12.67 l |
| **PLE 5** | 15.67 h-l | 16.00 h-l | 29.00 c-f | 31.67 bcd | 22.67 e-i |
| **$N^{+}$ control** | 0.00 m | 0.00 m | 0.00 m | 0.00 m | 0.00 m |
| **$N^{-}$ control** | 0.00 m | 0.00 m | 0.00 m | 0.00 m | 0.00 m |
| **CV (%)** | 26.10 | | | | |
| **Shoot dry weight (mg plant$^{-1}$)$^{x}$** | | | | | |
| **YMP 1** | 513.00 a-d$^{y}$ | 358.33 i-p | 498.33 a-e | 536.33 abc | 336.67 k-p |
| **YMP 7** | 515.00 a-d | 300.33 nop | 377.00 g-n | 483.33 a-f | 459.67 c-h |
| **YMP 11** | 563.67 ab | 497.00 a-e | 569.33 ab | 573.33 a | 536.33 abc |
| **PLE 1** | 394.67 f-n | 304.33 m-p | 428.67 d-k | 527.00 a-d | 299.00 nop |
| **PLE 5** | 438.00 c-j | 306.67 m-p | 517.00 a-d | 473.67 b-g | 407.67 e-l |
| **$N^{+}$ control** | 448.33 c-i | 344.33 j-p | 374.33 h-o | 455.00 c-i | 345.33 j-p |
| **$N^{-}$ control** | 328.33 l-p | 162.00 q | 277.00 op | 400.67 e-m | 275.00 p |
| **CV (%)** | 14.48 | | | | |

[x] Means of 3 replications

[y] Means followed by the same letter in the same column are not significantly different at 5% level

