CHARACTERIZATION OF INDIGENOUS GREEN GRAM RHIZOBIUM ISOLATES AND SURVIVAL ON DIFFERENT CARRIERS

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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

Nay Pyi Taw, Myanmar

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The thesis attached hereto, entitled "**Characterization of Indigenous Green Gram Rhizobium Isolates and Survival on Different Carriers**" 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 this or any other university.

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DEDICATED TO MY BELOVED PARENTS,

U MYINT AUNG AND DAW KHIN MA

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ABSTRACT

To evaluate the nodulation efficiency of indigenous rhizobium isolates, to examine morphology and biochemical properties of indigenous rhizobium isolates and to determine the survival of the most effective-indigenous rhizobium isolate on different carriers, the experiments were conducted at Department of Plant Pathology, Yezin Agricultural University from June 2018 to February 2019. A total of 45 soil samples were collected from green gram cultivated fields in Myaing, Pakokku and Yesagyo Townships of Magway Region. Nodulation and plant growth of 'Yezin-11´ green gram variety inoculated with 45 rhizobium isolates were evaluated by using sterile sand culture pots in a completely randomized design with five replications. The results indicated that all indigenous rhizobium isolates were significantly different in their effectiveness in terms of nodule dry weight and shoot dry weight. All indigenous rhizobium isolates were highly effective (> 80%) based on their symbiotic effectiveness (SE%). Based on nodule dry weight and shoot dry weight, 14 rhizobium isolates; M-1, M-9, M-10, M-11, M-12, P-8, P-9, P-12, P-13, P-15, Y-1, Y-6, Y-9 and Y-15 were selected and their nitrogen (N) uptakes, morphology and biochemical properties were tested. The N uptakes (mg plant^{-1}) were highly significant among rhizobium inoculated plants. All tested isolates showed significant differences in growth rate, colony size, colour and opacity on yeast extract mannitol agar medium. Seven isolates: M-10, M-12, P-8, P-12, P-13, P-15 and Y-6 were fast-growing *Rhizobium* spp. while the rest: M-1, M-9, M-11, P-9, Y-1, Y-9 and Y-15 were slow-growng *Bradyrhizobium* spp. All 14 rhizobium isolates were gram-negative and rod-shaped. They were able to grow in 2% sodium chloride concentration and on glucose peptone agar. Positive results were found in catalase and urease tests whereas negative reactions for methyl red except P-15. Starch hydrolysis test was negative except for M-1, M-12, P-8 and P-9. All of the fast-growing *Rhizobium* spp. and 57.14% of the slow-growing *Bradyrhizobium* spp. were able to fix more nitrogen than N (+) control. Survival of M-11 isolate which had the highest symbiotic effectiveness was assessed in different carriers: charcoal, clay soil, fly ash, Australia peat soil, Myanmar peat soil, press mud and saw dust at 15-day interval until 6 months at room temperature. Charcoal, clay soil, Australia peat soil and Myanmar peat soil retained the acceptable rhizobial population, 10^6 cfu g^{-1} at the end of the storage period. Therefore, charcoal and clay soil could be considered as the alternative carriers for the future rhizobium inoculant production.

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CHAPTER I
INTRODUCTION

Leguminous crops are one of the most important crops in both ecologically and agriculturally. Among them, green gram (*Vigna radiata* L.) constitutes the important group of grain legumes which form a major source of dietary proteins, minerals and vitamins (Taylor, Weavcer, Wood & Edzard, 2005). It was currently grown 6 million ha in worldwide (Chauhan & Williams, 2018). In Myanmar, it is also a very important crop not only for local consumption but also for export. Green gram covers about 1.24 million hectares (mil ha), with an average yield of 1.27 metric tons (MT) ha^{-1} and total production 1,595,000 MT in 2017-2018 (Ministry of Agriculture, Livestock and Irrigation [MOALI], 2018). The potential yield of the green gram in the world was 1.90 MT ha^{-1} (Chauhan & Williams, 2018). There are many factors such as application of chemicals, organic and biological fertilizers that can narrow down this yield gap (Elliott & Abbott, 2003).

Application of 30-50 kg mineral nitrogen (N) ha^{-1} resulted in significant increase of green gram productivity (Ashraf, Mueen & Warraich, 2003). Research has also shown that most farmers apply an inadequate amount of nitrogen fertilizers to their beans instead of recommended standards due to the high cost of the fertilizers (Mutuma, Okello, Karanja & Woomer, 2014). This means that beans will have to depend on biological nitrogen fixation from nitrogen fixing bacteria found in the soil in order to acquire the much-needed nitrogen for growth and development (Gilbert, 2016).

Legumes have a unique ability to establish symbiosis with nitrogen fixing bacteria called as rhizobia. These bacteria infect leguminous roots and induce formation of nodules, where nitrogen fixation takes place (Beattie & Handelsman, 1989). The bacteria's enzyme system provides constant source of reduced nitrogen to the host plant and the plant in turn supplies nutrients and energy for the activities of the bacteria (Shetta, Al-Shaharani & Abdel-Aal, 2011; Singh, Kaur & Singh, 2008; Rasool, Sharma & Rasool, 2015). Worldwide, legumes are grown on approximately 250 mil ha and fix about 90 MT of dinitrogen (N$_2$) annually from the vast free supply in the air (Kinzing & Socolow, 1994). The significance of rhizobium-legume symbiosis is a major contributor to biological nitrogen fixation (Zahran, 2001).

Although present in most of the soils, these bacteria vary in number, effectiveness in nodulation and N-fixation (Anjum, Ahmed & Rauf, 2006). One of the other most important effective factors is inoculation of legumes with rhizobial strains

selected for high symbiotic efficiency in increasing plant yield (Ashrafi & Seiedi, 2011). Besides supplying nitrogen to crop, enriches soil nitrogen content and maintains soil health and productivity, biological nitrogen fixation, a natural process offers a better alternative over chemical fertilizers as the synthetic process (Reddy & Reddy, 2004).

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). Green gram yield may increase from 10 to 37% by following rhizobium inoculation (Mansoor, 2007; Satter & Ahmed, 1992). Under unfavorable agro-ecological conditions, the yield of green gram is low because of its poor nodulation (Anjum et al., 2006). Under such conditions, it is necessary to inoculate the seeds or soil with highly effective rhizobial cultures (NifTAL, 1984). This helps increase nodulation, N uptake, growth and yield of legume crops (Erman et al., 2011; Henzell, 1988). Selection of suitable green gram-rhizobium is one of the most important means of obtaining higher yield (Uddin, Amin, Ullah & Asaduzzman, 2009).

Rhizobia are taxonomically very diverse, so efficient classification methods such as phenotypic, biochemical and molecular are needed to determine their diversity (Naz, Bano & Hussain, 2009). Wolde-Meskel, Berg, Peters and Frostegard (2004) emphasized on the classical phenotypic characterization of rhizobia which is helpful for primary classification of rhizobia. The phenotypic characterization of the rhizobia has been done by performing various biochemical tests (Kaur, Sharma, Kaur & Gill, 2012; Kucuk, Kivanc & Kinaci, 2006; Sharma, Srivastava & Sharma, 2010). Thus, biochemical tests are still one of the main criterions utilized to characterize the different rhizobium isolates including different species of *Rhizobium* (Rai, Bantawa & Sur, 2014).

There was no information on indigenous rhizobium isolates in different green gram cultivated soils in Magway Region prior to this study. The present study reported the isolation of rhizobium isolates from green gram plants and their characterization on the basis of morphological and biochemical characters. The importance of this information is that it will lead to the documentation of indigenous rhizobium isolates and as foundation to determine whether the strains are high nitrogen fixers or not.

Rhizobia are suitable to be used as biofertilizers or potential microbial inoculants (Otieno, Muthomi, Cheminingwa & Nderitu, 2009). For promoting success of inoculants, the native strains that are effective as well as competitive for nodulation

can be used as inoculants (Fening & Danso, 2002). Introduced strains also are often unsuccessful due to competition with well adapted indigenous population (Garcia, Probanza, Ramos, Burriuso & Manero, 2004). Rhizobia present in the soil are more adaptable to the soil as well as field conditions (Thies, Singleton & Bohlool, 1991).

Rhizobial inoculants are now widely used in various parts of the world because of inexpensive, environment-friendly, and easy to use with no side effects (Tena, Wolde-Meskel & Walley, 2016). Around 2000 tons of commercial rhizobial inoculants are produced annually in the world (Rebah, Prévost, Yezza & Tyagi, 2007) and applied on seed or in soil to improve crop productivity (Lupwayi, Clayton & Rice, 2006). In Myanmar, current inoculant production is contributed to only 3% of total legume growing areas (Than, Aung, San & Thein, 2003). Therefore, much more inoculants production for specific legume varieties corresponding to specific regions is urgently needed in Myanmar and high quality inoculants production is also essential for the farmers' confidence (Than et al., 2003).

Use of carrier materials for the microbial inoculants proves to be beneficial to prolong the bacterial shelf life and have long been practiced (Ardakani, Heydan, Tayebi & Mohammadi, 2010). Among various types of carrier materials, peat soil is the most commonly used carrier for commercial legume inoculants worldwide; however, high quality peat is not readily available in many developing countries especially in the tropics, and may be exhausted in the future (Halliday & Graham, 1978; Smith, 1992). Alternative materials such as sawdust (Arora, Khare, Naraian & Maheshwari, 2008), rice husk (Kaljeet, Keyeo & Amir, 2011), fly ash (Kumar, Chandra & Singh, 2010) and biochar (Saranya, Santhana Krishnan, Kumutha & French, 2011) have been tested for bio-inoculants preparation (Argal, Rawat, Aher & Rajput, 2015). Therefore, it is needed to find an alternative carrier that support large numbers of viable rhizobia for extended periods of time. The evaluation on the effectiveness of indigenous rhizobium isolate on specific host plant for inoculant production should be investigated.

The present study was, therefore, conducted with the following objectives**;**
(1) to evaluate the nodulation efficiency of indigenous rhizobium isolates from different green gram cultivated soils in Magway Region,
(2) to examine morphology and biochemical properties of indigenous rhizobium isolates, and
(3) to determine the survival of the most effective-indigenous rhizobium isolate on different carriers.

CHAPTER II
LITERATURE REVIEW

2.1 Green Gram Production in Myanmar

Green gram is an important and short duration crop of Myanmar. As green gram has high nutritive values, its consumption in most of the low income countries has increased from 22 to 66% (Shanmugasundaram, 2001). Green gram is used not only primarily as human food, but also 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, Soffes & Prine, 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 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. 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 (Ministry of Agriculture and Irrigation [MOAI], 2014). 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 Nitrogen in Soil and Plant System

Approximately 80% of the atmosphere is N_2 gas, but most plants cannot use this form of N. Decomposition of organic matter results in simple inorganic N forms such as ammonium (NH_4^+) and nitrate (NO_3^-). These are soluble in soil water and readily available for plant uptake (United State of Department of Agriculture [USDA], 2000). Ammonium is usually incorporated into organic compounds in the roots. Nitrate, however, is mobile in the xylem, and may also be stored in vacuoles and storage organs. The accumulation of nitrate in vacuoles may be important for the

cation-anion balance, for osmoregulation, and for the nutritional quality e.g. of vegetables (Marschner, 1995).

2.3 Importance of Biological Nitrogen Fixation (BNF) in Agriculture

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 represents almost 80% of the atmosphere (Abd-Alla, Issa & Ohyama, 2014), it is not readily available to plants (Unkovich et al., 2008) unless it is converted into a reduced form either biologically by bacteria or abiologically by lighting or industrial processes. Approximately 2 tons of industrially-fixed N is needed as fertilizer for crop production to equal the effects of 1 ton of biologically fixed N (Abd-Alla et al., 2014). Atmospheric nitrogen can be fixed through biological nitrogen fixation (BNF) (Mulongoy, 1992).

BNF through association with root-nodulating rhizobia effectively contributes to improve nitrogen nutrition of leguminous plants and is an ecologically sound and low-cost strategy for improving pulse productivity (Ramaekers, Galeeano, Garzon, Vanderleyden & Blair, 2013). BNF is an important source of N, and various legume crops and pasture species often fix as much as 200 to 300 kg N ha^{-1} (Ghosh et al., 2007). Globally BNF has been estimated at 70 million MT of N year^{-1}. The world production of fixed N from chemical fertilizer accounts for about 25%, whereas BNF accounts for about 60% (Zahran, 1999). Greater availability of legume inoculants offers the potential to better manage BNF (Herridge, Peoples & Boddey, 2008) and substitute for inorganic nitrogen fertilizer requirements (Sanginga & Woomer, 2009; Woyessa & Assefa, 2011). Therefore, BNF is an important and integral component of sustainable agricultural system (Sebbane et al., 2006).

2.3.1 Nitrogen fixing bacteria

Nitrogen fixing prokaryotes are able to make completely useful associations with plants: from loose associations to intercellular symbioses. 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, Zehr & Paerl, 2001). Nitrogen fixing organisms are generally active in plant root zone. Nitrogen fixing organisms are *Rhizobium*, *Azospirillum*, *Azotobacter*, *Azolla*, *Cyanobacteria* and *Gluconacetobacter diazotrophicus* (Shridhar, 2012).

Activity of nitrogen fixing microorganisms depends greatly upon excessive amount of carbon compounds 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 & Gyurjan, 1987). Plants that are capable of releasing exudates exhibit higher nitrogen fixation activity in soil (Egamberdieva & Kucharova, 2008). Besides, many microorganisms are able to produce hormones and these substances can influence plant growth effectively (Andrew et al., 2007). 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 & Gyurjan, 1987).

2.3.2 Rhizobium-legume symbiosis

The fixation of atmospheric N_2 by the rhizobium-legume symbiosis is a central element of the N-cycle in both types of ecosystems (Reichman, 2007). Therefore, rhizobium-legume symbiosis has been widely studied as the model of mutualistic evolution and the essential component of sustainable agriculture. Extensive genetic and recent genomic studies have led to the hypothesis that many distinct strategies, regardless of rhizobial phylogeny, contributes to the varied rhizobium-legume symbiosis (Tian et al., 2012). The interaction between plants and symbiotic soil microorganisms are important indicators of ecosystem productivity and diversity (Thrall et al., 2011). In rhizobium-legume symbiosis, macrosymbiont is the legume plant and microsymbiont is the prokaryotic bacteria (rhizobium). The macrosymbiont legume belongs to Leguminoseae, divided into three subfamilies comprising of around 20,000 plant species in about 750 different genera (Young & Haukka, 1996). Microsymbiont *Rhizobium* is nitrogen fixing motile prokaryote defined solely by their ability to nodulate legumes (Brahmaprakash & Pramod, 2012).

Effective nodulation requires compatibility between the host legume and specific strain of *Rhizobium.* Once symbiosis is established the host plant provides carbon substrate as source of energy and the bacteria reduces atmospheric nitrogen to ammonia which is exported to plant tissue for protein synthesis. The symbiosis between *Rhizobium* or *Bradyrhizobium* and legumes are usually more effective agronomic practice for ensuring an adequate supply of N for legume based crop and pasture production than the application of nitrogenous fertilizer (Aslam, Ahmad, Himayatullah & Ahmad, 2010).

2.3.3 Host specificity

Generally, the relation between rhizobia and legume is a selective one: each rhizobia species has a distinct host range allowing nodulation of a particular set of leguminous species, and each leguminous species nodulates only with a certain range of rhizobia. 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 8 species of *Mesorhizobium*. Red clover is nodulated by *Rhizobium leguminosarum* biovar. *trifolii* and alfalfa is nodulated by *Sinorhizobium meliloti*. *Bradyrhizobium japonicum* SAY3-7 was the most effective strain for nitrogen fixation in soybean (Htwe, 2016).

The correct matching or compatibility of the rhizobia and legume is extremely important. This matching system called, "cross-inoculation grouping" was necessary to organize the diverse legumes and their rhizobial partners. In summary, a cross-inoculation group consists of a collection of legume species that will develop nodules when inoculated with the rhizobia obtained from the nodules from any member of that legume group (Singleton et al., 1990). Nevertheless, the host range depends on the legume cultivar used and conditions tested (Martinez-Romero, 2003). However, a complex association between legume and rhizobia has been found and many leguminous species can nodulate with distinctive rhizobial species in different geographical regions (Han, Wang & Chen, 2005).

2.3.4 Nodule formation

The establishment of nodulation (Figure 2.1) 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).

In root nodule formation, bacteria can convert dinitrogen into ammonia and supply it to the host plant in exchange for carbohydrates (Young, 1992). 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 crucial 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 rhizobium-legume symbiosis requires the coordinate interaction of several major classes of genes present in rhizobia: the *nif* genes and *fix* genes (Kaminski, Batut & Boistard, 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 (Figure 2.2). Within the developing nodule, the rhizobia become swollen. At this stage, they are called bacteroids.