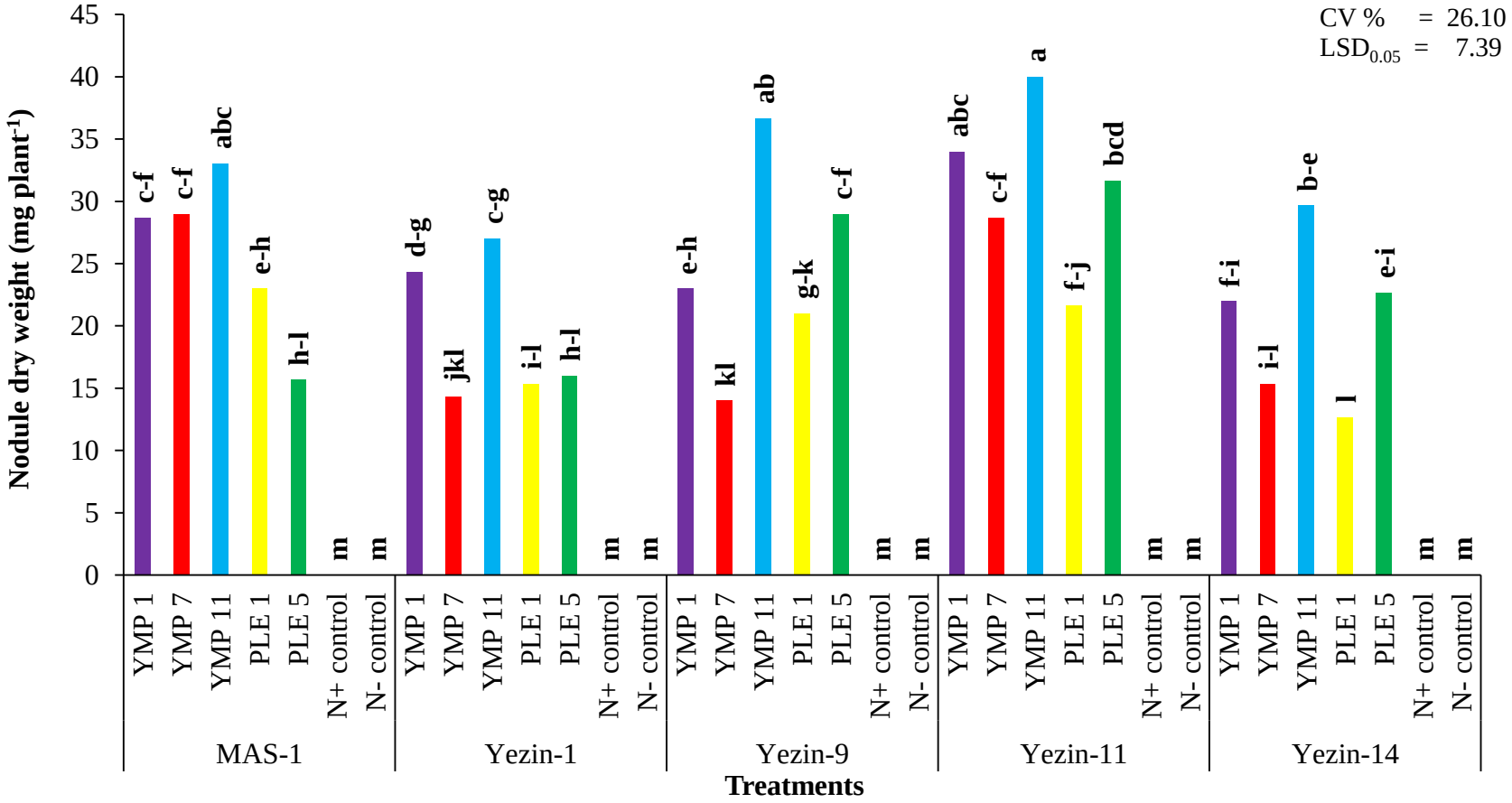



**Figure 4.6 Interaction effect of isolate and variety on nodule dry weight of green gram at 35 DAS**

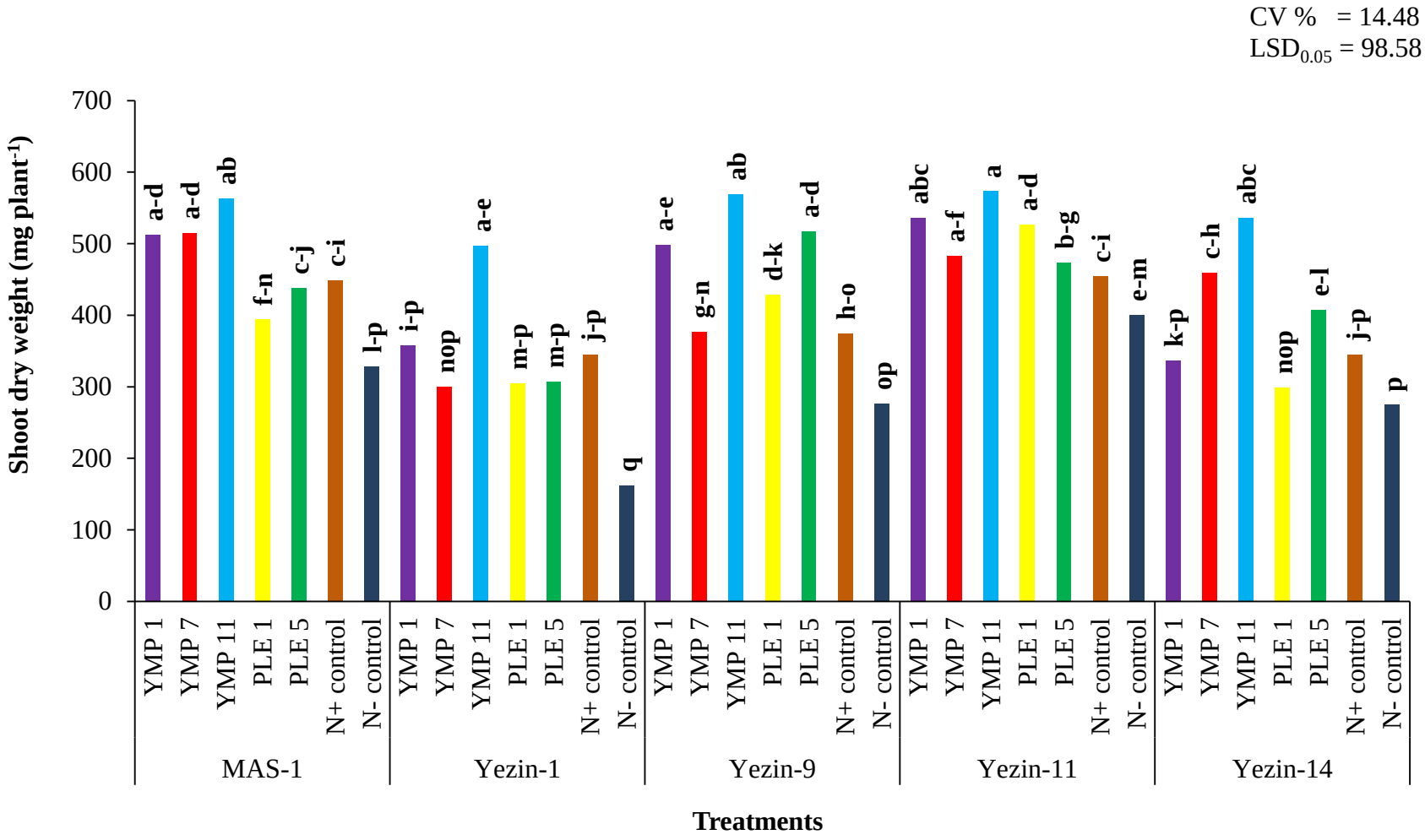



**Figure 4.7 Interaction effect of isolate and variety on shoot dry weight of green gram at 35 DAS**

control plant that significantly lower than that of $N^+$ control plant (393.47 mg plant$^{-1}$) (Figure 4.8). The difference in shoot dry weight observed with selected rhizobia isolates is due to the difference in the genetic but also in effectiveness of each isolate (Ndusha 2011). Among the five selected isolates, YMP 11 could be effective isolate.

Green gram varieties exhibited a significant effect on shoot dry weight at 1 % level. The shoot dry weights of green gram varieties ranged from 324.71 to 492.76 mg plant$^{-1}$ (Table 4.3). The maximum shoot dry weight (492.76 mg plant$^{-1}$) was observed in Yezin-11 variety that was statistically similar to that of MAS-1. Yezin-1 produced the minimum shoot dry weight (324.71 mg plant$^{-1}$) (Figure 4.9). The difference in shoot dry weight produced by different varieties is due to the genetic differences.

There was interaction effect between isolates and green gram varieties on shoot dry weight at 5 % level (Table 4.4). Aung (2007) found that significant interaction between the *Bradyrhizobium* strains and the soybean varieties in nodule dry weight and shoot dry weight. In this study, inoculation led to occurrence of higher shoot dry weight compared to un-inoculated $N^-$ control. $N^+$ control was also effective in obtaining higher shoot dry weight over $N^-$ control (Table 4.4). The maximum shoot dry weight (573.33 mg plant$^{-1}$) was observed in YMP 11 inoculated Yezin-11 variety while the minimum shoot dry weight (162.00 mg plant$^{-1}$) found in un-inoculated ($N^-$) Yezin-1 control plant. Among five tested isolates, YMP 11 isolate provided the highest shoot dry weight (573.33 mg plant$^{-1}$) in Yezin-11 and followed by Yezin-9 and MAS 1. PLE 1 isolate produced the lowest shoot dry weight (299.00 mg plant$^{-1}$) in Yezin-14 (Figure 4.7). In the present study, the nodulation efficiency depends on the compatibility between rhizobium isolates and green gram genotypes. Dhar (2010) described that symbiotic nitrogen fixation is a complex physiological process influenced by interaction of genetic elements in host genotype and rhizobia. Kahindi and Karanja (2009) explained the difference in preference may be due to the quality and quantity of exudates produced by different varieties. Among five tested isolates, YMP 11 gave relatively higher shoot dry weights on all tested varieties (Figure 4.7). Than (2010) found that YAU 65 gave the relatively higher shoot dry weights on Yezin-5, Yezin-7 and Yezin-8 chickpea varieties.

All tested rhizobium isolates gave higher SE% (>80%) ranging from 88.03% to125.73% on MAS-1, 87.22% to 144.34% on Yezin-1, 100.71% to 152.09% on Yezin-9, 104.10% to 126.01% on Yezin-11 and 86.58% to 155.31% on Yezin-14.

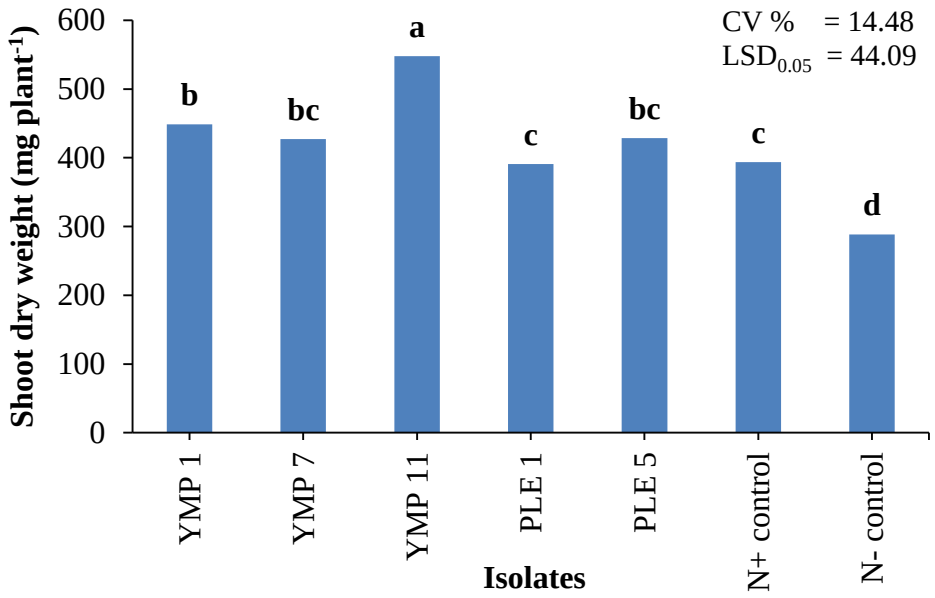



**Figure 4.8 Effect of different indigenous rhizobia isolates on shoot dry weight of green gram at 35 DAS**

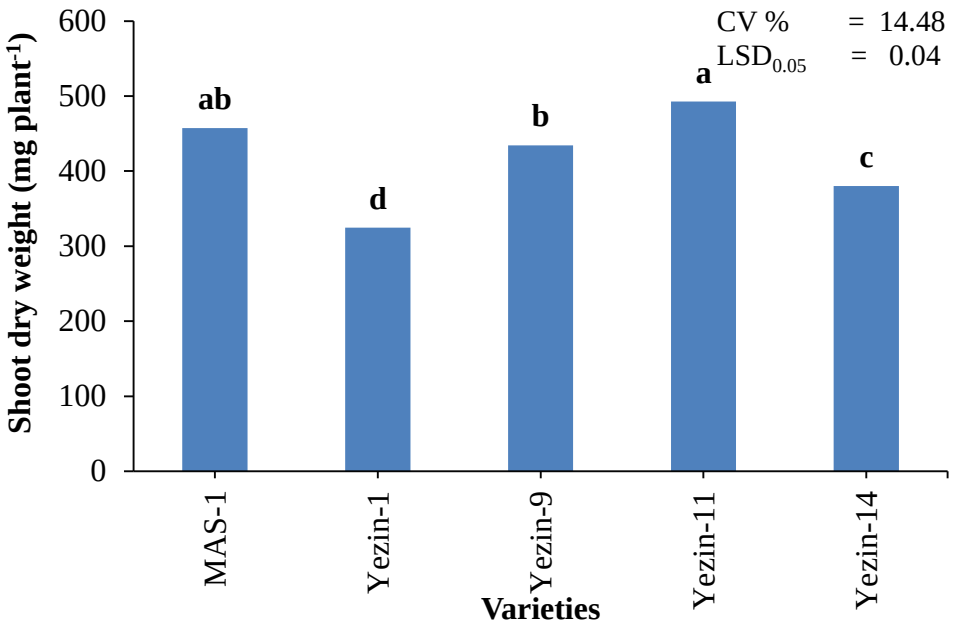



**Figure 4.9 Response of different green gram varieties to shoot dry weight at 35 DAS**

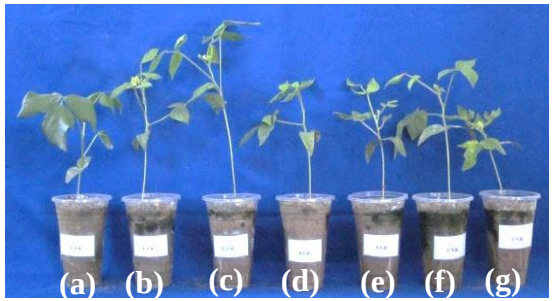
Almost YMP isolates were compatible to all green gram varieties. PLE isolates did not perform to increase in shoot dry weight over $N^+$ control in most of green gram varieties. Among the tested isolates, YMP 11 gave the highest SE % on all tested varieties (Table 4.5). Ogutcu et al. (2008) reported that among the 21 *Rhizobium* strains isolated from wild chickpea, the strain HF-1 provided the highest shoot dry weight (2020.00 mg plant$^{-1}$) and symbiotic efficiency (186.20%), although other tested strains also generally gave results better than un-inoculated control.

Based on the percentage differences in shoot dry weight of inoculated and $N^+$ control plants, the highest increased shoot dry weight percentage on all tested varieties was found in plants inoculated with YMP 11 isolate 25.73% on MAS-1, 44.34% on Yezin-1, 52.09% on Yezin-9, 26.01% on Yezin-11 and 55.31% on Yezin-14 (Table 4.5). Patra et al. (2008) reported that among nine rhizobial strains tested on soybean, the highest increase in shoot dry weight over the control (36.9%) was recorded with the strain SB-16. In the present study, there was no net increased shoot dry weight over $N^+$ control in Yezin-1 when inoculated with YMP 7 (-12.78%), PLE 1 (-11.97) and PLE 5 (-10.94%). No net increased shoot dry weight was also observed in MAS-1 which treated with PLE 1 (-11.97%) and PLE 5 (-2.30%). Moreover, net increased shoot dry weight was not occurred when YMP 1 (-2.51%) and PLE 1 (-13.42%) were inoculated in Yezin-14. Therefore, all tested green gram varieties preferred YMP 11 isolate to YMP 1, YMP 7, PLE 1 and PLE 5 isolates. Ghosh et al. (2006) reported that K-581 and MH-85 green gram cultivars were more response to M-1006 isolates than M-1005, M-11 and M-20 isolates. Similar trend was also found by Zaw (2014) who examined the interaction of five rhizobium isolates and three chickpea varieties. Only YAU 113 gave superior increased shoot dry weight in Yezin-4, Yezin-6 and Shweni Lone Gyi chickpea varieties while the rest isolates produced no net increased shoot dry weight in Yezin-6 variety.

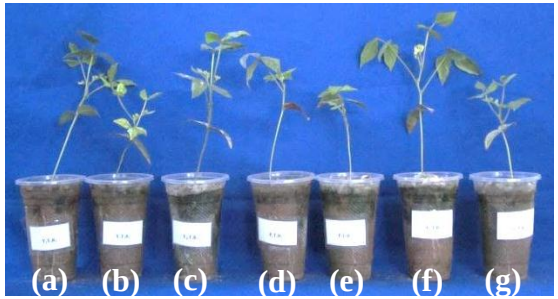
As effective rhizobia must have highly effective nitrogen fixing ability with the intended host species, YMP 11 isolate was compatible with tested green gram varieties based on the results of these parameters (nodule dry weight, shoot dry weight, symbiotic effectiveness and percent increased shoot dry weight over $N^+$ control) (Plate 4.6).Therefore, the best performing YMP 11 isolate can be used as a single inoculant isolate to effectively nodulate on all tested green gram varieties.

**Table 4.5 Percent increase in shoot dry weights over $N^+$ controls and symbiotic effectiveness percent**

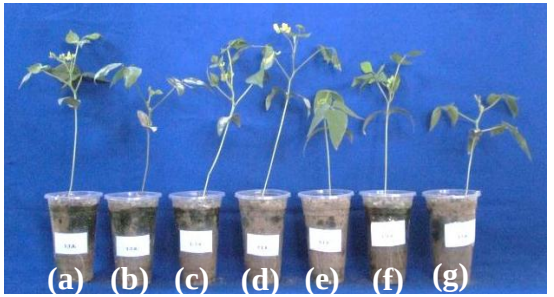
| Isolate | Variety | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Increased shoot dry weight (%) | | | | | Symbiotic effectiveness (SE %) | | | | |
| | **MAS-1** | **Yezin-1** | **Yezin-9** | **Yezin-11** | **Yezin-14** | **MAS-1** | **Yezin-1** | **Yezin-9** | **Yezin-11** | **Yezin-14** |
| **YMP 1** | 14.42 | 4.07 | 33.13 | 17.88 | -2.51 | 114.42 | 104.07 | 133.13 | 117.88 | 97.49 |
| **YMP 7** | 14.87 | -12.78 | 0.71 | 6.23 | 33.11 | 114.87 | 87.22 | 100.71 | 106.23 | 133.11 |
| **YMP 11** | 25.72 | 44.34 | 52.09 | 26.01 | 55.31 | 125.72 | 144.34 | 152.09 | 126.01 | 155.31 |
| **PLE 1** | -11.97 | -11.62 | 14.51 | 15.82 | -13.42 | 88.03 | 88.38 | 114.51 | 115.82 | 86.58 |
| **PLE 5** | -2.30 | -10.94 | 38.11 | 4.10 | 18.05 | 97.70 | 89.06 | 138.11 | 104.10 | 118.05 |
| **$N^+$control** | | | | | | 100 | 100 | 100 | 100 | 100 |



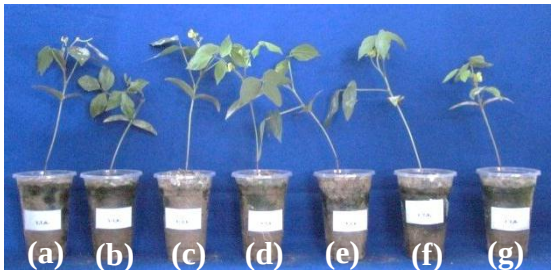

**MAS-1**



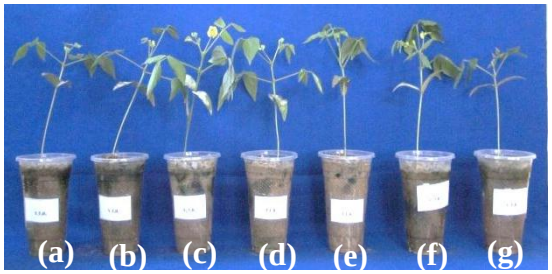

**Yezin-1**




**Yezin-9**




**Yezin-11**




**Yezin-14**

**Plate 4.6 Comparison of plant growth of green gram varieties namely MAS-1, Yezin-1, Yezin-9, Yezin-11 and Yezin-14 inoculated with five rhizobium isolates; (a) YMP 1, (b) YMP 7, (c) YMP 11, (d) PLE 1 and (e) PLE 5 and un-inoculated (f) $N^+$ and (g) $N^-$ controls**