2.3.5 Assessment of symbiotic efficiency

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, Rupela & Lee, 1995). Plants should be harvested during flowering period to estimate the amount of nitrogen fixed (Karaca & Uyanoz, 2012). Size and color of nodules should be examined for more accurate evaluation (Rebah, Tyagi & Prevost, 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 & Weaver, 1985). The number and mass of nodules can provide a rough indication of the amount of nitrogen fixed (Hardarson & Atkins, 2003). Shoot dry weight of plants harvested at floral initiation is generally accepted criterion for nitrogen-fixing ability (Prevost & Antoun, 2006). Total nitrogen accumulation in bean legumes is one of the best parameters that indicates the amount of nitrogen fixed under controlled conditions (Anglade, Billen & Garnier, 2015). Total nitrogen content and nodule dry weight frequently correlate well with shoot dry weight. Nodule dry weight provides an acceptable distinct factor for strain comparison (Somasegaran & Hoben, 2012).
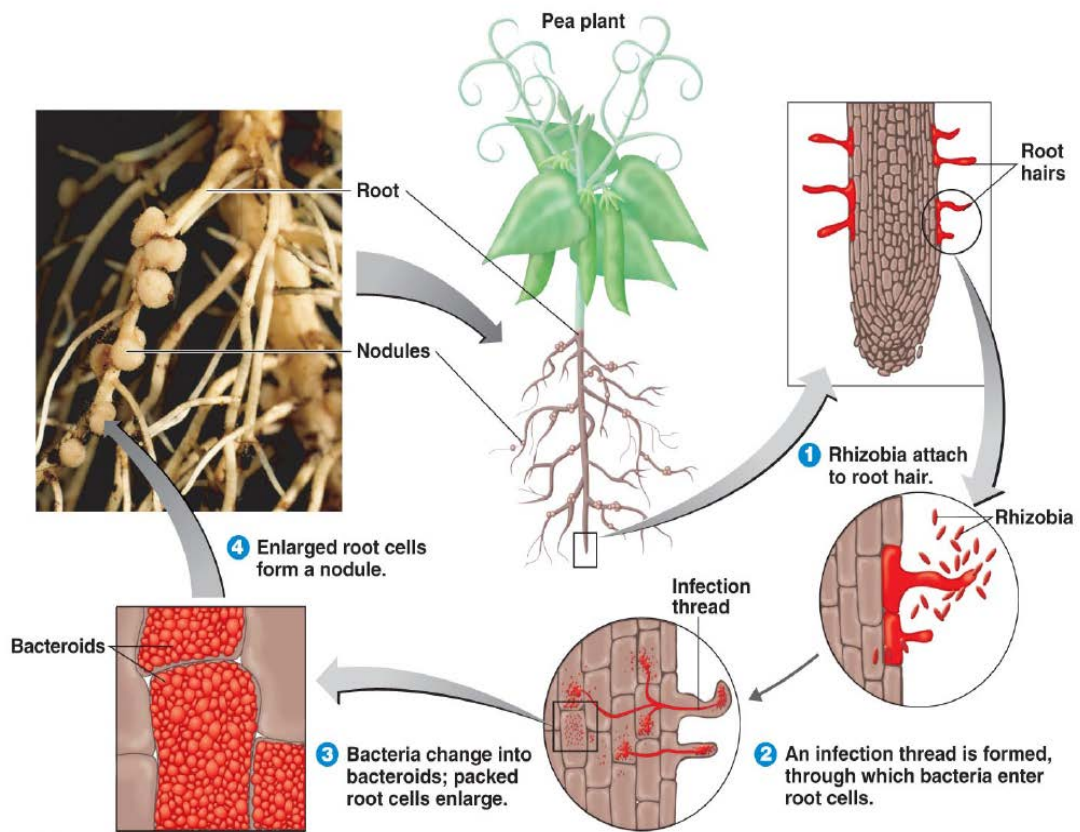
Figure 2.1 Interaction between rhizobia and their host plant

(http://eagri.org/eagri50/AMBE101/pdf/lec18.pdf)

Figure 2.2 Nodule formation on rhizobium inoculated root of green gram

2.3.6 Factors affecting the efficiency of symbiotic nitrogen fixation

Symbiotic nitrogen fixation (SNF) efficiency depends on rhizobium strain, plant host, the environmental factors, soil and their interaction. A number of biotic and abiotic factors affect SNF (Allito, Mensah & Alemneh, 2014). The most important abiotic factors include drought, salinity, water-logging, temperature, soil acidity, inadequate mineral nutrition and mineral toxicities (Abdel-Latef & Ahmed, 2015). The biotic factors include competition of ineffective-indigenous rhizobia, insect pest and diseases (Sofy, Attia, El-Monem, Sharaf & El-Dougdoug, 2014). These factors affects on survival of rhizobia in the soil, the infection process, nodule growth and nodule function and SNF (Serrage & Adu-Gyamfi, 2004).

The two important climatic determinants are temperature and light. Extreme temperatures affect nitrogen fixation adversely because nitrogen fixation is an enzymatic process (Ogutcu, Algur, Elkoca & Kantar, 2008). 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 & 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. Various agronomic practices influence the rate of nitrogen fixation (Siyeni, 2016). Seed inoculation with efficient strains of *Bradyrhizobium,* a starter dose of nitrogen through fertilizers, light irrigations to avoid water-logging and avoiding the use of plant protection chemicals that harm the microbes, positively influence the BNF and lead to greater amounts of nitrogen fixation. However, 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).

2.4 Morphological and Biochemical Characteristics of Rhizobia

Currently, rhizobia include at least 14 genera comprising more than 98 species (Weir, 2013). Morphological and biochemical characters; size, shape, colour, texture

of colonies and the ability to alter the pH of the medium are generally stable characteristics useful in defining strains or isolates (Kapembwa, 2014). Typical morphological colony characteristics, when grown on standard yeast-mannitol medium, may include; form, elevation, colour and margin (Somasegaran & Hoben, 2012). Biochemical characterization can be very helpful in confirming that isolates are rhizobium species. Among the biochemical tests, the glucose assay is the most important test which can be performed to determine the capability of microorganisms to utilize glucose as a sole carbon source for growth (Singh et al., 2008). Biochemical characterization plays an important role to differentiate between fast and slow growers of rhizobium species (Singh, Luthra & Desai, 2013).

2.5 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 (Than, San & Thein, 2006). Initially, rhizobial inoculant for groundnut, chickpea, green gram, black gram, soybean, pigeonpea and cowpea were produced by using exotic strains from NifTAL project (Than et al., 2006). 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 (Thu, 2018). Local strain (DAR 18) was used for green gram inoculants. Soybean inoculant was prepared by inoculating exotic strains; TAL 379, TAL 162 and local DAR 1 strain into sterilized peat. Rhizobial inoculant for pigeonpea 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 total legume growing areas (Than, 2010).

2.6 Types of Existing Carriers for Inoculants

The almost universal carrier for rhizobia (which is the only inoculant being sold in large volume today) is peat (Smith, 1987). It supports bacterial strain for long time and contains 13-31% organic matter (Burton, 1967). However, high quality peat

is not readily available in many developing countries especially in the tropics, and may be exhausted in the future (Smith, 1992). Alternatively, different carrier materials have been evaluated.

Carriers can be divided into five basic categories:

(i) Soils: peat, coal, clay, and inorganic soil (Kotb & Angle, 1986; Singh & Sharma, 1973; Smith, 1995).

(ii) Plant waste materials: composts, farmyard manure, soybean meal (Iswaran, Sen & Apte, 1972), soybean and peanut oil (Kremer & Peterson, 1982 a,b), wheat bran (Jackson, Whipps, & Lynch, 1991), press mud (a by-product from the sugar industry, Philip & Jauhri, 1984), agricultural waste material (Sadasivam, Tyagi & Ramarethinam, 1986), spent mushroom compost (Bahl & Jauhri, 1986), and plant debris (Richter, Ehwald & Conitz, 1989).

(iii) Inert malerials: vermiculite (Sparrow & Ham, 1983 a,b; Paau, Graham & Bennett, 1991),

(iv) Perlite, ground rock phosphate, calcium sulfate, polyacrylamide gels (Dommergues, Diem & Divies,1979), and alginate beads (Aino, Maekaua, Mayama & Kato, 1997).

(v) Plain lyophilized microbial cultures (Mohammadi, 1994 a,b) and oil-dried bacteria (Johnston, 1962).

CHAPTER III
MATERIALS AND METHODS

3.1 Nodulation Efficiency of Indigenous Rhizobium Isolates on Green Gram

The experiment was carried out in the screen house of Department of Plant Pathology, YAU, during rainy season (June to July 2018).

3.1.1 Study sites and soil samples collection

Sampling site of the present study was focused on Magway Region, the largest green gram growing areas of Myanmar. Townships selected for soil sample collection were Myaing, Pakokku and Yesagyo. Forty five soil samples were collected during November 2017 after harvesting of green gram (Appendix 1). They were collected from farmers' fields in which green gram had been grown with no history of rhizobium inoculation and with a long history of green gram cultivation. Thirty grams of soil samples were collected from the depth of 0-20 cm by using cross diagonal pattern. These soil samples were mixed and homogenous composite samples (150 g) were put in sterilized polyethylene bags (Jida & Assefa, 2011). They were transported to the Department of Plant Pathology Laboratory, Yezin Agricultural University to determine the nodulation efficiency.

3.1.2 Rhizobium isolates

Forty five indigenous rhizobium isolates were named based on collection sites and were shown in Table 3.1.

3.1.3 Preparation of test plant

Green gram seeds, var. 'Yezin-11', provided from the Food Legumes Section, Department of Agricultural Research (DAR), were surface-sterilized with 95% ethyl alcohol and suspended in 5% NaOCl solution for 3-4 minutes. They were then thoroughly washed with sterilized distilled water for 5 times and sterilized seeds were germinated in petridishes. River bank sands were washed well in tap water and immersed in 10% dilute hydrochloric acid for 2 days. The acid was decanted off and the sand was washed in tap water and finally distilled water to get rid of the last traces of acid. Sands were sterilized by autoclaving for 2 hours for three consecutive days (Legesse, 2016).

Table 3.1 Designation of isolates, host varieties and collection sites

Sr. No	Isolate	Host variety	Collection site	
			Village	Township
1	M-1	Yezin-11	Ywarthitphya	Myaing
2	M-2	Yezin-11	Ywarthitphya	Myaing
3	M-3	Yezin-14	Ywarthitphya	Myaing
4	M-4	Yezin-11	Ywarthitphya	Myaing
5	M-5	Yezin-11	Ywarthitphya	Myaing
6	M-6	Yezin-14	Kyauksauk	Myaing
7	M-7	Yezin-14	Kyauksauk	Myaing
8	M-8	Yezin-11	Kyauksauk	Myaing
9	M-9	Yezin-11	Kyauksauk	Myaing
10	M-10	Yezin-14	Kyauksauk	Myaing
11	M-11	Yezin-11	Magyisu	Myaing
12	M-12	Yezin-11	Magyisu	Myaing
13	M-13	Yezin-11	Magyisu	Myaing
14	M-14	Yezin-11	Magyisu	Myaing
15	M-15	Yezin-11	Magyisu	Myaing
16	P-1	Yezin-11	Gyiywar	Pakokku
17	P-2	Yezin-11	Gyiywar	Pakokku
18	P-3	Yezin-11	Gyiywar	Pakokku
19	P-4	Yezin-11	Gyiywar	Pakokku
20	P-5	Yezin-11	Gyiywar	Pakokku
21	P-6	Yezin-14	Meethwaekan	Pakokku
22	P-7	Yezin-14	Meethwaekan	Pakokku
23	P-8	Yezin-14	Meethwaekan	Pakokku
24	P-9	Yezin-14	Meethwaekan	Pakokku
25	P-10	Yezin-14	Meethwaekan	Pakokku
26	P-11	Yezin-11	East Chaukkan	Pakokku
27	P-12	Yezin-14	East Chaukkan	Pakokku
28	P-13	Yezin-11	East Chaukkan	Pakokku
29	P-14	Yezin-11	East Chaukkan	Pakokku
30	P-15	Yezin-14	East Chaukkan	Pakokku

Table 3.1 (Continued)

Sr. No	Isolate	Host variety	Collection site	
			Village	Township
31	Y-1	Yezin-11	Sinchaung	Yesagyo
32	Y-2	Yezin-11	Sinchaung	Yesagyo
33	Y-3	Yezin-11	Sinchaung	Yesagyo
34	Y-4	Yezin-11	Sinchaung	Yesagyo
35	Y-5	Yezin-11	Sinchaung	Yesagyo
36	Y-6	Yezin-11	Ywarthit	Yesagyo
37	Y-7	Yezin-11	Ywarthit	Yesagyo
38	Y-8	Yezin-11	Ywarthit	Yesagyo
39	Y-9	Yezin-11	Ywarthit	Yesagyo
40	Y-10	Yezin-11	Ywarthit	Yesagyo
41	Y-11	Yezin-11	Gwaycho	Yesagyo
42	Y-12	Yezin-14	Gwaycho	Yesagyo
43	Y-13	Yezin-11	Gwaycho	Yesagyo
44	Y-14	Yezin-14	Gwaycho	Yesagyo
45	Y-15	Yezin-11	Gwaycho	Yesagyo

The culture pots were sterilized using 95% ethanol and then washed with distilled water. The pots were filled with 500 g of sterilized sand and 50 ml of nitrogen-free nutrient solution (Appendix 2). The 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 of potassium nitrate (KNO_3) in nutrient solution with was supplied (Somasegaran & Hoben, 2012).

3.1.4 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 solution (Appendix 3) in a 200 ml conical flask (Htwe, 2016). The flask was shaken on a rotary shaker at 120 rpm for one hour to prepare a well-mixed soil suspension. Five milliliters of incubated aliquot soil suspension were inoculated beside the pre-germinated seed. Then the surface of the substrate was covered with sterilized gravel (about 2 cm) as an anti-contamination layer.

3.1.5 Data recording

Plants were harvested at 38 days after sowing (DAS) (Karaca & 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, Muthomi & Theuri, 2007). Nodule dry weight (mg), shoot dry weight (mg) and nitrogen uptake (mg plant[-1]) were recorded.

Total shoot N content was estimated by Kjeldahl's method using Modern Kjeldahl apparatus (VELP SCIENTIFIC UDK 159 digestion, distillation and titration system) (Bremner, 1960). Chemical reagents were shown in Appendix 4.

The nitrogen content was calculated by the following equation (Bremner, 1960; Kapembwa, 2014).

$$\text{Nitrogen content (\%) in sample} = \frac{V_S - V_B \times N \times 1.4007}{W}$$

Where, V_S = Titre volume of sample (ml)

V_B = Titre volume of blank (ml)

N   = Normality of standard acid taken

W   = Weight of sample taken

The total N uptake (mg plant^{-1}) in plant was calculated with the following equation (Roy, 2007).

$$\text{N uptake (mg plant}^{-1}) = \frac{\text{Nitrogen content (\%)} \times \text{Dry matter yield}}{100}$$

Symbiotic effectiveness percent (SE%) of the isolates was also calculated by using the following formula (Elkan, 1987).

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

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, Materon & Afandi, 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).

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

3.1.6 Experimental design and statistical analysis

The experiment was laid out in a Completely Randomized Design (CRD) with five replications. 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.

3.2 Study on Morphology and Biochemical Properties of Indigenous Rhizobium Isolates

The experiment was conducted in the laboratory of the Department of Plant Pathology, Yezin Agricultural University (YAU) from August to September 2018.

3.2.1 Isolation of green gram-nodulating rhizobia

Rhizobia were isolated from root nodules of green gram. A sterile paper towel was spread under the lamina flow on which the cleaned nodules were cut from the root system using sterile sharp scalpel blade and forceps. Nodules were initially washed with 95% ethanol for 5-10 seconds followed by 5% sodium hypochlorite

solution for 3-5 minutes. Nodules were then rinsed five times with sterile distilled water. Each nodule was then crushed in a sterile petri plate with a drop of sterilized distilled water using sterile glass rods until the contents of the nodule are released into the water drop. A loopful of bacterial suspension was streaked on yeast extract mannitol agar (YMA) medium (Appendix 5). The plates were then inverted and incubated for 3-7 days at 27°C (Somasegaran & Hoben, 2012). The bacterial isolates were purified twice until single colonies were obtained. Sub-cultures of single cell colonies were streaked in YMA slants for further studies.

3.2.2 Rhizobium isolates

Fourteen indigenous rhizobium isolates; M-1, M-9, M-10, M-11, M-12, P-8, P-9, P-12, P-13, P-15, Y-1, Y-6, Y-9 and Y-15 were selected based on the results of the previous experiment.

3.2.3 Identification of selected indigenous rhizobium isolates

The single colony of isolated bacteria appeared on YMA media plate was characterized on the basis of colony form, elevation, margin and color (Tiwari, Hoondal & Tewari, 2009).

3.2.4 Biochemical properties of indigenous rhizobium isolates

Rhizobia have different biochemical properties (Appendix 12). Among them, some biochemical properties such as growth on bromothymol blue agar, gram staining reaction, growth in 2% sodium chloride concentration, growth on glucose peptone agar, catalase activity test, starch hydrolysis test, urease hydrolysis and methyl red test were tested with 3 replications.

(a) Growth on Bromothymol Blue (BTB) Agar

The cultures were streaked on BTB (Appendix 6) agar plates for differentiating slow-growing *Bradyrhizobium* spp. from fast-growing *Rhizobium* spp. (Maruekarajtinpleng, Homhaulb & Chansa-ngaveja, 2012). The plates were then incubated at 28±2°C for 2-5 days. Change in colour of the medium was observed. The isolates were classified as slow growers if the media turned blue and fast growers if they turned the medium yellow (Chen et al., 2001).

(b) Gram Staining Reaction

Selected pure rhizobium isolates were sub-cultured on YMA media slant and incubated at 27°C for 48 hours. A loopful of the bacteria was spread on a slide containing a drop of water and allowed to dry in air. The slide was fixed by heating on a very low flame to obtain the heat fix smear. It was allowed to cool and then stained with crystal violet solution for 1 minute followed by washing with water for 5 seconds. The smear was covered with one drop of concentrated iodine solution and left for 1 minute followed by decolourized with alcohol for 5 minutes. The slide was gently washed with sterilized water for 5 seconds and counterstained with dilute safranin solution for 3 minutes. Finally the smear was washed with water and allowed to air-dry and examined under the high power microscope (100X) using oil immersion (Somasegaran & Hoben, 2012).

(c) Growth in 2% Sodium Chloride (NaCl) Concentration

The ability of rhizobium isolates to grow at 2% NaCl concentration was tested by culturing them on YMA medium containing 2% NaCl (Appendix 7). Observation of growth was done after 3-5 day of incubation (Graham & Parker, 1964).

(d) Growth on Glucose Peptone Agar (GPA)

Rhizobium isolates were cultured in glucose peptone agar containing bromocresol purple (Appedix 8) to determine the capability of the rhizobium to utilize glucose as the sole carbon source for its growth (Singh et al., 2008) and then incubated for 48 hours. Presence of growth was observed (Kleczkowska, Nutman, Skinner & Vincent, 1968).