## 4.2 Effect of Indigenous Rhizobium Inoculant on Yield and Yield Components of Different Green Gram Varieties

### 4.2.1 Grain yield

Significant difference in grain yield was found among inoculation treatments at 1 % level. However, there was no significant difference in grain yield between rhizobium inoculation (3.03 g plant$^{-1}$) and un-inoculated $N^+$ control (2.88 g plant$^{-1}$) treatments (Table 4.6). Anjum et al. (2006) reported that seed inoculation with rhizobium and 30 kg N ha$^{-1}$ application significantly increased grain yield of green gram as compared to control but there was no significant difference among them. In order to obtain normal plant growth and grain yield, an adequate N supply for green gram is essential (Delic et al. 2011). Rhizobium inoculation significantly enhanced the grain yield 5.21% over un-inoculated $N^+$ control and 37.10% over un-inoculated $N^-$ control. Delic et al. (2011) found that inoculation with *Bradyrhizobium* strains increased the grain yield of mungbean by 23-53% in Fluvisol over control. Anjum et al. (2006) confirmed that inoculation of mungbean with effective rhizobial strains increase grain yield. This could be assumed that rhizobium inoculation increased the nodulation and hence yield increased due to the enhanced availability of nitrogen.

Grain yields of green gram varieties were not significant different to each other at 5 % level (Table 4.6). However, Yezin-11 gave the highest grain yield (2.97 g plant$^{-1}$) while Yezin-9 gave the lowest grain yield (2.48 g plant$^{-1}$). There was no interaction between inoculation and green gram varieties (Table 4.6). However, in all tested green gram varieties, the higher grain yield was obtained from rhizobium inoculation, followed by un-inoculated $N^+$ and $N^-$ control treatments respectively (Figure 4.10). The highest grain yield (3.23 g plant$^{-1}$) was recorded in rhizobium inoculated Yezin-11 variety while the lowest grain yield (1.73 g plant$^{-1}$) found in un-inoculated ($N^-$) Yezin-14 control variety. Tena et al. (2016) also reported that rhizobium inoculation has a pronounced effect on grain yield of chickpea. The results indicated that rhizobium inoculation increased grain yield of green gram. A significant increase in grain yield over un-inoculated (N-) control was observed in Yezin-14 due to rhizobium inoculation. Response of YMP 11 isolate used in this study on grain yield of green gram varieties did not vary. YMB 11 was found to be the effective isolate to enhance the grain yield of green gram varieties (Plate 4.7).

**Table 4.6 Effect of indigenous rhizobium inoculation on yield and yield components of different green gram varieties**

| Treatments | Grain yield (g plant$^{-1}$) | No. of pods plant$^{-1}$ | No. of grains pod$^{-1}$ | Hundred grain weight (g) | Total dry matter per plant (g) |
|---|---|---|---|---|---|
| **Inoculation (I)** | | | | | |
| Inoculation | 3.03 a | 7.85$^{x}$ a$^{y}$ | 7.68 a | 5.97 a | 6.67 a |
| $N^+$ control | 2.88 a | 7.05 ab | 7.37 ab | 5.84 ab | 5.99 b |
| $N^-$ control | 2.21 b | 6.35 b | 6.69 b | 5.73 b | 5.23 c |
| **$LSD_{0.05}$** | **0.42** | **0.91** | **0.78** | **0.16** | **0.21** |
| **Variety (V)** | | | | | |
| MAS-1 | 2.92 a | 7.25 ab | 7.73 a | 5.94 ab | 6.08 ab |
| Yezin-1 | 2.56 a | 7.08 ab | 6.73 a | 5.67 c | 5.83 bc |
| Yezin-9 | 2.48 a | 6.25 b | 6.95 a | 5.72 c | 5.76 c |
| Yezin-11 | 2.97 a | 7.67 a | 7.71 a | 6.07 a | 6.22 a |
| Yezin-14 | 2.60 a | 7.17 ab | 7.11 a | 5.83 bc | 5.94 bc |
| **$LSD_{0.05}$** | **0.53** | **1.17** | **1.01** | **0.21** | **0.27** |
| **Pr>F** | | | | | |
| Inoculation (I) | ** | * * | * | * | ** |
| Variety (V) | ns | ns | ns | ** | * |
| I × V | ns | ns | ns | ns | ns |
| **CV%** | **23.90** | **20.13** | **16.87** | **4.37** | **5.59** |

[x] Means of 4 replications

[y] Means in the same column followed by the same letters are not significantly different at 5 % level of LSD.

*Significant at 5 % level, ** Significant at 1 % level, [ns] Not significant

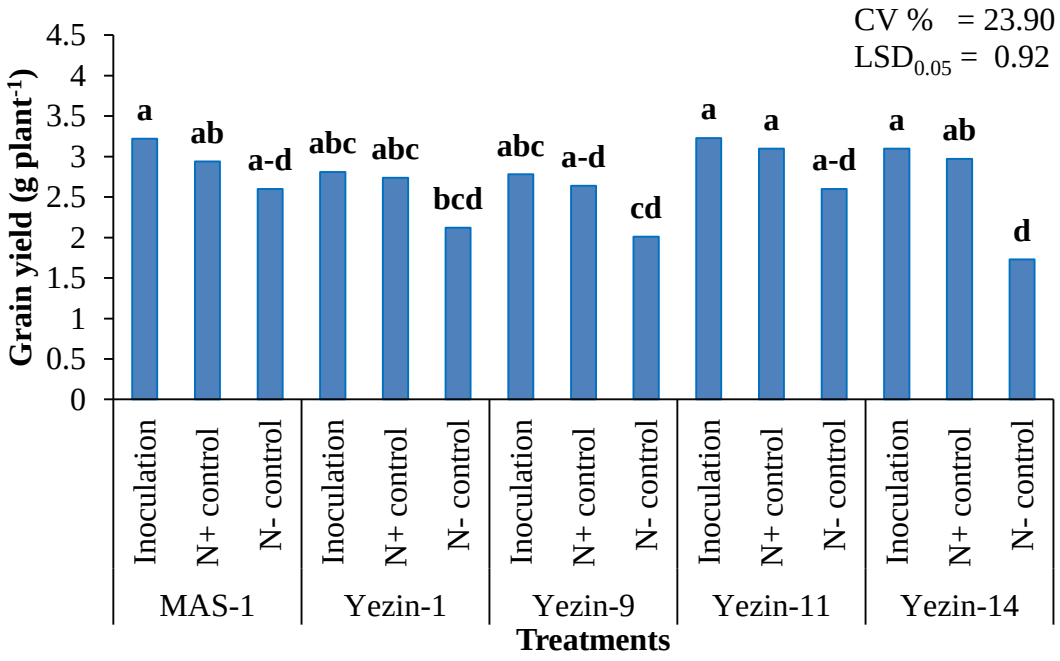
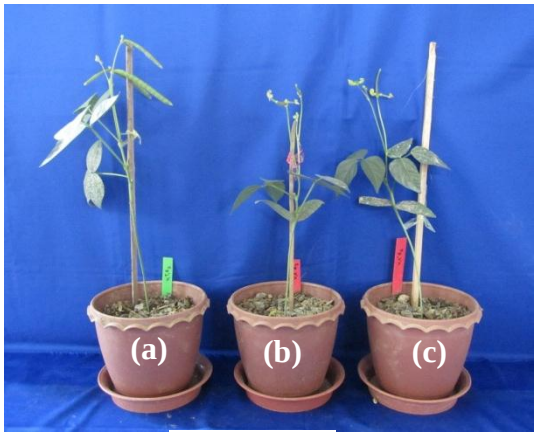
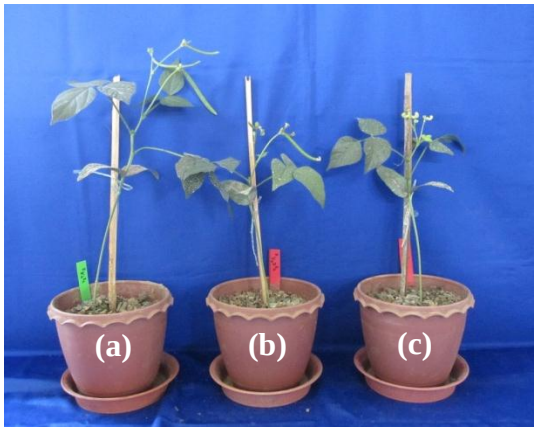