(e) Catalase Activity Test

Two or three drops of 3% H_2O_2 was placed on a glass slide and small amount of bacterial culture were mixed with the inoculation loop. Production of gas bubbles was observed (Cappuccino & Sherman, 1992).

(f) Starch Hydrolysis Test

Nutrient Agar (NA) (Appendix 9) medium containing 0.2% starch powder was used to determine the starch hydrolysis by the isolates. Positive test indicates that isolates have the competence to solubilise starch by producing amylase enzyme. After incubation period, plates were flooded with Gram's iodine and presence or absence of halos around the bacterial colonies was recorded (de Oliveira, de Oliveira, Andrade & Chagas, 2007).

(g) Urease Hydrolysis Test

Yeast extract mannitol (YEM) broth was prepared by adding 2% (w/v) urea and 0.012% phenol red (Appendix 10) to check the ability of rhizobium that produces an exoenzyme, called urease. The broth was inoculated with log phase cultures and incubated for 48 hours. The production of color was observed according to Lindstrom and Lehtomaki (1988). If the urea in the broth is degraded, ammonia is produced. Thus, an alkaline environment is created, and the media turns pink.

(h) Methyl Red (MR) Test

A tube of glucose phosphate peptone (Appendix 11) was inoculated with pure culture of test organism and incubated at $28\pm2°C$ for 2 days. At the end of incubation, 5 drops of MR reagent were directly added to the tube. The tube was slightly shaken and the color result was immediately interpreted. Development of stable red color indicated the positive reaction while yellow or intermediate orange colour was negative (Bhatt, Vyas, Shelat & Mistry, 2013).

3.3 Survival of the Most Effective-Indigenous Rhizobium Isolate on Different Carriers

This experiment was conducted in the period from August 2018 to February 2019 at the Department of Plant Pathology, Yezin Agricultural University (YAU). The evaluation of the survival of selected isolate on different carriers was done *in vitro*.

3.3.1 Rhizobium isolate

The tested isolate was M-11, the most effective isolate obtained from the experiment I.

3.3.2 Collection and preparation of different carriers

Australia peat soil and Myanmar peat soil were provided from Australian Centre for International Agricultural Research (ACIAR) Organization and Agricultural Microbiology Research Section, DAR, respectively. Fly ash was collected from Tigyit coal mine and power plant which is situated at Tigyit village, Pinlaung Township, Shan State, Myanmar. Charcoal was produced by slow heating or burning wood (tamarinds). Clay soil was collected from Nay La Ban Stream in Yezin. Press mud and saw dust were purchased from Sugarcane and Timber Industries in Nay Pyi Taw Union Territory.

All substrates (carriers) were air-dried and grounded in a hammer mill and sifted to pass 200-mesh sieve (Plate 3.1). Moisture content was kept at 20% maximum water holding capacity level. One hundred gram of each carrier was packed in plastic bag and then the bags were sealed and sterilized for 20 minutes at 121°C. Sterility of carriers was examined by plating small samples of autoclaved carriers in YMA and making observation of colony growth.

3.3.3 Physicochemical properties of carrier materials

Moisture content, pH and water holding capacity of all tested carriers were measured. The pH of the carriers was determined in 1:5 carrier material:water ratio using a pH meter. The moisture content of the sample was calculated by using the following equation (Bhavya, 2016):

$$\text{Moisture content (\%)} = \frac{\text{Weight of wet inoculant} - \text{Weight of dry inoculant}}{\text{Weight of wet inoculant}} \times 100\%$$

Water holding capacity of each carrier material was determined according to Keen-Rackzowski box (Keen & Raczkowski, 1921).

$$\text{Water holding capacity (\%)} = \frac{\text{Total water in the wet soil}}{\text{Oven dry weight of total soil}} \times 100\%$$

3.3.4 Preparation of broth cultures

Sterilized yeast mannitol broth was inoculated with 2 days old culture of M-11 in 250 ml conical flask in a rotary shaker at 28°C for 72 hours at 100 rpm (Ghazi, 2017). The cell count of rhizobial broth culture after incubation was measured by serial dilution plate method and also absorbance was measured at 600 nm using a spectrophotometer. The culture containing a bacterial population of about 2.4×10^9 cells ml^{-1} at optical density (OD) 1 was used for mixing with the carrier preparations.

3.3.5 Inoculation of carriers with M-11

Three days old broth culture of M-11 was used for inoculation. The initial rhizobial population in each inoculated test carrier were quantified by following usual dilution plate method using YMA medium and observations were recorded.

| Charcoal | Clay soil | Fly ash | Australia peat soil |

| Myanmar peat soil | Press mud | Saw dust |
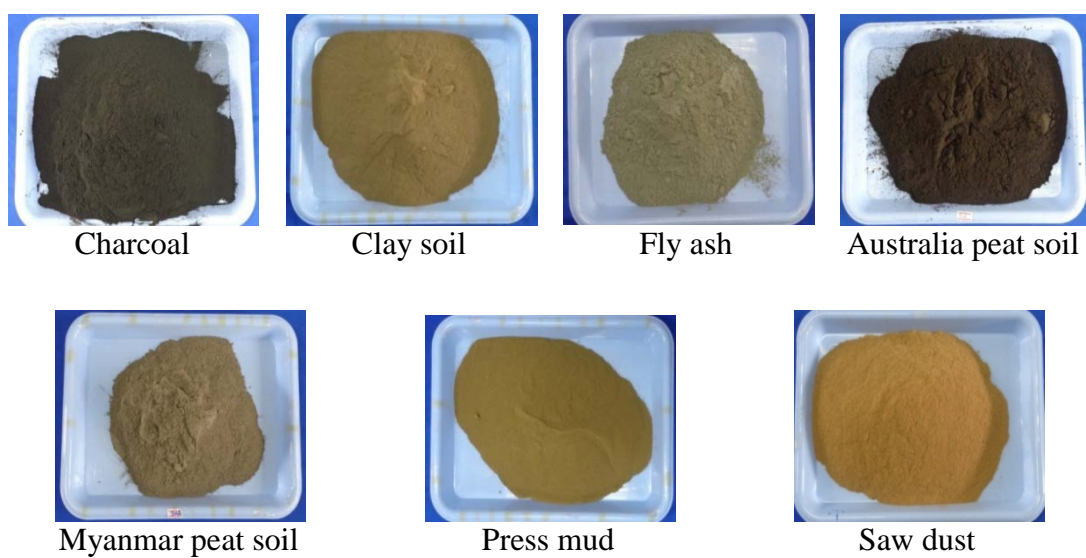
Plate 3.1 Seven carriers after sieving with 200-mesh sieve for rhizobial inoculant

The punctured area was wiped with 70% isopropyl alcohol. Then the bacterial suspension was injected to the sterilized carriers with sterile plastic disposable syringe fitted with a sterile needle guage in the proportion of 1:2 (broth:carrier) i.e. to the moisture content is nearly around 40-50 percent. The punctured hole was then covered with the sticky seal patch (Okereke & Okeh, 2007). The bags were evenly mixed to ensure similarity in distribution and absorption of the broth culture into the carrier. Then the inoculants were carefully labeled with isolate name, treatment, replication and date of injection (Plate 3.2). To evaluate the survival of the rhizobium, the packages were incubated at room temperature. Un-inoculated controls which were used to check any contamination showed a negligible bacterial growth on YMA plate in serial dilution during the study.

3.3.6 Determination of viable cell count of rhizobia in carrier materials

A total of 1 g of inoculant from each bag was placed into test tubes containing 9 ml of sterilized distilled water. It was then mixed thoroughly to ensure complete separation of the microorganism from the carrier. Next, 10-fold serial dilution was performed until 10^{-8}. Firstly, 0.1 ml of substrate solution from each dilution was placed on YMA and spread with sterilized glass rod. Two replications from each dilution were prepared (Hoben & Somasegaran, 1982). Then, all the plates were incubated at 28˚C for 3-5 days and the numbers of colony appeared (colony forming unit, cfu g^{-1}) was determined. From this value, the $\log_{10}$ cfu g^{-1} was calculated.

3.3.7 Data recording

The survival of rhizobia in different carrier inoculants was checked periodically at 15 days interval up to 6 months of storage by serial dilution plate count method using yeast extract mannitol agar containing Congo-red as indicator. Moisture content and pH of carrier inoculants were recorded at the initial and final stages after injection.

3.3.8 Experimental design and statistical analysis

The experiment was laid out in a Completely Randomized Design (CRD) with 3 replications. Analysis of variance (ANOVA) was performed by using Statistix Version 8.0 program and means were separated by Least Significant Difference (LSD) test at 5% probability level.

| Charcoal | Clay soil | Fly ash | Myanmar peat soil |

| Australia peat soil | Press mud | Saw dust |
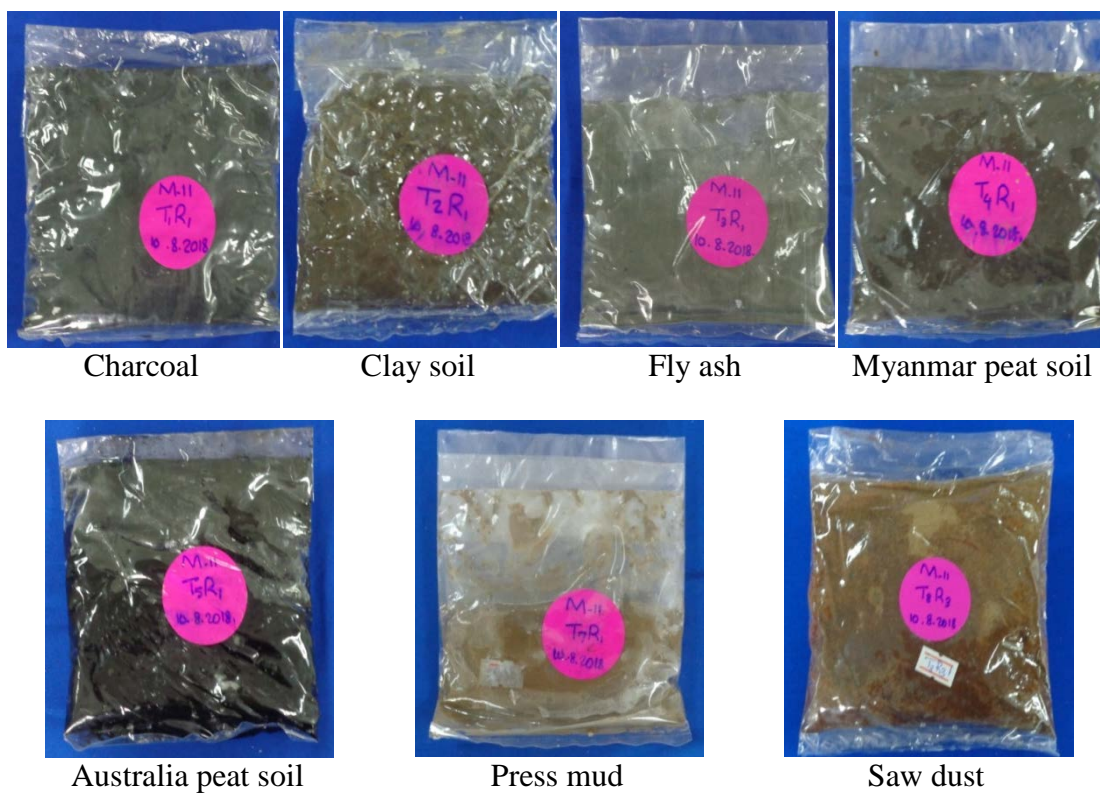
Plate 3.2 Rhizobium inoculant bags containing 100 g each carrier

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Nodulation Efficiency of Different Indigenous Rhizobium Isolates on Green Gram

All 45 indigenous rhizobium isolates collected from different green gram cultivated soils in Myaing, Pakokku and Yesagyo Townships were able to nodulate on 'Yezin-11'. When inoculated with each isolate, nodule formations were observed on the root of 'Yezin-11' variety. However, the plants treated with N (+) and N (-) controls did not form any nodules (Plate 4.1, 4.2 and 4.3) and showed nitrogen deficiency symptoms in N (-) control. In this study, the colour of nodule was pink to red. Most of nodules were large and clustered on the primary and upper lateral roots. And also the patterns of nodule were not distributed throughout the root system. The effective nodules were usually large and clustered on the primary and upper lateral roots while ineffective nodules are small, numerous and distributed throughout the root system. Nodules harboring effective rhizobia are usually large and they contain leghemoglobin and are colored pink to red while those formed by ineffective rhizobia are small and white (Society for General Microboilogy [SGM], 2002).