**Figure 4.10 Interaction effect of inoculation and variety on grain yield of green gram**

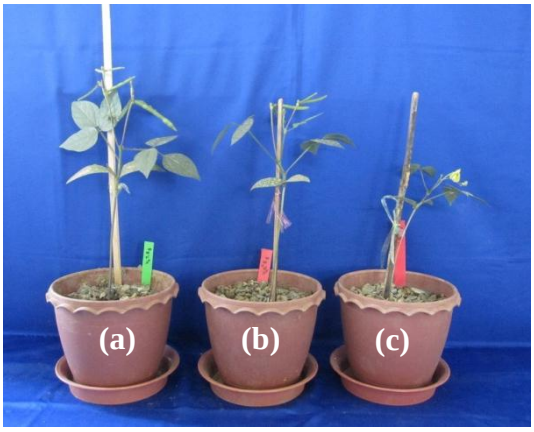



**MAS-1**

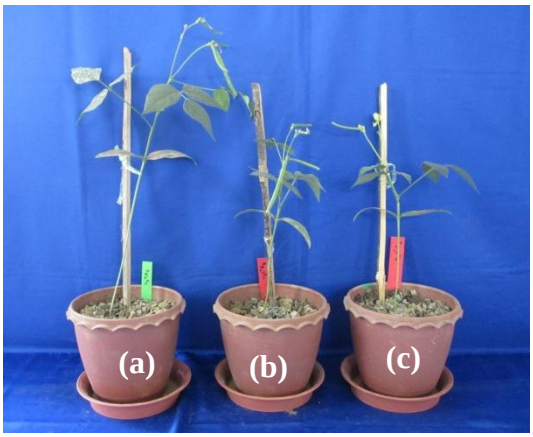

**Yezin-1**




**Yezin-9**




**Yezin-11**




**Yezin-14**

**Plate 4.7 Comparison of plant growth of green gram varieties namely MAS-1, Yezin-1, Yezin-9, Yezin-11 and Yezin-14 inoculated with (a) rhizobium, un-inoculated (b) $N^+$ and (c) $N^-$ controls**

### 4.2.2 Yield components

#### 4.2.2.1 Number of pods plant$^{-1}$

Yield and yield components were significantly affected by application of rhizobium inoculant in green gram varieties (Table 4.6). Rhizobium inoculation exhibited a significant effect on number of pods plant$^{-1}$ at 1 % level. Number of pods plant$^{-1}$ of rhizobium inoculated treatment was found to be maximum (7.85) that not significantly different from that of un-inoculated $N^{+}$ control treatment (7.05). The minimum number of pods plant$^{-1}$ (6.35) was found in un-inoculated $N^{-}$ control treatment (Table 4.6). In this experiment, it was observed that seed inoculation with rhizobium inoculant and application of nitrogen increased number of pods plant$^{-1}$ of green gram (Figure 4.10). This could be assumed that during pod filling stage, production of photosynthesis decreases but demand for N nutrient increases (Uddin et al. 2009). This demand could be satisfied if the nodules in the plant are vigorous to supply N constantly for growth cycle, which could be obtained by rhizobium inoculation. These results are in line with that of Bhuiyan et al. (2008) who concluded that number of pod plant$^{-1}$ of green gram and soybean is significantly increased by inoculating with *Bradyrhizobium*.

There was no significant difference in number of pods plant$^{-1}$ among the green gram varieties at 5% level (Table 4.6). Average number of pods plant$^{-1}$ of Yezin-11 variety was found to be maximum (7.67) and the lowest (6.25) was found in Yezin-9 variety. Interaction of rhizobium inoculation and green gram varieties were non-significant. The highest number of pods plant$^{-1}$ (9.00) was observed in Yezin-11 variety when seeds were treated with rhizobium inoculant. The lowest number of pods plant$^{-1}$ (5.50) was found in Yezin-9 variety without inoculation (Figure 4.11). Avais et al. (2017) found that rhizobium inoculation significantly improved number of pods plant$^{-1}$ (9.33%) but the interaction of chickpea advanced lines with rhizobium inoculation was non-significant.

#### 4.2.2.2 Number of grains pod$^{-1}$

Number of grains pod$^{-1}$ showed significant response to inoculation at 5 % level. (Table 4.6). The maximum number of grains pod$^{-1}$ (7.68) was observed in rhizobium inoculated plant and it was statistically similar to un-inoculated $N^{+}$ control treatment (7.37). Un-inoculated $N^{-}$ control gave the minimum number of grains pod$^{-1}$ (6.69). It was observed that the number of grains pod$^{-1}$ was significantly affected by