Nodule dry weight and shoot dry weight of green gram inoculated with 45 rhizobium isolates were significantly different from each other (Table 4.1 and 4.2). These variations may be due to differences in efficiency of individual rhizobium isolates on nodulation and plant growth of green gram variety 'Yezin-11'. Than (2010) and Zaw (2014) described that the effectiveness of different indigenous rhizobium isolates varied significantly with each other in terms of nodule number, nodule dry weight and shoot dry weight, respectively in chickpea. Thu (2018) and Vijila and Jebaraj (2008) also reported that all tested strains nodulated their host very well with different levels of effectivity in green gram. In this study, rhizobium isolates from Myaing and Yesagyo Townships gave better performance in plant growth than those from Pakokku Township. Thu (2018) also mentioned that the distinct differences in nodulation and plant growth of green gram were observed when the plants were inoculated with different indigenous rhizobium isolates. Rhizobial strains within a species vary in their ability in nodulation and N-fixation (Kucey et al., 1988).
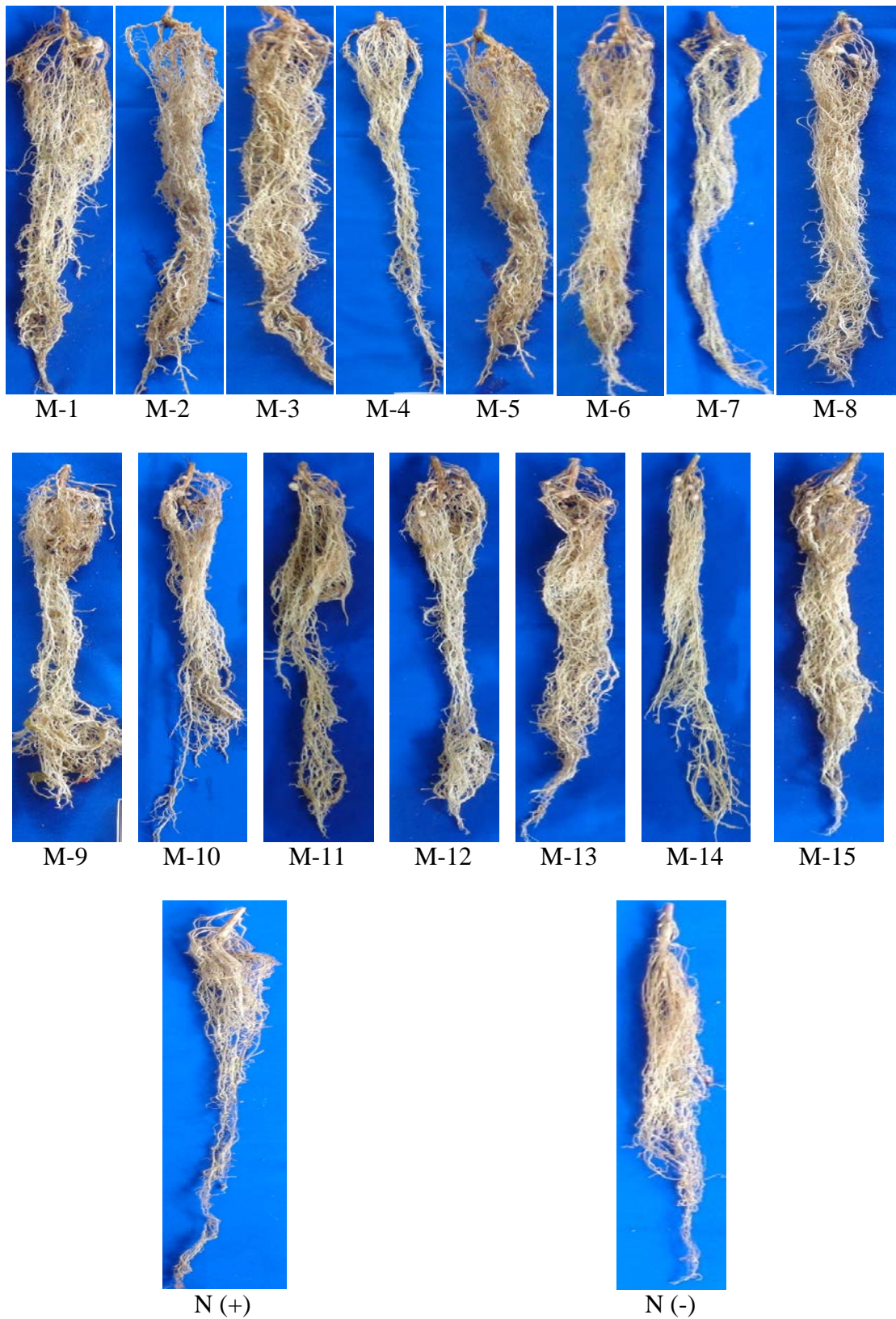
Plate 4.1 Nodule formation of green gram variety, 'Yezin-11' inoculated with 15 rhizobium isolates collected from Myaing Township and un-inoculated controls at 38 DAS
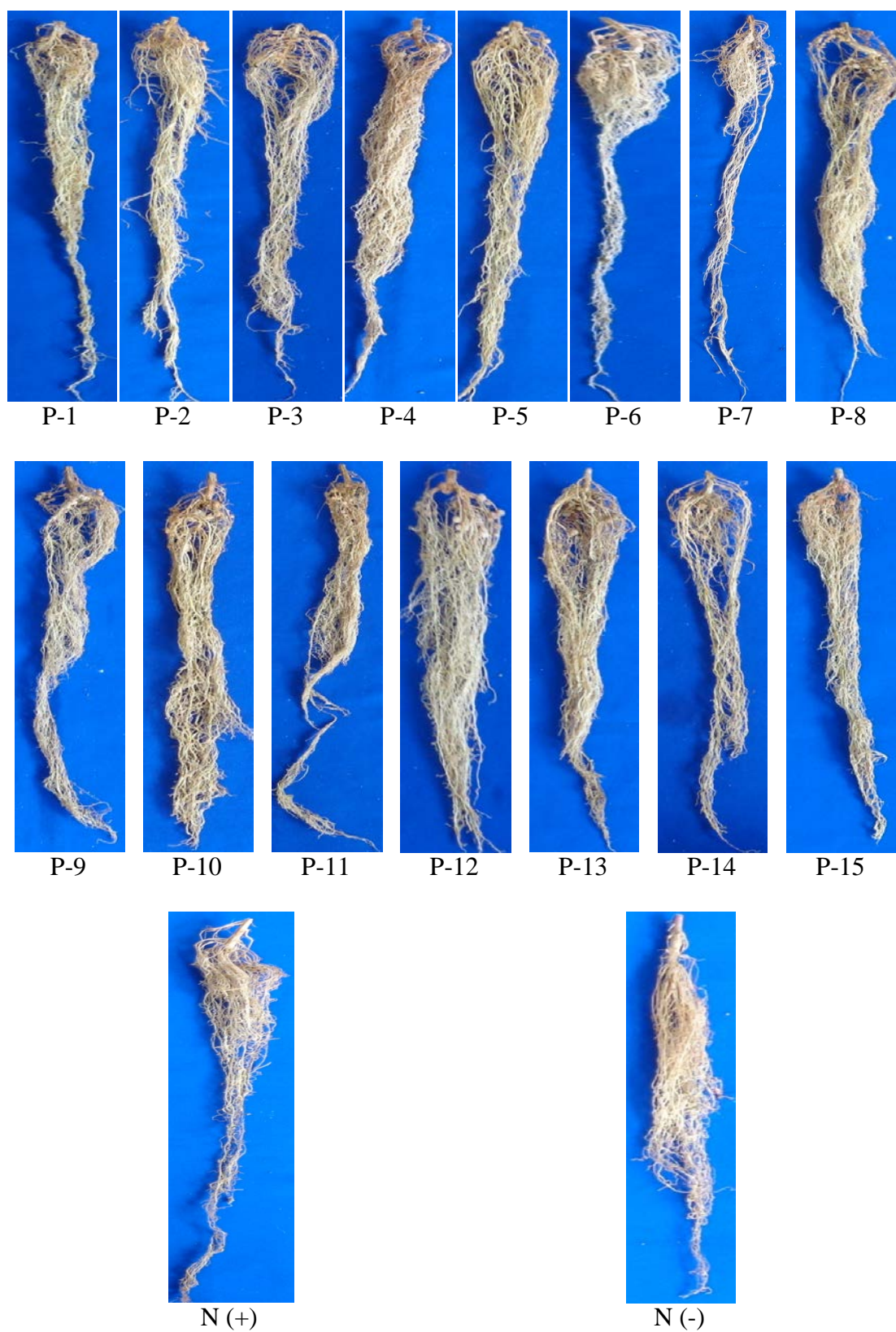
Plate 4.2 Nodule formation of green gram variety, 'Yezin-11' inoculated with 15 rhizobium isolates collected from Pakokku Township and un-inoculated controls at 38 DAS
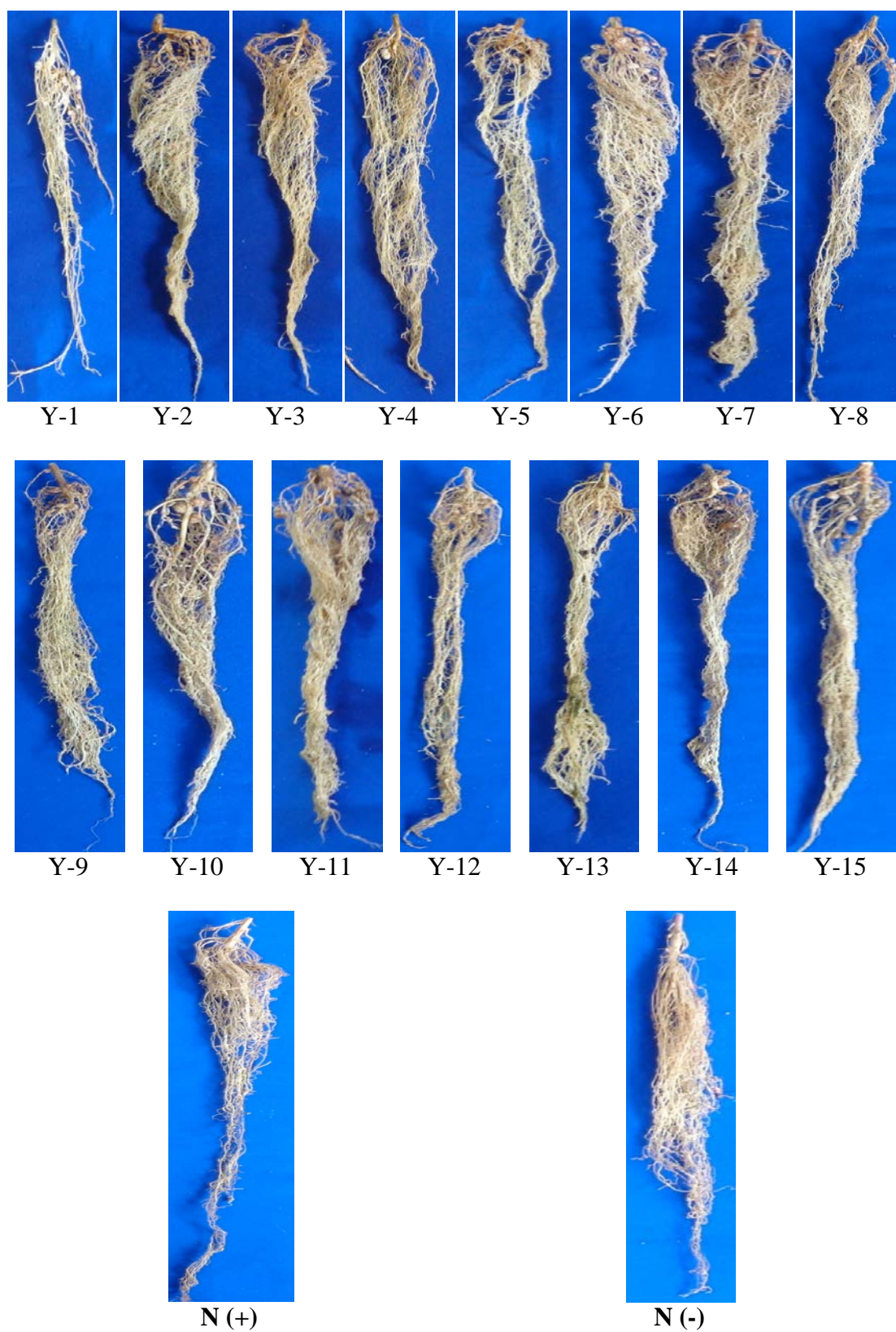
Plate 4.3 Nodule formation of green gram variety, 'Yezin-11' inoculated with 15 rhizobium isolates collected from Yesagyo Township and un-inoculated controls at 38 DAS

Table 4.1 Analysis of variance of nodule dry weight and shoot dry weight of 'Yezin-11' green gram inoculated with 45 rhizobium isolates at 38 days after sowing (DAS)

Source of variation	Degree of freedom	Mean square	
		Nodule dry weight (mg plant^{-1})	Shoot dry weight (mg plant^{-1})
Treatments	46	653.032**	440329**
Error	188	124.817	116907
Total	234		

** Significant at 1% level

Table 4.2 Nodule dry weight, shoot dry weight, symbiotic effectiveness (SE%) and percent increase in shoot dry weight (SDW) over N (+) control of 'Yezin-11' green gram inoculated with 45 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	M-1	32.2 i-n[y]	576.6 n[y]	88.4 l[y]	-11.7
2	M-2	36.6 f-n	655.8 k-n	100.5 j-l	0.5
3	M-3	34.6 h-n	696.2 j-n	106.7 i-l	6.7
4	M-4	33.4 h-n	902.2 f-n	138.3 f-l	38.3
5	M-5	36.0 f-n	1064.2 e-l	163.1 e-j	63.1
6	M-6	42.6 c-l	1063.4 e-l	163.0 e-j	63.0
7	M-7	46.2 b-h	1278.8 c-g	196.0 c-f	96.0
8	M-8	43.6 b-k	874.0 f-n	133.9 f-l	33.9
9	M-9	50.6 a-e	1747.6 ab	267.8 ab	167.8
10	M-10	28.4 mn	1426.8 b-e	218.7 b-e	118.7
11	M-11	51.6 a-d	1886.2 a	288.9 a	188.9
12	M-12	40.4 d-n	1398.0 b-e	214.2 b-e	114.2
13	M-13	35.0 f-n	1176.0 d-i	180.2 d-h	80.2
14	M-14	44.6 b-i	1081.0 d-i	165.6 d-i	65.6
15	M-15	35.8 f-n	1028.8 e-m	157.7 e-k	57.7
16	P-1	30.0 k-n	791.6 i-n	121.3 h-l	21.3
17	P-2	36.6 f-n	688.4 j-n	105.5 i-l	5.5
18	P-3	39.6 d-n	786.6 i-n	120.5 h-l	20.5
19	P-4	37.6 e-n	837.4 h-n	128.3 g-l	28.3
20	P-5	39.8 d-n	690.0 j-n	105.7 i-l	5.7
21	P-6	40.2 d-n	830.0 h-n	127.2 g-l	27.2
22	P-7	34.8 g-n	944.0 f-n	144.7 f-l	44.7
23	P-8	62.2 a	1082.4 d-j	165.9 d-i	65.9
24	P-9	57.0 ab	1123.0 d-i	172.1 d-h	72.1
25	P-10	33.0 h-n	844.2 h-n	129.4 g-l	29.4
26	P-11	35.2 f-n	832.0 h-n	127.5 g-l	27.5
27	P-12	48.8 a-f	1136.0 d-i	174.2 d-h	74.2

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
28	P-13	56.4 a-c^y	1163.6 d-i^y	178.3 d-h^y	78.3
29	P-14	30.0 k-n	1016.8 e-m	155.8 e-k	55.8
30	P-15	48.6 a-g	1188.8 d-i	182.2 d-h	82.2
31	Y-1	40.0 d-n	620.2 mn	95.0 kl	-5.0
32	Y-2	46.2 b-h	1058.4 e-l	162.2 e-j	62.2
33	Y-3	41.4 d-m	1018.4 e-m	156.1 e-k	56.1
34	Y-4	32.4 h-n	1017.2 e-m	155.9 e-k	55.9
35	Y-5	28.8 l-n	924.4 f-n	142.1 f-l	42.1
36	Y-6	48.6 a-g	1491.2 a-d	228.5 a-d	128.5
37	Y-7	31.8 i-n	1287.8 c-f	197.3 c-f	97.3
38	Y-8	33.8 h-n	1164.4 d-i	178.4 d-h	78.4
39	Y-9	33.4 h-n	1629.6 a-c	249.7 a-c	149.7
40	Y-10	30.4 j-n	1274.6 c-g	195.3 c-f	95.3
41	Y-11	36.0 f-n	1062.8 e-l	190.7 c-g	90.7
42	Y-12	28.2 mn	1184.6 d-i	181.5 d-h	81.5
43	Y-13	31.2 i-n	1083.2 d-j	166.0 d-i	66.0
44	Y-14	44.2 b-j	1222.2 c-h	187.3 c-g	87.3
45	Y-15	27.2 n	852.8 g-n	130.7 g-l	30.7
	N (+) control	0.0 o	652.6 l-n	100.0 j-l	-
	N (-) control	0.0 o	558.2 n	-	-
	Pr > F	**	**	**	
	LSD$_{0.05}$	**13.94**	**426.58**	**63.72**	
	CV(%)	**29.94**	**32.85**	**31.90**	

^{x} = Means of 5 replications

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

** Significant at 1% level

The nodule dry weight and shoot dry weight of the plants inoculated with 15 isolates from Myaing Township were ranged from 28.4 to 51.6 mg plant^{-1} and 576.6 to 1886.2 mg plant^{-1}, respectively. Among 15 rhizobium isolates, M-9 and M-11 gave the highest nodule dry weight (50.6 and 51.6 mg plant^{-1}) and shoot dry weight (1747.6 and 1886.2 mg plant^{-1}) when compared with those of other tested isolates and controls. The nodule dry weights of the plants inoculated with M-9 and M-11 were significantly different from those of M-1, M-2, M-3, M-4, M-5, M-10, M-13 and M-15 but not significantly different from the rest isolates. Similarly, M-11 isolate gave significantly higher in shoot dry weight than M-1, M-2, M-3, M-4, M-5, M-6, M-7, M-8, M-13, M-14, M-15 and control plants. It was observed that there was no significant differences among shoot dry weight of the plants inoculated with 4 rhizobium isolates; M-9, M-10, M-11 and M-12. M-1 isolate gave the lowest shoot dry weight (576.6 mg plant^{-1}) which was even less than un-inoculated N (+) control but not significantly different from each other (Table 4.2).

Nodule dry weight and shoot dry weight of the plants inoculated with 15 tested isolates from Pakokku Township were recorded between 30.0 and 62.2 mg plant^{-1}, and 688.4 and 1188.8 mg plant^{-1}, respectively. There were no significant differences among nodule dry weight and shoot dry weight of the plants inoculated with 5 rhizobium isolates; P-8, P-9, P-12, P-13 and P-15. Out of 15 tested isolates, P-8 gave the highest nodule dry weight which was significantly different when compared with those of the plants inoculated with P-1, P-2, P-3, P-4, P-5, P-6, P-7, P-10, P-11, and P-14. It was noted that the lowest nodule dry weight (30.0 mg plant^{-1}) was observed from the plants inoculated with P-1 but it was not statistically significant from P-2, P-3, P-4, P-5, P-6, P-7, P-10, P-11 and P-14. P-15 isolate produced the highest shoot dry weight (1188.8 mg plant^{-1}) which was not significantly different from those of P-8, P-9, P-12 and P-13. The lowest shoot dry weight (688.4 mg plant^{-1}) was obtained from the plants inoculated with P-2 which was not significantly different from N (+) control (Table 4.2).

The nodule dry weights of the plants inoculated with 15 rhizobium isolates from Yesagyo Township were significantly different from each other ranging from 27.2 to 48.6 mg plant^{-1}. The highest nodule dry weight was recorded in plants inoculated with Y-6 which was significantly varied compared to other remaining isolates except Y-1, Y-2, Y-3, Y-11 and Y-14. Out of 15 tested isolates, Y-9 gave the

highest shoot dry weight followed by Y-6, Y-7, Y-10 and Y-14 (Table 4.2). But these rhizobium isolates varied significantly from the rest isolates and N (+) control in shoot dry weight. The lowest shoot dry weight was observed in Y-1 which was not significantly different from that of un-inoculated N (+) control (Table 4.2).

The correlation analysis result showed that nodule dry weight was significantly different and positively correlated (r = 0.43**) with shoot dry weight (Figure 4.1). Regardless of it, some isolates (e.g. Y-9 and Y-10 collected from Yesagyo Township) gave higher shoot dry weight although they produced lower nodule dry weight. Therefore, it was noted that the nodule formation and plant growth responses by inoculation with individual isolates were generally varied in each Township. Similar relationships were reported by Manalku, Gebrekidan and Assefa (2009) in faba bean, Than (2010) and Zaw (2014) in chickpea and Thu (2018) in green gram. Many scientists reported that positive and significant correlation observed between nodulation and plant biomass, and nodule dry weight and shoot dry weight (Ngo-Nkot, Nwaga, Ngakou, Henri Fankem & Etoa, 2011). Particularly, shoot dry weight and nodule dry weight were routinely used as indicators of relative strain effectiveness (Somasegaran & Hoben, 2012).

Symbiotic efficiency differed significantly among the isolates tested. All tested isolates gave higher SE (> 80%), which ranged from 88.4 to 288.9% (Table 4.2 and 4.3). Such findings suggest that these isolates enhanced nitrogen fixation, which consequently increased SDW and nitrogen accumulation. And also indicated that rhizobium isolates collected from different green gram cultivated soils seem to be compatible with 'Yezin-11'. These results agree with the findings of Kawaka et al. (2014) who reported that SE ranging from 67 to 170% when common beans were inoculated with native rhizobia in Western Kenya. The results indicated that M-11 collected from Myaing Township produced the highest SE% (288.9%) followed by M-9 (267.8%), Y-9 (249.7%) and Y-6 (228.5%). Only two isolates, M-1 and Y-1 gave the lowest SE values (88.4% and 95.0%). The results are in accordance with the findings of Thu (2018), who observed that rhizobia isolated from green gram cultivated soil had higher SE% than that of positive control. Karaca and Uyanoz (2012) also reported that SE% of all tested rhizobium isolates from dry bean cultivated soils were higher than that of positive control.
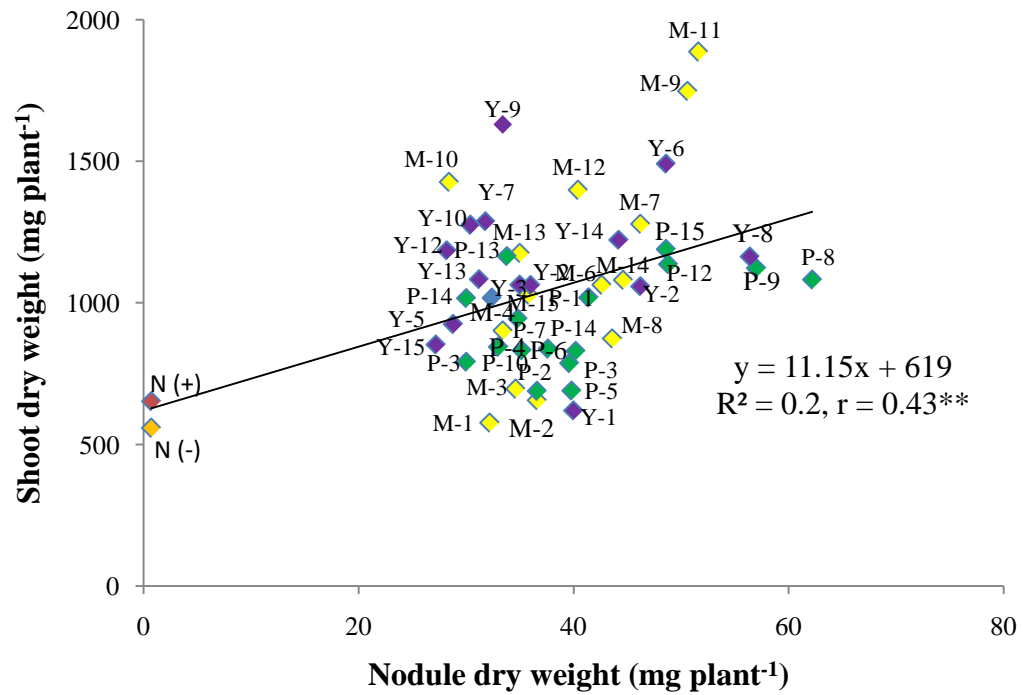
Figure 4.1 Correlation between nodule dry weight and shoot dry weight of 'Yezin-11' green gram inoculated with 45 rhizobium isolates

Table 4.3 Comparative analysis of relative symbiotic effectiveness of the isolates

Symbiotic Effectiveness (SE%)	Rhizobium isolates			% of total isolates
	Myaing isolates	**Pakokku isolates**	**Yesagyo isolates**	
Ineffective	-	-	-	0
Lowly effective	-	-	-	0
Effective	-	-	-	0
Highly effective	M-1	P-1	Y-1	100
	M-2	P-2	Y-2	
	M-3	P-3	Y-3	
	M-4	P-4	Y-4	
	M-5	P-5	Y-5	
	M-6	P-6	Y-6	
	M-7	P-7	Y-7	
	M-8	P-8	Y-8	
	M-9	P-9	Y-9	
	M-10	P-10	Y-10	
	M-11	P-11	Y-11	
	M-12	P-12	Y-12	
	M-13	P-13	Y-13	
	M-14	P-14	Y-14	
	M-15	P-15	Y-15	

SE%

Highly effective = > 80%

Effective = 51-80%

Lowly effective = 35-50%

Ineffective = < 35% (Beck et al., 1993)

Based on the percentage differences in shoot dry weight of rhizobium inoculated and N (+) control plants, M-1 and Y-1 were inferior to N (+) control but superior to N (-) control (Table 4.2). The other isolates performed symbiotically well on 'Yezin-11' and their contributions to shoot biomass were higher than that of un-inoculated N (+) control. Thus, only 4% were lower than un-inoculated N (+) control but about 96% of tested isolates performed better (Table 4.2). These observations are coherent with the study of Thu (2018) who reported that 92% of indigenous rhizobium isolates nodulating green gram were highly effective and 8% were lower than positive control. Kawaka et al. (2014) also mentioned that 42% of the native rhizobia isolates nodulating common bean were effective nitrogen fixers and 16% performed as good as the positive control.

Saleh, Zaman and Kabir (2013) explained that nodule dry weight may be considered as a usual character to select effective rhizobium isolates. Shoot dry weight is a good indicator of relative isolate effectiveness (Peoples, Giller, Herridge & Vessey, 2002; Somasegaran & Hoben, 2012). In this experiment, it was found that Y-15 was the lowest in nodule dry weight while the highest value was recorded in plants inoculated with P-8, but not statistically significant from those with M-9, M-11, P-9, P-12, P-13, P-15 and Y-6 inoculated plants. Ali (2010) observed that the comparison of inoculated and un-inoculated plants in respective to nodulation was highly significant. Moreover, the highest shoot dry weight was recorded from plants inoculated with M-11 which was not significantly different from those treated with M-9, M-10, M-12, Y-6 and Y-9 isolates. On the other hand, M-1 and Y-1 gave the lowest shoot dry weights. According to these, 14 rhizobium isolates; M-1, M-9, M-10, M-11, M-12, P-8, P-9, P-12, P-13, P-15, Y-1, Y-6, Y-9 and Y-15 were selected for the determination of nitrogen uptake (mg plant^{-1}) from the shoot samples.

Nitrogen uptakes of the inoculated plants with 5 rhizobium isolates from Myaing Township were ranged from 21.0 to 70.8 mg plant^{-1} (Table 4.4). The highest nitrogen uptake was found in M-11 but not significantly different from M-12. M-11 and M-12 produced significantly higher nitrogen uptake than M-1, M-9 and M-10. The lowest nitrogen uptake (21.0 mg plant^{-1}) was observed from the inoculated plants with M-1 which was not significantly different from N (+) control (Figure 4.2). Therefore, among 5 rhizobium isolates, 4 isolates (80%) increased more N uptake of plants as compared with N (+) control and the rest (20%) was effective as N (+) control.

Table 4.4 Nodule dry weight, shoot dry weight, nitrogen uptake and symbiotic efficiency of indigenous rhizobium isolates

Isolates	Nodule dry weight (mg plant^{-1})^x	Shoot dry weight (mg plant^{-1})^x	Nitrogen uptake (mg plant^{-1})^x	Symbiotic efficiency (SE%)^x
M-1	32.2 d^y	576.6 f^y	21.0 g^y	88.4 f^y
M-9	50.6 ab	1747.6 ab	54.4 b-d	267.8 ab
M-10	28.4 d	1426.8 b-d	37.3 ef	218.6 b-d
M-11	51.6 a	1886.2 a	70.8 a	288.9 a
M-12	40.4 b-d	1398.0 b-d	68.2 ab	214.2 b-d
P-8	62.2 a	1082.4 de	43.1 cd	165.9 c-e
P-9	57.0 a	1123.0 c-e	38.5 ef	172.1 c-e
P-12	48.8 a-c	1136.0 c-e	43.3 c-e	174.1 c-e
P-13	56.4 a	1163.6 c-e	38.3 ef	178.3 c-e
P-15	48.6 a-c	1188.8 c-e	38.5 ef	182.2 c-e
Y-1	40.0 b-d	620.2 f	25.4 fg	95.0 f
Y-6	48.6 a-c	1491.2 a-c	42.8 de	228.5 bc
Y-9	33.4 c-d	1629.6 a-c	58.7 a-c	249.7 ab
Y-15	27.2 d	852.8 a-c	26.9 fg	130.7 ef
N (+)	0.0 e	652.6 f	15.8 gh	100 f
N (-)	0.0 e	558.2 f	3.7 h	-
Pr > F	**	**	**	**
LSD$_{0.05}$	15.59	399.89	15.76	60.17
CV (%)	31.56	27.49	24.20	26.83

^{x} = Means of 5 replications

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

** Significant at 1% level
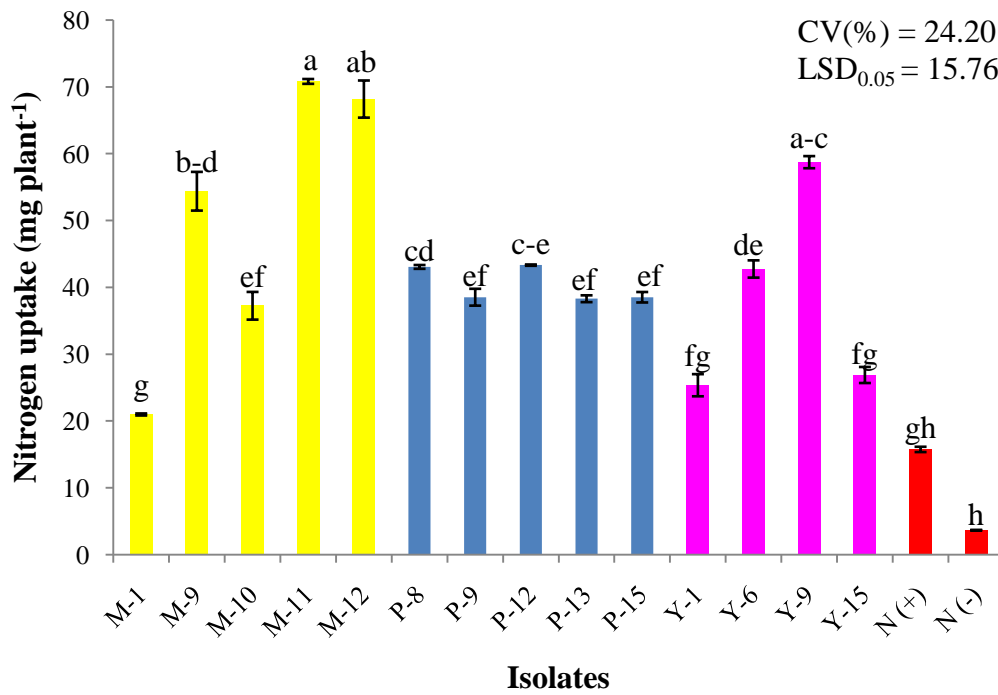
Figure   4.2   Effect of 14 rhizobium isolates on nitrogen uptake of green gram

There was no significant difference among nitrogen uptakes of the plants inoculated with 5 rhizobium isolates; P-8, P-9, P-12, P-13 and P-15 from Pakokku Township. But these isolates were significantly higher than N (+) control (Table 4.4 and Figure 4.2). These rhizobium isolates had higher efficiency for nitrogen fixation.

Among 4 rhizobium isolates collected from Yesagyo Township, Y-9 gave the highest value, 58.7 mg plant^{-1} in nitrogen uptake which was significantly higher than other 3 rhizobium isolates and N (+) control (Table 4.4 and Figure 4.2). The lowest nitrogen uptake was obtained from the plants inoculated with Y-1 and Y-15 which were not statistically different from N (+) control. They were significantly different from those of Y-6 (Table 4.4). It was noted that 50% of the isolates were more effective in N uptake of the plants than N (+) control.

Nitrogen uptakes of the inoculated plants with 14 rhizobium isolates in green gram were significantly different from each other ranging from 21.0 to 70.8 mg plant^{-1} (Table 4.4). The highest N uptake was obtained from inoculated plants with M-11 (70.8 mg plant^{-1}) but not significant from those with M-12 (68.2 mg plant^{-1}) and Y-9 (58.7 mg plant^{-1}) which were significantly different compared to other remaining isolates and un-inoculated N (+) control (Figure 4.2). Jain, Kumar and Panwar (2007) reported that *Rhizobium* along with micronutrients significantly enhanced the N and P uptake as compared to control in mungbean. M-1 (21.0 mg plant^{-1}), Y-1 (25.4 mg plant^{-1}) and Y-15 (26.9 mg plant^{-1}) produced the lowest N uptakes which were not significantly different from un-inoculated N (+) control. Therefore, it was found that 78.57% of the isolates were effective nitrogen fixers and other (21.43%) performed as good as N (+) control.

Correlation coefficient between nitrogen uptake and shoot dry weight in green gram was shown in Figure 4.3. It was found that shoot dry weight was highly correlated with nitrogen uptake affected by 14 rhizobium isolates in green gram (r = 0.99**). Aung (2018) also reported that positive and significant correlation (r = 0.91**) between yield and N uptake in chinese cabbage. Inoculation with bacterial biofertilizer may reduce the application of N fertilizer by increasing N uptake by plants (Kennedy, Choudhury & Kecskes, 2004). Hussain, Ali and Noorka (2012) also reported that the uptake of nutrient is a function of their content and yield. Increase in seed and straw yield along with higher content of N might have resulted in higher uptake of this nutrient by mungbean. Moreover, the coefficient of determination (R^2) in nitrogen uptake was 0.98 (Figure 4.3). According to the above characteristics, 14 indigenous rhizobium isolates were selected to examine their morphology and biochemical properties.
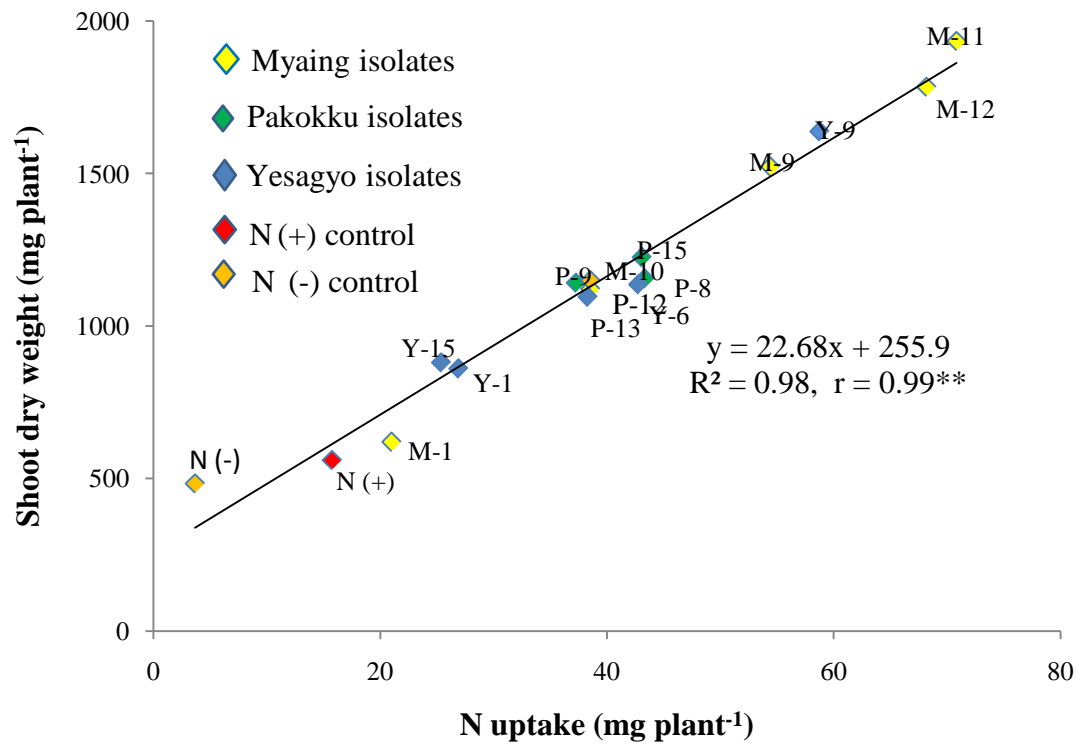
Figure 4.3 Relationship between nitrogen uptake and shoot dry weight of inoculated plants with 14 rhizobium isolates

4.2 Morphology and Biochemical Properties of Indigenous Rhizobium Isolates

4.2.1 Morphology of indigenous rhizobium isolates

(a) Growth on Yeast Extract Mannitol Agar (YMA) Medium

All selected indigenous rhizobium isolates from root nodules of green gram grew well on YMA but did not show chromogenesis. Therefore, they were identified as rhizobia based on their colony morphology and ability of not taking up Congo-red stain (Keen & La Rue, 1983) when grown on YMA medium (Vincent, 1970). All isolates showed typical colonies which were circular with convex surface, milky white, whitish pink colored, translucent, opaque showing production of mucus (Plate 4.4 and 4.5) with marked distinction from red colored colonies of *Agrobacterium.*

The isolates showed significant differences in growth rate, colony size, colony colour and opacity on YMA. Among selected isolates, some isolates, M-10, M-12, P-8, P-12, P-13, P-15 and Y-6 grew on YMA media on 48 hours developing into punctiform (1-2 mm diameter) colony size. On 3rd day, the colonies of these isolates observed 2-4 mm dia. and then took 5-7 days to attain 4-5 mm dia. (Table 4.5). This indicated that they were able to grow fast. The colonies of these isolates were milky white, translucent, circular in shape, shiny, raised with entire smooth surface (Plate 4.4). These morphology approaches closer to the genus *Rhizobium* as described by Jordan and Allen (1974).