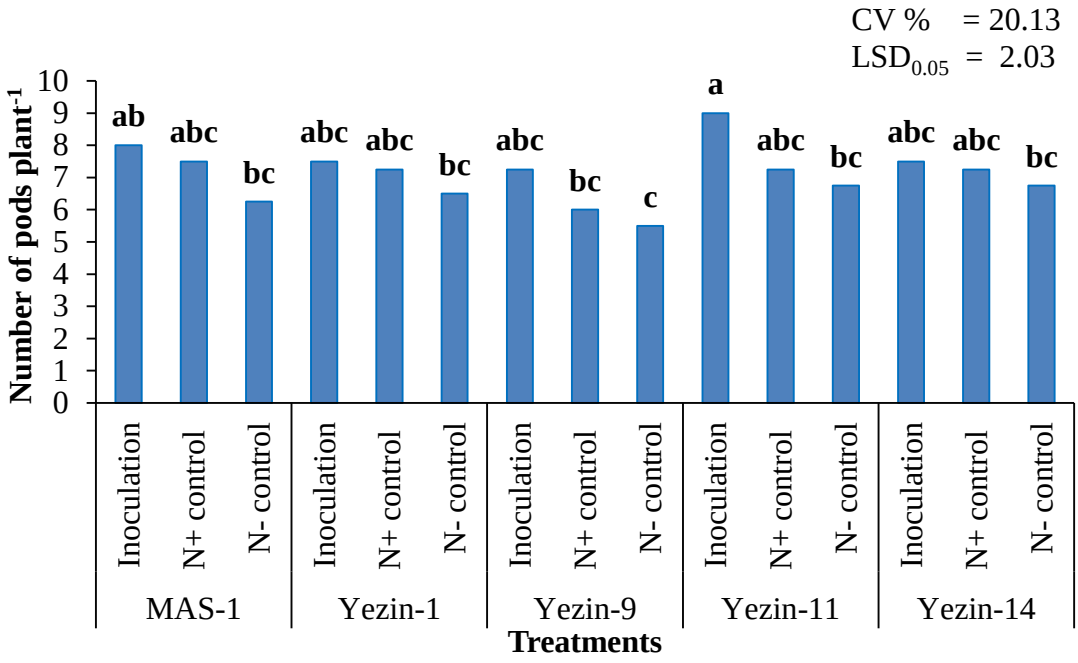



**Figure 4.11 Interaction effect of inoculation and variety on number of pods plant$^{-1}$**

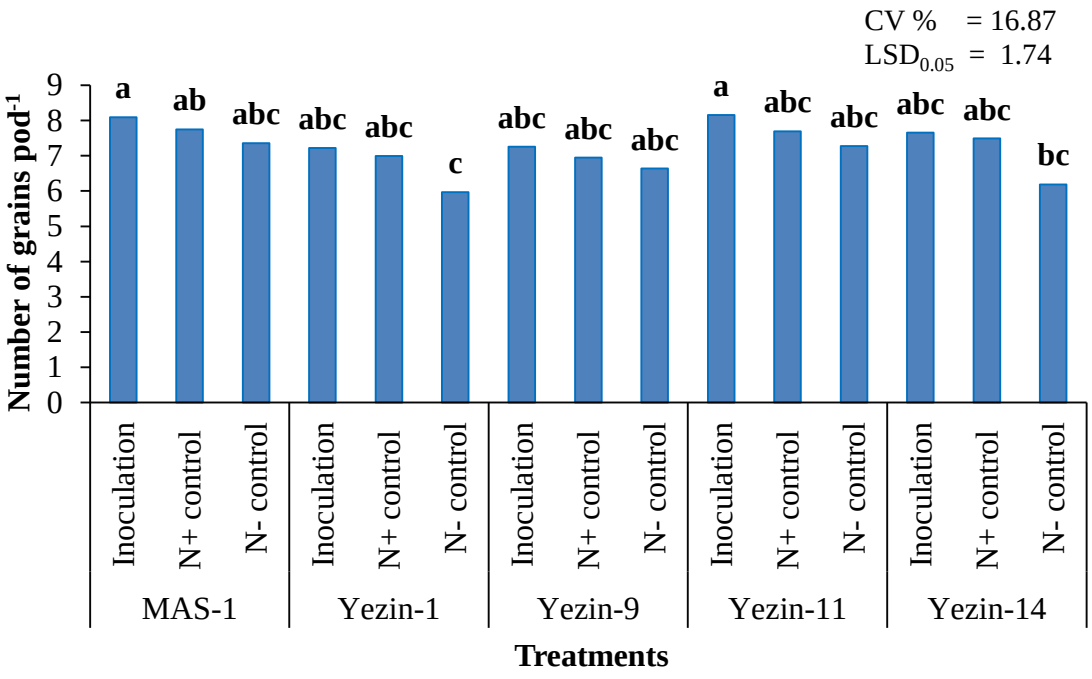



**Figure 4.12 Interaction effect of inoculation and variety on number of grains pod$^{-1}$**

rhizobium inoculation and this finding was also similar with Han (2012). Anjum et al. (2006) also reported that seed inoculation with rhizobium and nitrogen fertilizer increased the number of grains pod$^{-1}$ in comparison to check ($N^-$) control in green gram.

On comparing the means for green gram varieties, it was observed that the number of grains pod$^{-1}$ of green gram varieties did not differ significantly from one another at 5 % level (Table 4.6). The maximum number of grains pod$^{-1}$ (7.73) was observed in MAS-1 variety while the minimum number of grains pod$^{-1}$ (6.73) was found in Yezin-1 variety. There was no significant effect observed on number of grains pod$^{-1}$ due to rhizobium inoculation in green gram varieties. Antonio and Virgilio (1981) reported that the number of grains pod$^{-1}$ of different mungbean cultivars was not significantly altered by rhizobium inoculation and nitrogen fertilization. These results indicated that interaction effect of rhizobium inoculation and green gram varieties did not affect on number of grains pod$^{-1}$. Rhizobium inoculated varieties gave higher number of grains pod$^{-1}$ than un-inoculated control treatments (Figure 4.12).

#### 4.2.2.3 Hundred grains weight

Rhizobium inoculation significantly increased hundred grains weight in green gram at 5 % level (Table 4.6). Rhizobium inoculated green gram produced the maximum hundred grains weight (5.97 g) and minimum hundred grains weight (5.73 g) was found in un-inoculated $N^-$ control. Aslam et al. (2010) stated that rhizobium inoculation significantly increased hundred grains weight in chickpea.

Among green gram varieties, hundred grains weights were significantly different from one another at 5 % level. Maximum hundred grains weight (6.07 g) of Yezin-11 variety was statistically similar to that of MAS-1 and significantly differed from the other green gram varieties which had non-significant differences among themselves. Interaction among the inoculation and green gram varieties was non-significant (Table 4.6). Avais et al. (2017) found that thousand grains weight of chickpea variety/ advanced lines were significantly different but non-significant differences among chickpea genotypes and bacterial inoculation. In this study, rhizobium inoculation gave larger hundred grains weight than un-inoculated $N^+$ and $N^-$ control treatments (Figure 4.13).