At 3-5 days, the other isolates; M-1, M-9, M-11, P-9, Y-1, Y-9 and Y-15 showed moderate rate of growth. These isolates found 1-3 mm dia. by this time and phenotypically were the slow growing among 14 isolates. The colonies of slow-growing isolates obtained 3-5 mm dia. on 7th day of incubation (Table 4.5). The colony of slow-growing rhizobium isolates varied from translucent to opaque (Plate 4.5). M-1 and Y-1 produced whitish pink, translucent, circular in shape with entire smooth surface and 1-3 mm in diameter. Similarly, Gachande and Khansole (2011) isolated *Rhizobium japonicum* and *Bradyrhizobium japonicum* colonies, which were circular in shape with whitish-pink color on YMA medium. The colonies of M-9, M-11, P-9 and Y-9 were rounded with entire margin, milky-white, translucent and smooth surface. Only Y-15 isolate observed small colonies with opaque. Bhatt et al. (2013) also described that fast and slow-growing rhizobium isolates in mungbean had similar morphological characters.
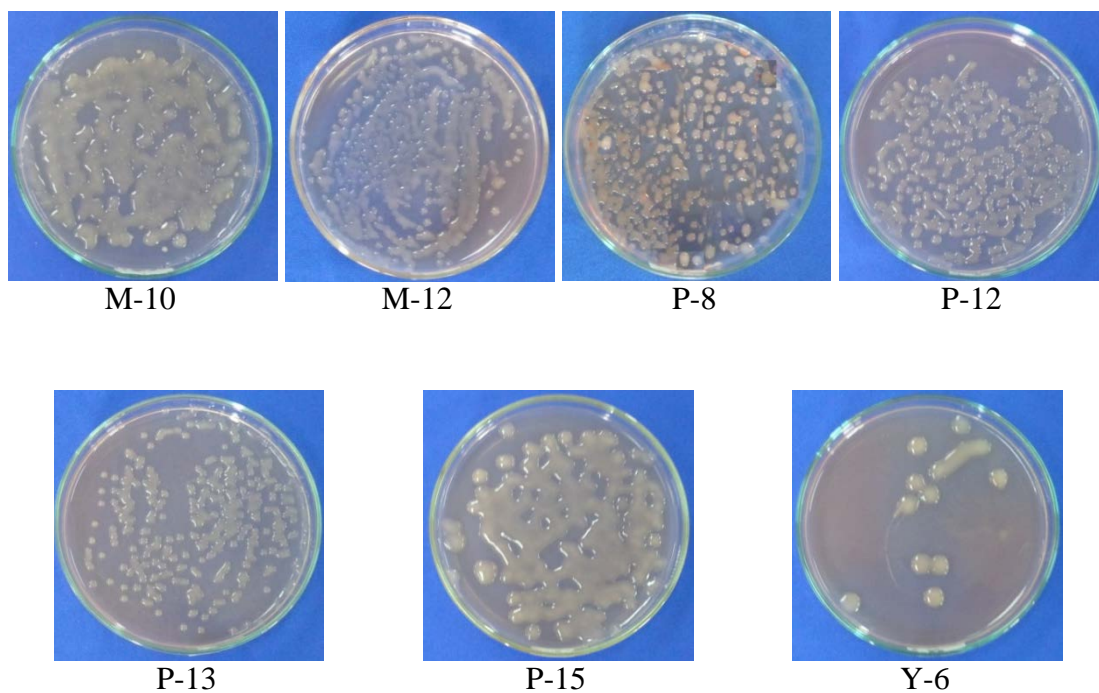
Plate 4.4 Colonies of different rhizobium isolates on Yeast Extract Mannitol Agar (YMA) medium at 3 days after incubation
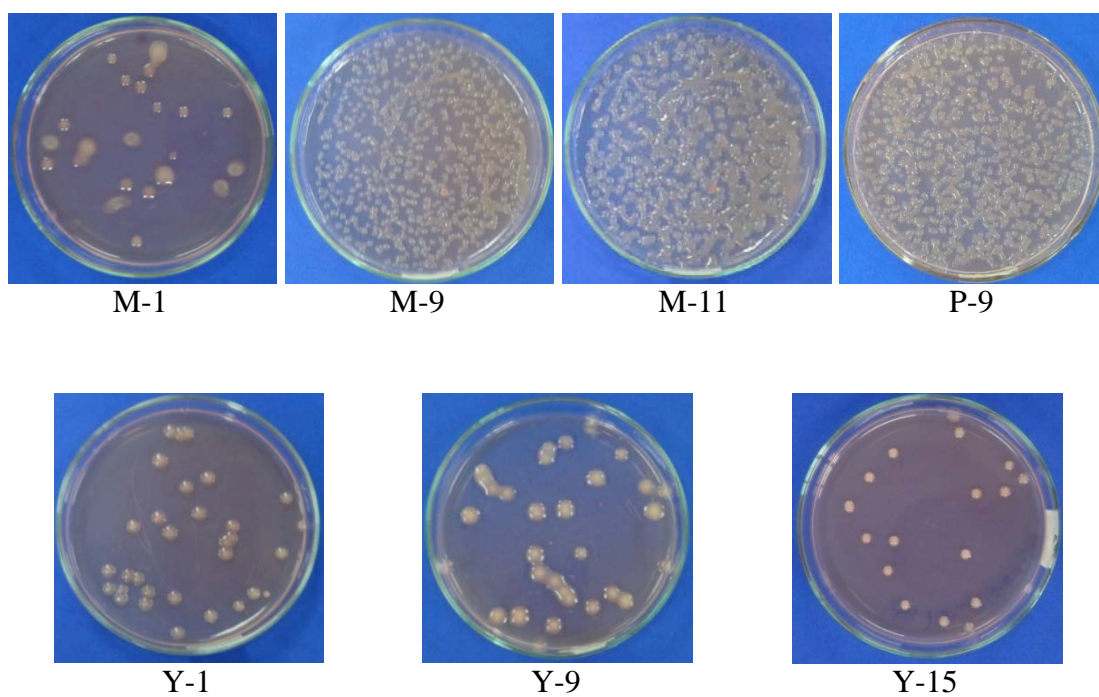
Plate 4.5 Colonies of different rhizobium isolates on Yeast Extract Mannitol Agar (YMA) medium at 5 days after incubation

Table 4.5 Morphology of 14 indigenous rhizobium isolates on Yeast Extract Mannitol Agar (YMA) medium

Sr. No	Isolate	Colony size (mm)	Form	Color	Opacity	Surface	Elevation	Margin
1	M-10	5	C	Mw	Trl	S	C	E
2	M-12	4	C	Mw	Trl	S	C	E
3	P-8	5	C	Mw	Trl	S	C	E
4	P-12	4	C	Mw	Trl	S	C	E
5	P-13	4	C	Mw	Trl	S	C	E
6	P-15	5	C	Mw	Trl	S	C	E
7	Y-6	5	C	Mw	Trl	S	C	E
8	M-1	2	C	Wp	Trl	S	C	E
9	M-9	2	C	Mw	Trl	S	C	E
10	M-11	3	C	Mw	Trl	S	C	E
11	P-9	2	C	Mw	Trl	S	C	E
12	Y-1	2	C	Wp	Trl	S	C	E
13	Y-9	3	C	Mw	Trl	S	C	E
14	Y-15	2	C	Mw	Op	S	C	E

Form (C = circular), Color (Wp = whitish pink, Mw = milky white), Opacity (Trl = translucent, Op = opaque), Surface (S = smooth), Elevation (C = convex), Margin (E = entire)

4.2.2 Biochemical properties of indigenous rhizobium isolates

(a) Growth on Bromothymol Blue (BTB) Agar Medium

All the isolates were able to grow on BTB amended medium. It was identified that M-10, M-12, P-8, P-12, P-13, P-15 and Y-6 changed the media color from green to yellow at 2-3 days after incubation. These isolates were fast growers, *Rhizobium* spp. due to acid production (Table 4.6 and Plate 4.6). On the other hand, remaining 7 isolates were able to produce alkaline reaction with or without changing the color of media from green to blue after 3-day incubation, which considered as slow-growing rhizobia, *Bradyrhizobium* spp. (Table 4.6 and Plate 4.6). The slow-growing *Bradyrhizobium* spp. remained blue coloured on BTB agar medium whereas the medium turned yellow due to acid production by fast-growing *Rhizobium* spp. by Vincent (1970). Also Jordan (1984) observed that slow-growing rhizobia produce alkaline while fast-growing rhizobia produce acid in BTB agar medium. Datta, Singh and Tabassum (2015) also found that *Bradyrhizobium japonicum* was negative to bromothymol blue test, as there was no color change in the medium.

Out of 14 isolates of this study, it was found that about 50% of the isolates were fast-growing *Rhizobium* spp. and other 50% isolates were slow-growing *Bradyrhizobium* spp. Both fast and slow-growing strains can infect the same host (Broughton, Bohlool, Shaw, Bohnert & Pankhurst, 1985; Pankhurst, 1977). Ghosh (2013) also reported that among 23 root nodule bacteria isolated from summer mungbean rhizosphere, about 73% of the strains were fast-growing *Rhizobium* spp. while only 27% of the strains were slow-growing *Bradyrhizobium* spp. In this study, the colonies of the fast growers were between 2.0 and 6.0 mm in diameter, whereas the colonies of the slow growers were approximately 0.5 to 3.0 mm in diameter. On BTB agar plates, both fast and slow-growing rhizobia formed circular, convex colonies.

(b) Gram Staining Reaction

On gram staining, all the isolates were identified as gram negative, short, rod-shaped and were non-spore forming (Plates 4.7). It was also observed that there was no variation in shape, motility, and reaction to gram stain among the isolates while the size of the isolates varied within the range of 0.6-0.96 x 1.25-2.75 μm (Table 4.7). Dakora, Joseph and Phillips (1993) stated that the fast-growing *Rhizobium* spp., the slow-growing *Bradyrhizobium* spp. and other root-nodulating bacteria are medium-sized and rod-shaped. Cells are 0.5-0.9 μm in width and 1.2-3.0 μm in length.

Table 4.6 Identification of fast (*Rhizobium* spp.) and slow-growing (*Bradyrhizobium* spp.) green gram rhizobium isolates on Bromothylmol Blue (BTB) agar medium

Sr. No	Isolates	Color produced on BTB agar (reaction)	Fast/Slow grower
1	M-10	Yellow (+)	Fast
2	M-12	Yellow (+)	Fast
3	P-8	Yellow (+)	Fast
4	P-12	Yellow (+)	Fast
5	P-13	Yellow (+)	Fast
6	P-15	Yellow (+)	Fast
7	Y-6	Yellow (+)	Fast
8	M-1	Blue (-)	Slow
9	M-9	Blue (-)	Slow
10	M-11	Blue (-)	Slow
11	P-9	Blue (-)	Slow
12	Y-1	Blue (-)	Slow
13	Y-9	Blue (-)	Slow
14	Y-15	Blue (-)	Slow

+ = positive reaction , - = negative reaction
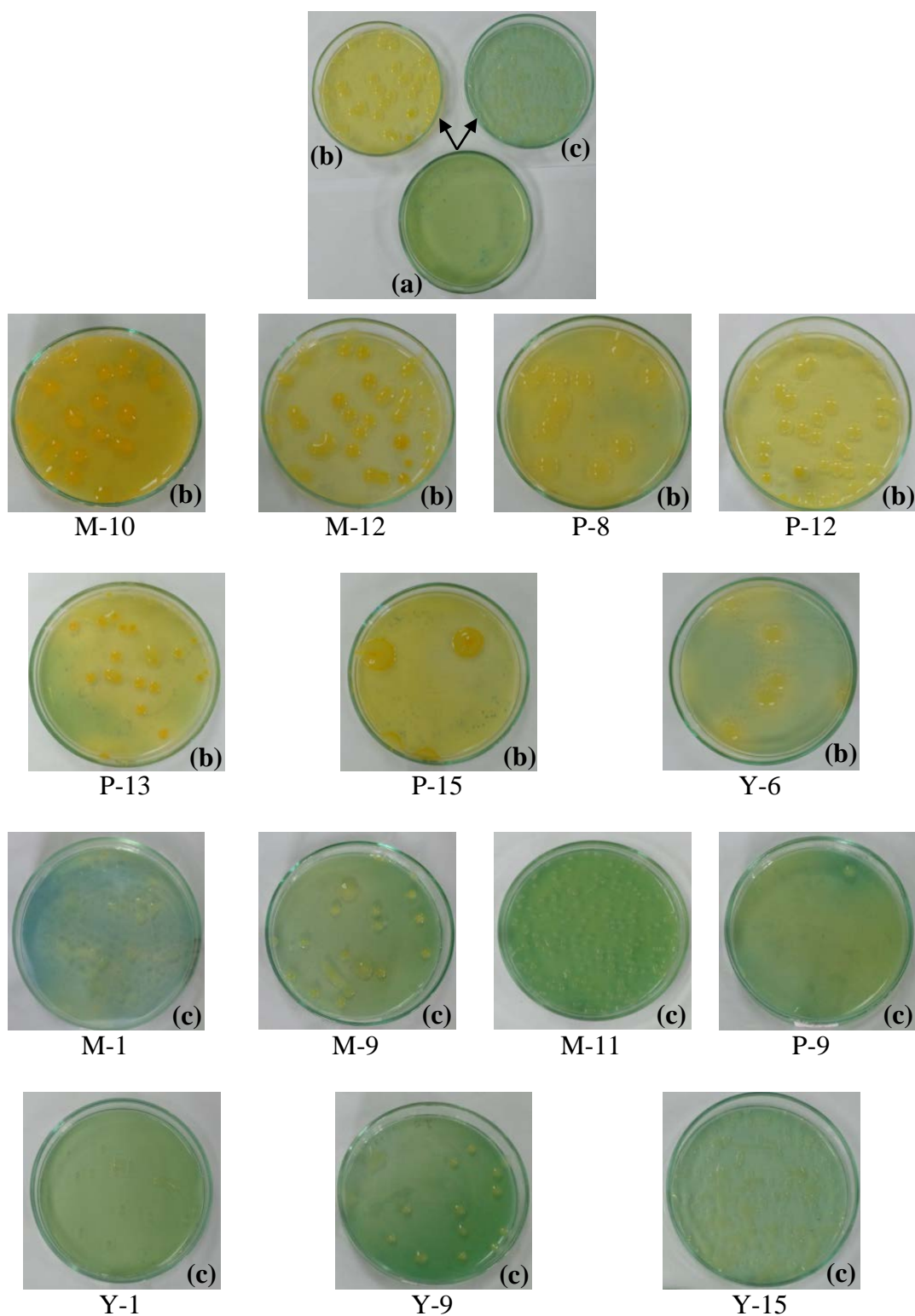
Plate 4.6 **Fast growers (*Rhizobium* spp.) and slow growers (*Bradyrhizobium* spp.) on Bromothymol Blue (BTB) agar medium (a) green plate (control; neutral pH 6.8); (b) yellow plate (acid production); (c) green or greenwish blue plate (alcohol production)**
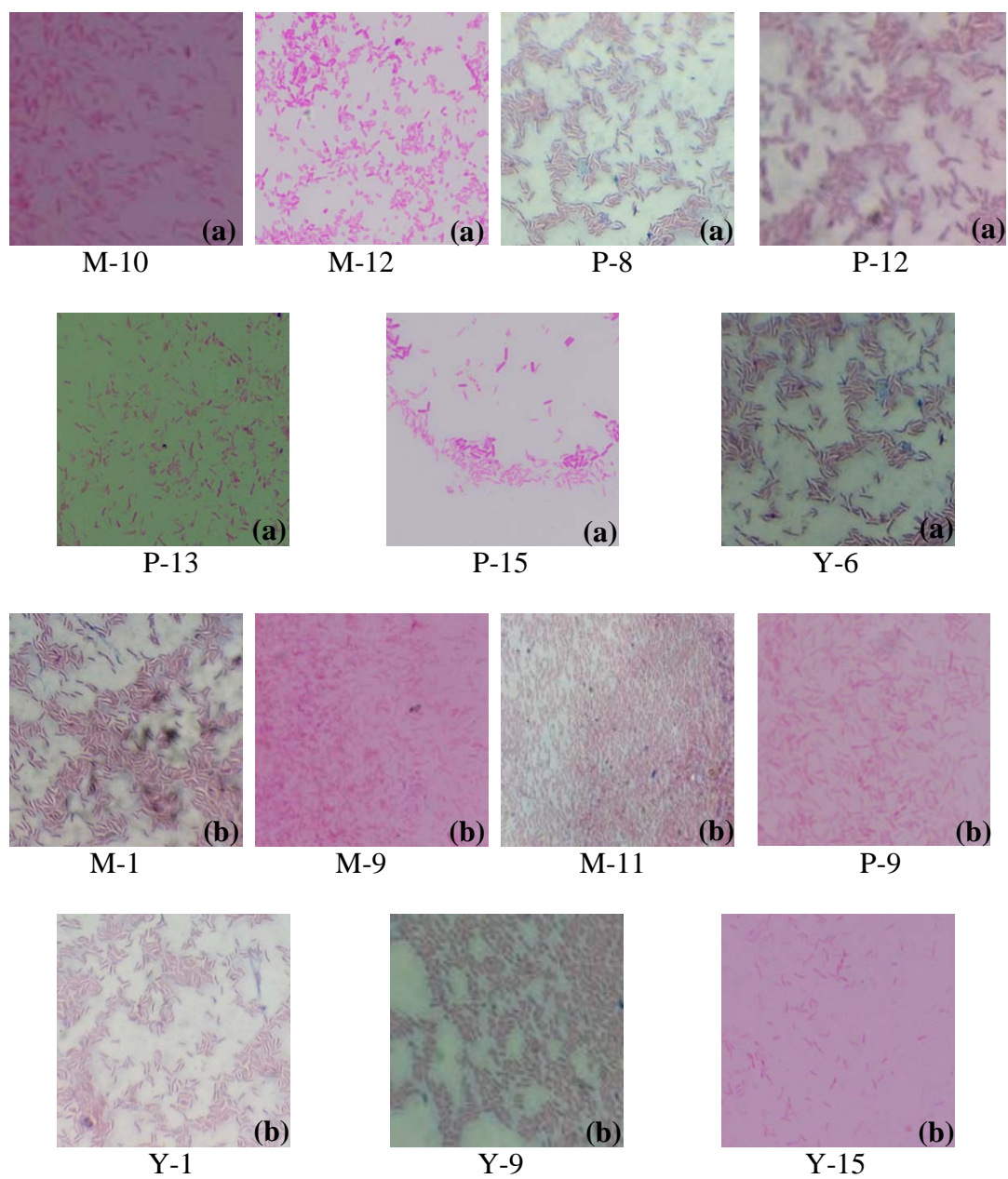
Plate 4.7 Gram staining reactions of 14 indigenous rhizobium isolates (a) fast-growing *Rhizobium* spp.; (b) slow-growing *Bradyrhizobium* spp.

Table 4.7 Gram staining reactions of 14 indigenous rhizobium isolates

Sr. No.	Isolates	Gram staining			
		Gram's reaction	Cell shape	Cell size (µm)	Cell color
1	M-10	negative	rod	0.96×2.45	pink
2	M-12	negative	rod	0.95×2.20	pink
3	P-8	negative	rod	0.65×1.55	pink
4	P-12	negative	rod	0.80×1.60	pink
5	P-13	negative	rod	0.65×1.25	pink
6	P-15	negative	rod	0.70×2.20	pink
7	Y-6	negative	rod	0.75×2.25	pink
8	M-1	negative	rod	0.68×2.25	pink
9	M-9	negative	rod	0.70×2.33	pink
10	M-11	negative	rod	0.70×1.90	pink
11	P-9	negative	rod	0.67×2.74	pink
12	Y-1	negative	rod	0.60×2.65	pink
13	Y-9	negative	rod	0.88×2.50	pink
14	Y-15	negative	rod	0.68×1.58	pink

(c) Growth in 2% Sodium Chloride Concentration

It was observed that all the isolates were able to grow in the medium containing 2% NaCl (Table 4.8). Almost all of the isolates exhibit growth on 2% NaCl which support the investigation carried out by Bhatt et al. (2013) and Dubey, Maheshwari, Kumar and Choure (2010).

(d) Growth on Glucose Peptone Agar (GPA) Medium

All tested isolates showed growth on glucose peptone agar medium containing bromocresol purple dye with or without pH changes (Table 4.8). At 2 days after incubation, all fast-growing rhizobium isolates; M-10, M-12, P-8, P-12, P-13, P-15 and Y-6 showed the growth on GPA media with little change (yellow color) in pH (Plate 4.8) which indicated that they could utilize glucose as carbon source. Similar finding was reported by Deora and Singhal (2010) that rhizobial cells were able to grow on the GPA media by changing pH showing the utilization of glucose as the carbon source by the *Rhizobium*. However, Panwar et al. (2012) also stated that none of the isolates showed growth on glucose peptone agar medium containing bromocresol purple dye.

Almost all slow-growing rhizobium isolates were able to grow on glucose peptone agar medium indicating the utilization of glucose as carbon source at 2 days after incubation. But these isolates did not change the colour of bromocresol purple (Plate 4.8). Pervin et al. (2017) recorded that all the isolates they observed showed little or no growth on the media of GPA without altering the pH. Idris, Abdel and Suleiman (2015) reported that all isolates in groundnut including the reference strain were grown well on glucose peptone agar after incubation for three days.

(e) Catalase Activity Test

In catalase test, all tested isolates showed positive reaction by the liberation of free oxygen as gas bubbles after adding hydrogen peroxide into the bacterial cultures (Table 4.8 and Plate 4.9). This result indicated that these isolates presented catalase enzyme in the culture. Naz et al. (2009) also recorded that all the isolates were found positive for catalase test indicating the presence of catalase enzyme. Datta et al. (2015) revealed that bubble formation around bacterial colonies showed positive catalase test.

Table 4.8 Biochemical properties of 14 indigenous rhizobium isolates

Sr. No	Isolates	2% NaCl	GPA test	Catalase test	Starch hydrolysis test	Urease test	Methyl red test
1	M-10	+	+	+	-	+	-
2	M-12	+	+	+	+	+	-
3	P-8	+	+	+	+	+	-
4	P-12	+	+	+	-	+	-
5	P-13	+	+	+	-	+	-
6	P-15	+	+	+	-	+	+
7	Y-6	+	+	+	-	+	-
8	M-1	+	+	+	+	+	-
9	M-9	+	+	+	-	+	-
10	M-11	+	+	+	-	+	-
11	P-9	+	+	+	+	+	-
12	Y-1	+	+	+	-	+	-
13	Y-9	+	+	+	-	+	-
14	Y-15	+	+	+	-	+	-

GPA = Glucose Peptone Agar, + = positive reaction, - = negative reaction
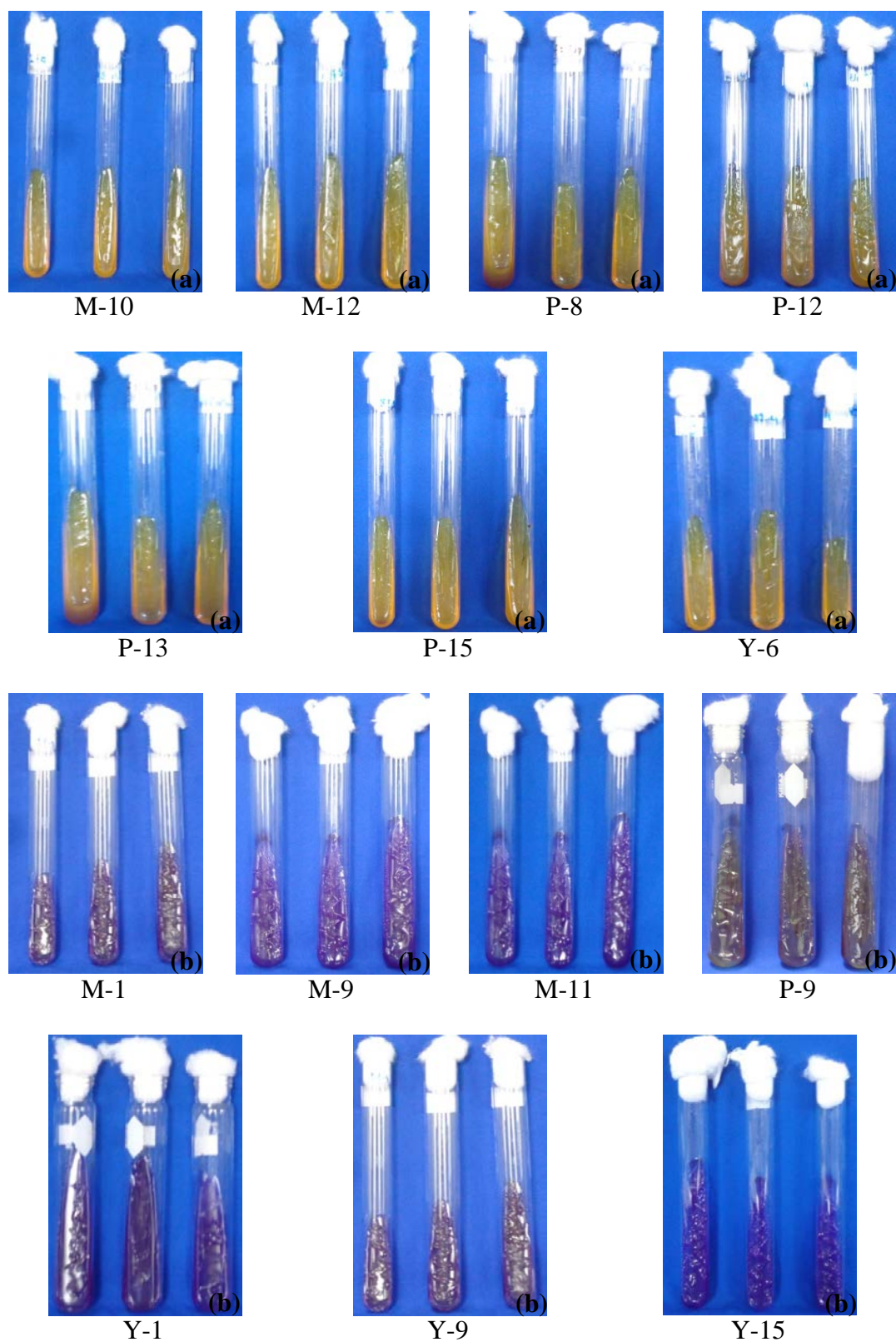
Plate 4.8 Growth of (a) fast and (b) slow-growing rhizobium isolates on Glucose Peptone Agar (GPA) medium at 3 days after incubation
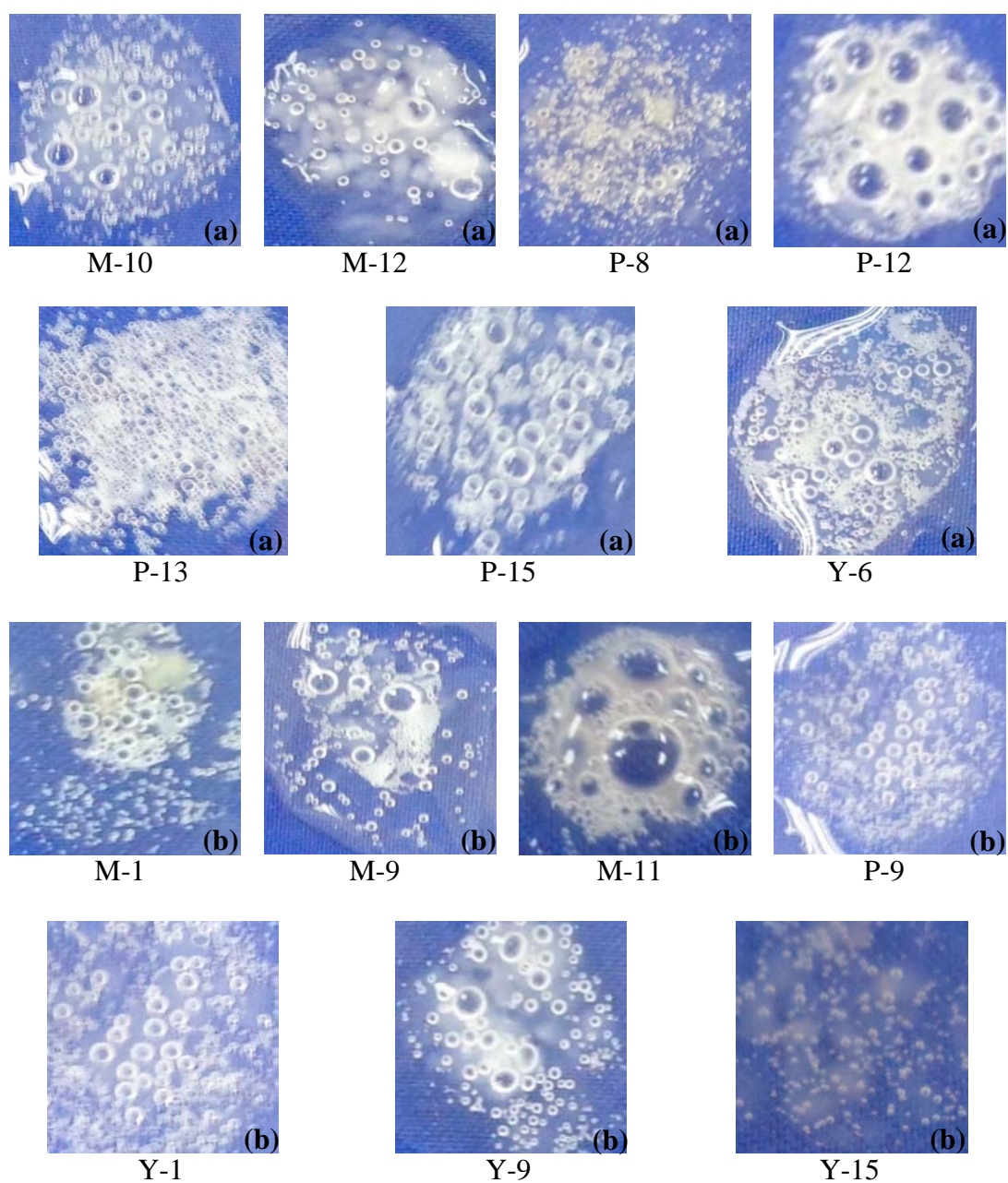
Plate 4.9 Positive catalase reactions of (a) fast and (b) slow-growing rhizobium isolates by liberation of free oxygen as gas bubbles after adding hydrogen peroxide into the culture

(f) Starch Hydrolysis Test

In this test, among the fast-growing rhizobium isolates, M-10, P-12, P-13, P-15 and Y-6 isolates did not show halos around the colonies. This was showing negative reaction for starch hydrolysis (Table 4.8 and Plate 4.10). This suggested that they did not possess the potential to hydrolyze starch present in the medium. Aneja (1996) reported that presence of clear halos around the colonies shows a positive reaction whereas, absence of such halos and persistence of dark blue coloration of the medium indicates negative reaction for starch hydrolysis. Similarly, Datta et al. (2015) also observed that no clear zones around colonies were observed in *Rhizobium leguminosarum* and *Rhizobium trifolii*. However, fast-growing isolates; M-12 and P-8 produced clear zones around colonies showing that they had amylase enzyme to hydrolyze starch (Table 4.8 and Plate 4.10).

The slow-growing rhizobium isolates, M-1 and P-9, showed positive starch hydrolysis (Table 4.8 and Plate 4.10). Similar results were observed by Datta et al. (2015) who recorded *Bradyrhizobium japonicum* and *Rhizobium phaseoli* can utilize starch from different carbon sources. The results also agree with those of de Oliveira et al. (2007), who observed that *Rhizobium* strains obtained from different sources have the potential to use starch. The other remaining slow-growing isolates: M-9, M-11, Y-1, Y-9 and Y-15 were found to be negative starch hydrolysis (Table 4.8 and Plate 4.10). Similar variation of growth of *Bradyrhizobium* in different carbon sources was found by Deka and Azad (2006) and Padmanabhan, Hirtz and Broughton (1990).

(g) Urease Hydrolysis Test

In this study, results of urease test revealed that all tested isolates showed positive reaction by development of deep pink color due to the presence of urease enzyme produced by the isolates (Table 4.8 and Plate 4.11) which hydrolyzes urea to ammonia and carbon dioxide. Therefore, all of fast and slow-growing rhizobium isolates showed positive results. These finding is also supported by Ghosh (2013) who reported that isolates of *Rhizobium* spp. and *Bradyrhizobium* spp. from rhizosphere and root nodules of mungbean were found to be positive for urease test. Similar observation was reported by Gauri et al. (2011).

Rasool, Sharma and Rasool (2015) also found that urea broth had turned pink after incubation suggesting alkaline nature of medium because of ammonia production indicating that the test organism is urease positive. These findings are in contradiction to the results of Datta et al. (2015) and Rassem and David (2017), who observed that all isolates showed the utilization of urease enzyme but *Rhizobium leguminosarum* had no growth.
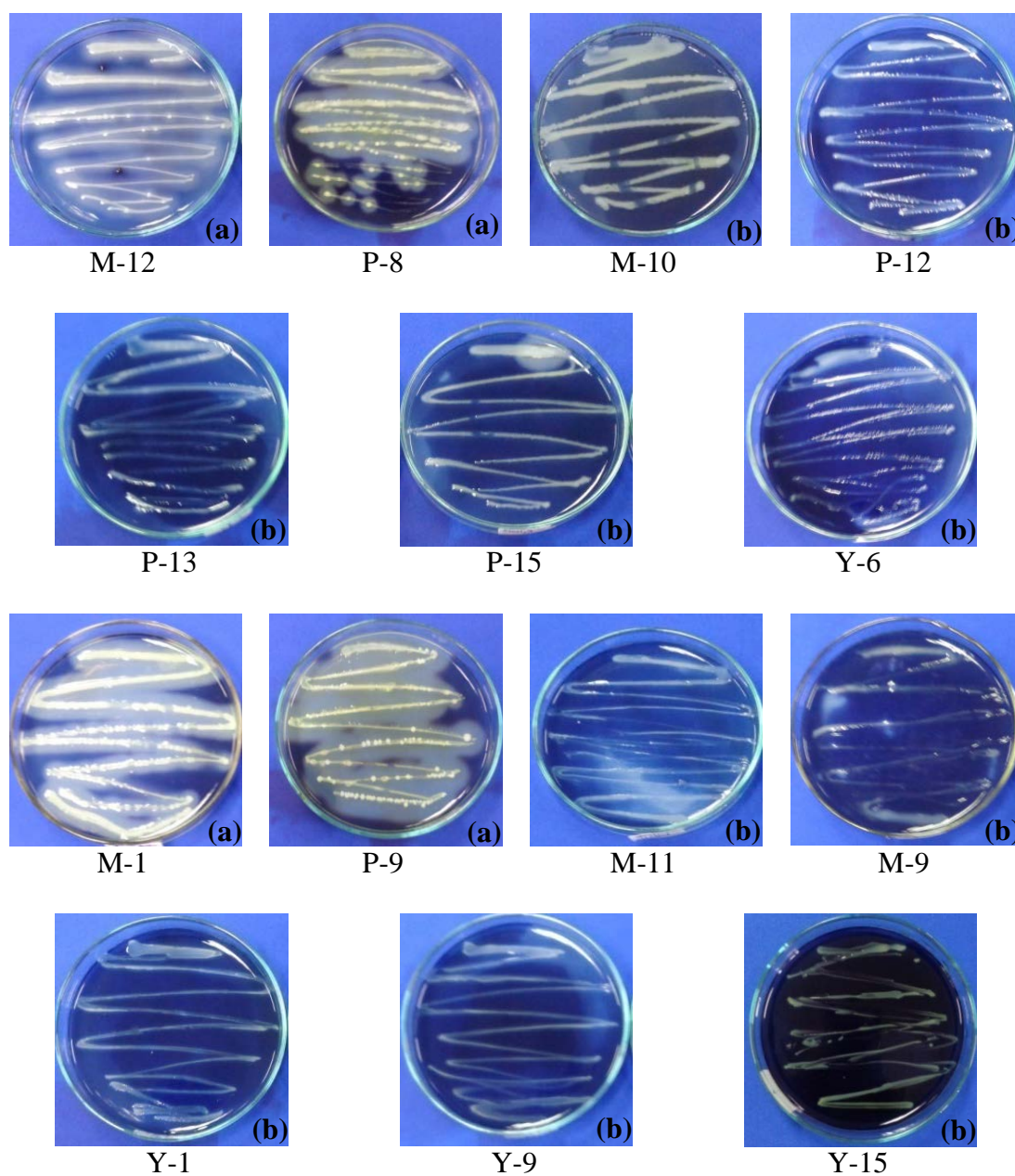
Plate 4.10 (a) Positive and (b) negative starch hydrolysis reactions of fast and slow-growing rhizobium isolates by the formation of clear halos and dark blue color surrounding the bacterial growth after pouring iodine solution into the culture
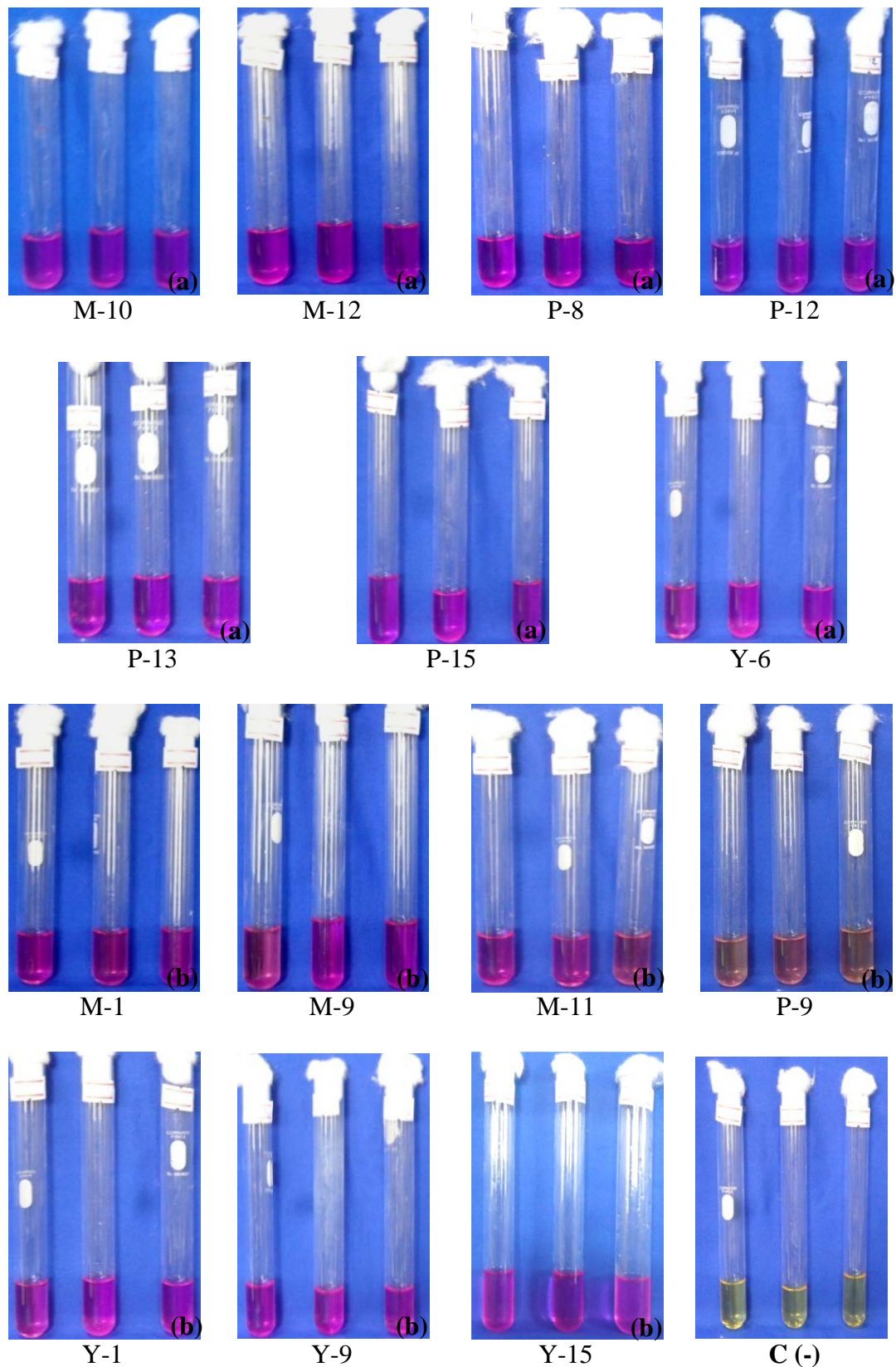
Plate 4.11 Deep pink color development of positive urease reactions at 24-48 hours after incubation (a) fast and (b) slow-growing rhizobium isolates and C (-) without rhizobium