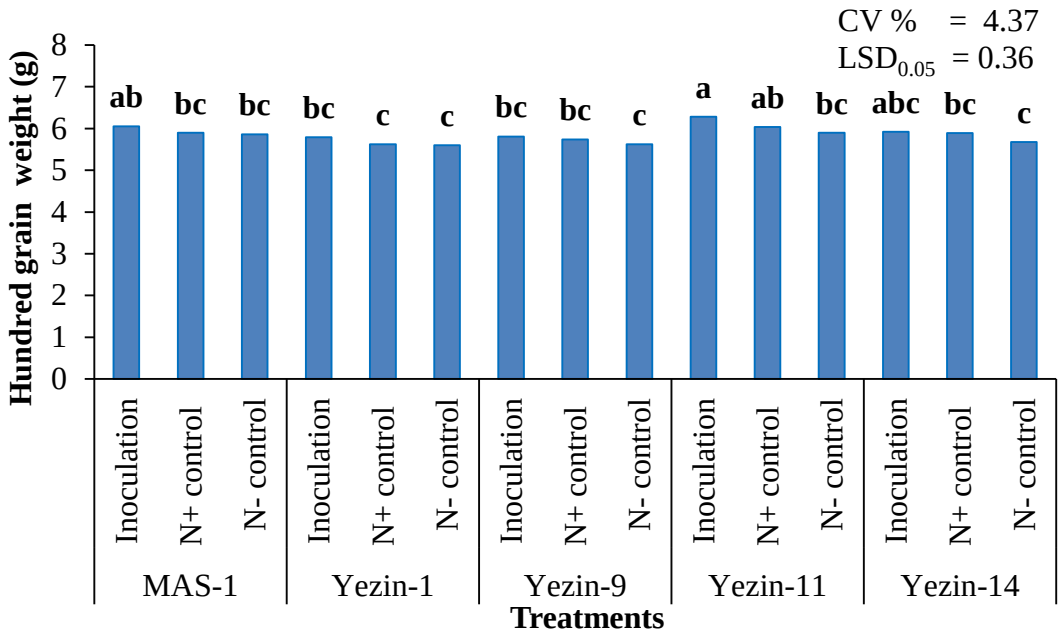



**Figure 4.13 Interaction effect of inoculation and variety on hundred grains weight of green gram**

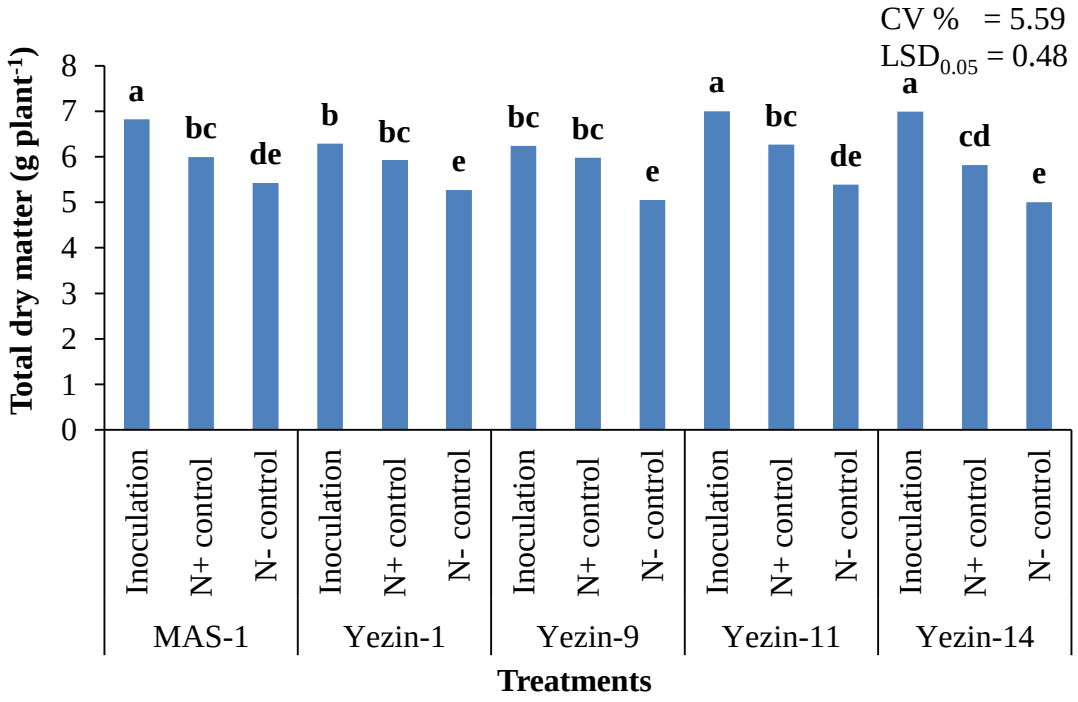



**Figure 4.14 Interaction effect of inoculation and variety on total dry matter of green gram**

### 4.2.3 Total dry matter

Total dry matter in rhizobium inoculation was significantly higher than un-inoculated control treatments. Results revealed that the highest amount of total dry matter (6.67 g plant$^{-1}$) was obtained from rhizobium inoculation followed by un-inoculated $N^+$ control. The lowest amount of total dry matter was received from un-inoculated $N^-$ control (Table 4.6). Inoculation with rhizobial strains significantly increased dry matter over un-inoculated control (Vijila and Jebaraj 2008). Uddin et al. (2009) also reported that total dry matter of mungbean was significantly influenced by *Bradyrhizobium* inoculum.

Yezin-11 variety produced the highest amount of total dry matter (6.22 g plant$^{-1}$) while the production of dry matter of Yezin-9 was the lowest (5.76 g plant$^{-1}$). There was no significant difference among the treatments due to the interaction of inoculation and varieties (Table 4.6). However, the highest amount of total dry matter was observed in Yezin-11 inoculated plant and, the lowest amount of total dry matter was observed in Yezin-14 un-inoculated plant (Figure 4.14). Among inoculation treatments, rhizobium inoculation gave the higher dry matter production in all green gram varieties.

### 4.2.4 Plant height

Effect of rhizobium inoculation on the plant height of green gram varieties was shown in Figure 4.15. Rhizobium inoculation significantly increased the plant height. Maximum plant height (24.54 cm) was found in rhizobium inoculation while minimum plant height (18.60 cm) was obtained from un-inoculated control. Similar trend was also found by Akhtar and Siddiqi (2009) who reported that seed inoculation of chickpea with *Rhizobium* spp. caused a significant increase in plant height as compared to without inoculation under field conditions. Significant difference in plant height was noticed in green gram varieties. Yezin-9 variety produced the highest plant height (23.63 cm) and the lowest plant height (19.38 cm) was observed in Yezin-11 variety. But, there was no interaction between rhizobium inoculation and five green gram varieties at 35 DSA. The maximum plant height (26.98 cm) was found with rhizobium inoculation in Yezin-9 variety which was significantly higher than those of un-inoculated $N^+$ control (22.38 cm) and $N^-$ control (21.53 cm) (Figure 4.15). The results are in line agreement with the findings of Avais

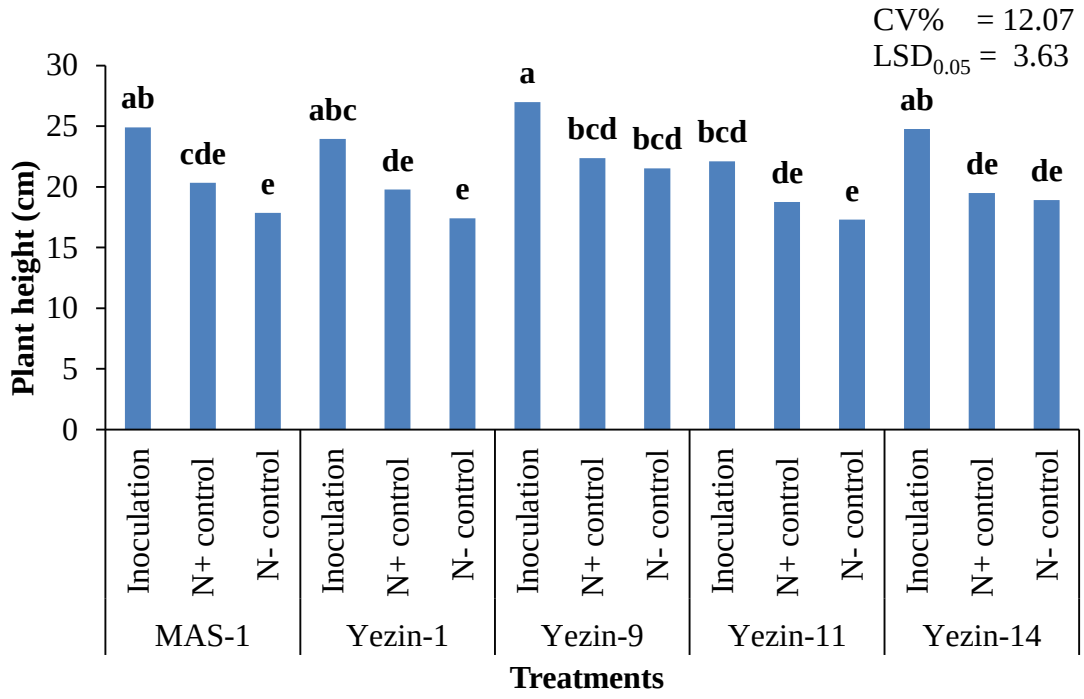



**Figure 4.15 Interaction effect of inoculation and variety on plant height of green gram**

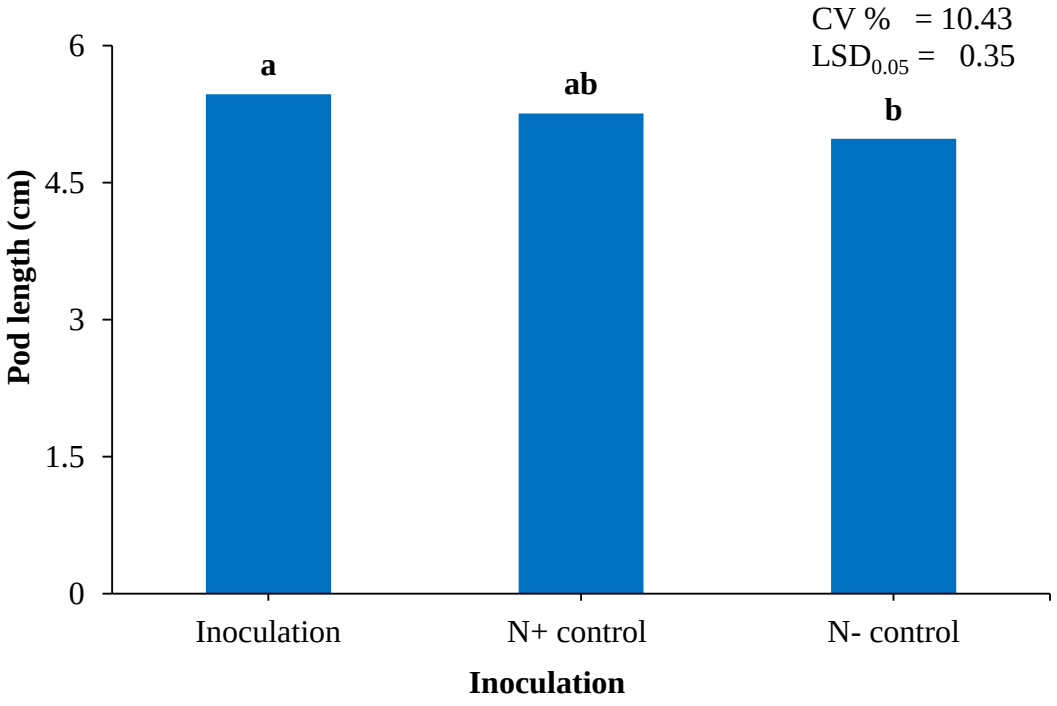



**Figure 4.16 Mean value of pod length as affected by inoculated and un-inoculated control ($N^+$ and $N^-$)**

et al. (2017) who concluded that the increase in plant height may be attributed to the symbiotic nitrogen fixation, release of plant nutrients and growth hormones due to microbial activity.

### 4.2.5 Pod length

Pod length showed significantly different among rhizobium inoculation treatments at 5 % level (Figure 4.16). Rhizobium inoculation gave the maximum mean value of pod length (5.47 cm) followed by un-inoculated $N^+$ control (5.26 cm). Un-inoculated $N^-$ control produced the minimum mean value of pod length (4.98 cm). Khaing (2016) reported that pod length was significantly higher in plants with rhizobium inoculation than plants without inoculation. Pod length showed no significant difference among green gram varieties. There was no interaction between rhizobium inoculation and green gram varieties.

# CHAPTER V
# CONCLUSION

The present study revealed that inoculation of 52 indigenous rhizobium isolates showed positive effects on nodulation and plant growth of Yezin-11 green gram. As all tested isolates were highly effective, the soil in Yinmarpin, Palae, Salingyi, Butalin and Monywa Townships of Sagaing Region harboured green gram nodulating rhizobia with good symbiotic properties. Among tested isolates, five rhizobium isolates; YMP 1, YMP 7, YMP 11, PLE 1 and PLE 5 isolates were found to be the most effective isolate to enhance the growth of Yezin-11 variety. Response of five green gram varieties; MAS-1, Yezin-1, Yezin-9, Yezin-11 and Yezin-14 varied with five rhizobium isolates. In all tested green gram varieties, YMP 11 isolate resulted in higher symbiotic effectiveness and % increased shoot dry weight over $N^+$ control than other tested isolates. The higher symbiotic effectiveness of YMP 11 isolate is an indication that such isolate may be better compatible and thus may have potential as source of rhizobium inoculant. Performance in a pot experiment where application of rhizobium inoculant (YMP 11) with five green gram varieties showed that application of rhizobium inoculant significantly enhanced the grain yield 5.21% over un-inoculated $N^+$ control and 37.10% over un-inoculated $N^-$ control. Among tested varieties, Yezin-11 produced the highest grain yield. Interaction studies indicated that rhizobium inoculation increased in grain yield from 23.65% to 79. 68% over un-inoculated $N^-$ control. The highest grain yield (3.23 g $plant^{-1}$) was recorded in rhizobium inoculated Yezin-11 while the lowest (1.73 g $plant^{-1}$) in un-inoculated ($N^-$) Yezin-14 variety. Yezin-14 variety was found to be responsive to rhizobium inoculation because a significant increase in grain yield of 79.68% over un-inoculated $N^-$ control was only observed in Yezin-14. On the basis of findings of the present study, it is recommended to inoculate seeds of tested green gram varieties with rhizobium inoculant (YMP 11) for cropping a good yield.

Further studies are needed to evaluate the response of green gram varieties to five rhizobium isolates under open field conditions. The best performing YMP 11 indigenous rhizobium inoculant need to be evaluated under diverse edaphic adaptation.

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# APPENDICES

## Appendix 1 Number of samples collected from each Township

| Sr. No. | Township | Sown area (acre) | No of samples* |
|---|---|---|---|
| 1 | Yinmarpin | 21,788 | 14 |
| 2 | Palae | 12,993 | 12 |
| 3 | Salingyi | 13,427 | 10 |
| 4 | Butalin | 14,296 | 4 |
| 5 | Monywa | 12,423 | 11 |
| | Total | 74,927 | 52 |

*Number of samples from each township was based on green gram growing areas of 2014-2015

## Appendix 2 Composition of Yeast Mannitol Agar (YMA) (Vincent 1970)

| | |
|---|---|
| Mannitol | 10.000 g |
| $K_2HPO_4$ | 0.500 g |
| $MgSO_4.7H_2O$ | 0.200 g |
| NaCl | 0.100 g |
| Agar | 15.000 g |
| Congo-red | 0.025 g |
| Distilled water | 1.000 L |

**Appendix 3 Composition of N free nutrient solution** (Broughton and Dillworth 1970)

| Stock Solutions | Element | Form | g $L^{-1}$ |
|---|---|---|---|
| 1 | Ca | $CaCl_2.2H_2O$ | 294.100 |
| 2 | P | $KH_2PO_4$ | 136.100 |
| 3 | Fe | Fe-citrate | 6.700 |
| | Mg | $MgSO_4.7H_2O$ | 123.300 |
| | K | $K_2SO_4$ | 87.000 |
| | Mn | $MnSO_4.H_2O$ | 0.338 |
| 4 | B | $H_3BO_3$ | 0.247 |
| | Zn | $ZnSO_4.7H_2O$ | 0.288 |
| | Cu | $CuSO_4.5H_2O$ | 0.100 |
| | Co | $CoSO_4.7H_2O$ | 0.056 |
| | Mo | $Na_2MoO_2.2H_2O$ | 0.048 |

For each 10 L of full strength culture solution, take 5.0 ml each of solutions 1 to 4, then add to 5 L of water, then dilute to 10 L. For positive ($N^+$) control treatments, $KNO_3$ (0.05%) is added giving an N concentration of 70 ppm.