(h) Methyl Red Test

None of the tested isolates gave positive results except P-15 (Table 4.8). Only fast-growing isolate, P-15, indicated positive result suggesting that it was able to produce and maintain the stable acid end products (lactic, acetic, formic) from glucose fermentation. Bright red color at pH 4.2 indicated a positive reaction while a negative result was indicated cloudy color or pale yellow color (Plate 4.12). According to Rassem and David (2017), *Rhizobium leguminosarum* and *Sinorhizobium melitoli* were found negative to methyl red but *Rhizobium pusense* was positive. Ghosh (2013) also suggested that all of the fast-growing *Rhizobium* spp. and slow-growing *Bradyrhizobium* spp. were found to be negative for methyl test.

4.3 Survival of the Most Effective-Indigenous Rhizobium Isolate on Different Carriers

4.3.1 Physicochemical properties of carrier materials

The physicochemical properties of carrier materials used for the preparation of rhizobial inoculants revealed that the pH of the materials ranged from 5.8 to 10.4 (Table 4.9). The pH of press mud and saw dust were acidic while Australia peat soil was neutral. Charcoal, clay soil, fly ash and Myanmar peat soil were alkaline. The water holding capacity (WHC) of carrier materials ranged from 27.36% to 204.33% with the highest WHC in charcoal followed by Australia peat soil, Myanmar peat soil, fly ash, clay soil and press mud. The lowest WHC (27.36%) was in saw dust. Date (1976) suggested that high organic carbon, high water holding capacity and neutral in reaction were the desirable characters of carriers.

4.3.2 Survival of M-11 on different carriers at room temperature

An inoculant should meet the quality standard of at least one billion (1×10^9) cells g^{-1} inoculant, as reported in Australia (Herridge et al., 2002). However, in many countries, the accepted standard for rhizobia inoculants is 10^7 or more viable cells g^{-1} (Burton, 1978). The standards set by most countries as being acceptable, ranged from 1×10^7 to 1×10^9 rhizobia g^{-1} inoculant (Rebah et al., 2007; Smith, 1992). The number of viable rhizobia should not fall below specified values; this number varies from 10^6 to 10^9 rhizobia g^{-1} of carrier (Hardy, 2016; Vincent, 1965).

| P-15 (a) | M-10 (b) | M-12 (b) | P-8 (b) |

| P-12 (b) | P-13 (b) | Y-6 (b) |

| M-1 (b) | M-9 (b) | M-11 (b) | P-9 (b) |

| Y-1 (b) | Y-9 (b) | Y-15 (b) |
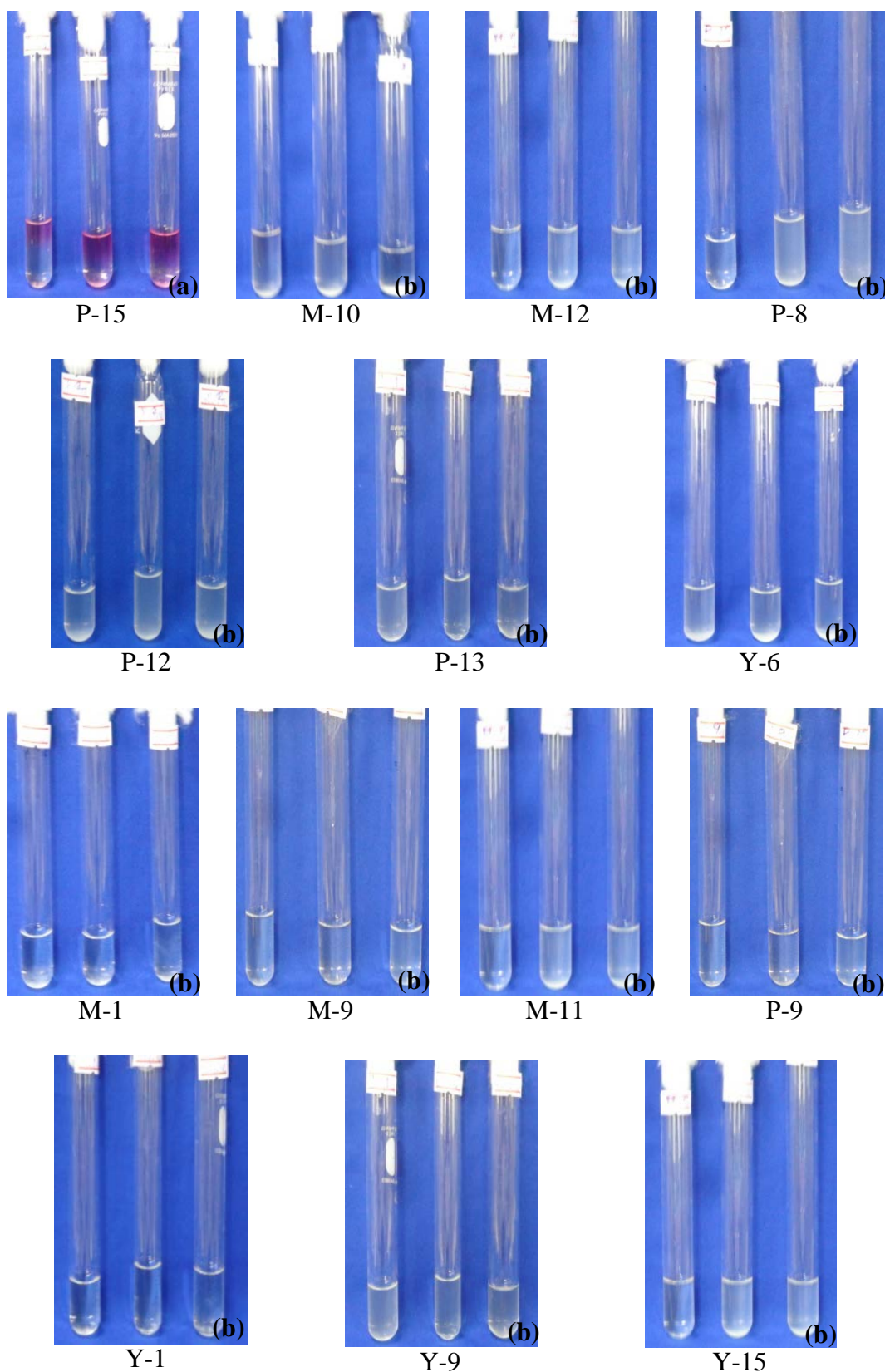
late 4.12 (a) Positive and (b) negative Methyl Red (MR) reactions of fast and slow-growing rhizobium isolates by the development of stable red color and pale yellow color after pouring 5 drops of MR indicator into liquid culture

Table 4.9 Physicochemical properties of carrier materials

No.	Carrier materials	Water holding capacity (%)	pH			Moisture content (%)	
			Before injection	After injection		Initial (0 day)	Final (180days)
				Initial (0 day)	Final (180 days)		
1	Charcoal	204.3	8.1	8.3	8.3	44.5	36.0
2	Clay soil	68.5	7.5	7.3	7.9	40.2	28.8
3	Fly ash	69.7	10.4	10.5	9.9	44.1	30.9
4	Australia peat soil	164.8	6.8	6.9	7.1	43.6	34.4
5	Myanmar peat soil	83.9	7.5	7.3	8.3	41.8	33.8
6	Press mud	44.2	6.1	6.3	6.6	41.9	19.6
7	Saw dust	27.4	5.8	5.9	5.7	43.2	26.0

(a) Charcoal

The initial population of M-11 was log 9.4 cfu g^{-1}. The microbial analysis revealed that there was a decline in the population of M-11 from log 9.4 to 7.1 cfu g^{-1} (Table 4.10 and Figure 4.4). There was an increment in the population of the bacteria at 15 days, followed by a decrease in the population after 30 days. So, charcoal carrier inoculant gave the highest population number until 30 days after injection. Up to 75.53% of the cells survived at 180 days. But, it retained the optimum population (1×10^7 rhizobia g^{-1}) which is accepted as standard for rhizobia inoculants in many countries until the end of incubation period. Sparrow and Ham. (1983b) reported that charcoal and vermiculite can be successfully used as carriers for *Rhizobium phaseoli* inoculants. Pandher, Gupta, Bhandal and Gupta (1993) have recorded that lignite and charcoal favoured higher growth and survival of *Rhizobium* as compared to other carriers. Elsalahi, Mohamed, Sherif and Osman (2016) observed that charcoal was found to be superior in terms of rhizobial counts ($> 1.0 \times 10^8$ rhizobia g^{-1}), availability and abundance besides its high water holding capacity, and its least contamination liability and storage ability of 60 days at room temperature.

(b) Clay soil

The initial population of M-11 in clay soil was log 9.4 cfu g^{-1}. A significant increase in rhizobial population was observed at 30 days. After 30 days, the rhizobial population was gradually decreased from log 8.5 to 6.1 cfu g^{-1} (Table 4.10 and Figure 4.4). About 65% of the cells survived at 180 days. However, it maintained enough rhizobial population at the end of storage period. Tabassam, Sultan, Akhtar, Hassan and Ali (2015) reported that clay is a widely distributed and abundant mineral resource that high adsorption power, good colloidal properties and high amending capability that make it the most suitable material for solid carrier formulation.

(c) Fly ash

In fly ash, the initial population of M-11 was log 9.2 cfu g^{-1}. In this carrier inoculant, bacterial multiplication did not completely occur. The microbial analysis revealed that there was a decline in the population of M-11 from log 9.2 to 4.2 cfu g^{-1} (Table 4.10 and Figure 4.4). Significant decline in rhizobial population number was observed at almost all intervals except 0 day when compared with those of other carrier materials. The population remained nearly constant or decline steadily after 15 days onwards. In addition, only 45.65% of the cells survived at 180 days. It was noted that until 15 days, fly ash supported more than 1×10^8 cell g^{-1} which can be

acceptable for use as biofertilizers. Kumar and Gupta (2010) formulated carriers for *Azotobacter chroococcum* in fly ash alone and in combination with lignite (1:0, 1:1 and 0:1) and evaluated the viability up to 9 months. Viability was in the order: fly ash > lignite and fly ash > lignite (1:1) > lignite.

(d) Australia peat soil

In this carrier inoculant, the initial population was log 9.4 cfu g^{-1}. There was an increment in the bacterial population during 30 days followed by a slightly decrease in the population from log 8.8 to 7.6 cfu g^{-1} (Table 4.10 and Figure 4.4). These results indicated that this carrier maintained the rhizobial population which is equal to or more than 10^7 cfu g^{-1} during the incubation period. These results are consistent with the cells having entered stationary phase after about 30 days in peat soil. In addition, up to 80.40% of the cells survived when Australia peat soil was used as the carrier material at the end of 180 days.

(e) Myanmar peat soil

The initial population of M-11 in Myanmar peat soil was log 9.5 cfu g^{-1}. Bacterial multiplication was occurred at 15 days and then the population slowly dropped after 30 days from log 8.5 to 6.5 cfu g^{-1} (Table 4.10 and Figure 4.4). Up to 180 days, about 68% of the cells survived. However, until 180 days, it retained the rhizobial population which is equal to the accepted standard for rhizobial inoculant. Kaljeet, Keyeo and Amir (2011) observed that peat soil was able to sustain the number of bacteria of more than 10^7 cells g^{-1} even after eight weeks of storage. In a report of Feng, Roughley and Copeland (2002), the viability of bacteria in peat soil as carrier material existed and maintained in high number at 10^8 cells g^{-1} even after 85 days. As can be observed from the result, Myanmar peat soil showed better results as compared to clay soil, fly ash, press mud and saw dust even after 180 days.

(f) Press mud

The initial population in press mud was log 9.3 cfu g^{-1}. It was observed that up to 30 days, the highest rhizobial population was maintained. After 30 days, there was a decline in the population from log 7.7 to 6.6 cfu g^{-1}. Up to 70.97% of the cells survived till 90 days. Rhizobial population decreased to zero after 90 days, towards the end of incubation period (Table 4.10 and Figure 4.4). Tabassam et al. (2015) reported that sugarcane press mud carrier based inoculants have lower tolerance for physical stress during storage for bacteria. Sugarcane press mud suitability as a carrier for the production of bacterial inoculants was studied in various countries and found

not a perfect carrier for bacterial inoculants production, its effectiveness can be enhanced by amending it with soil and charcoal. Jauhri (1990) studied that the combination of press mud with charcoal was found the best for rhizobium survival.

(g) Saw dust

The initial population of M-11 in saw dust was log 9.5 cfu g^{-1}. This carrier sustained optimum rhizobial population till 45 days and then fell down sharply (Table 4.10 and Figure 4.4). It was found that no viable cells were detected after 60 days at room temperature. Ratnayake, Van Holm, Moors and Jayalath (1978) also described that the tannin in the saw dust might be the main factor retarding the growth and survivability of *Rhizobium leguminosarum* in it. Phiromtan, Mala and Srinives (2013) also reported that the saw dust blocked bacterial respiration and reduced water absorption media compost resulting in less available water and inhibiting the bacterial population during incubation period.

4.3.3 Effect of pH on growth of M-11 isolate

In this study, the initial pH of the carrier inoculants evaluated ranged between 5.9 and 10.5 (Table 4.9). Jordan (1984) reported that some *Rhizobium* strains may grow on lower pH range between pH 4.5 to pH 9.5. Singh, Manchanda, Singh, Srivastava and Dubey (2015) also observed that some species grow at pH 4.5 and pH 11. Similarly, M-11 isolate showed good growth at all pH levels in this study. The highest bacterial count was maintained at pH 7-8 along the storage period. In contrast, at pH 10, the rhizobial population was drastically dropped at 15 days and afterwards remained to stationary phase. This indicated that pH 10 was not suitable for rhizobial growth for inoculant production. Similarly, at around pH 6, bacterial count was reached to zero during storage. These pH 6 and 10 were not appropriate for the rhizobial survival to produce a biofertilizer.

By Correa and Barneix (1997), legumes and rhizobia can be extremely sensitive to low soil pH. Gauri, Singh and Bamania (2012) observed that *Rhizobium* has been reported to grow the best at neutral pH i.e. 7.0. Among specific quality requirements of biofertilizers-*Rhizobium* described in Yadav (2009), pH value is in the range of 6.5-7.5. According to Somasegaram and Hoben (2012), the optimal pH levels for growth of the rhizobia in culture are variable (pH 5.8- 7.2) depending on the rhizobia species. According to also these results in this study, the optimum pH for M-11 isolate was found to be 7 and 8 for inoculant productions at room temperature.

4.3.4 Effect of moisture content on growth of M-11 isolate

Other than viable cell count and pH, another parameter used for standard quality assurance of biofertilizer was moisture content at the time of its manufacture. Roughley and Vincent (1967) have reported that optimum moisture levels of 40–50% for maximum survival. Mishra (2002) also described that the minimum moisture content of the carrier material should be 35-40%. Kaljeet et al. (2011) recorded that the optimal moisture content for rhizobia was in the range of 40-60%. According to Yadav (2009), the carrier based biofertilizer for rhizobia should be in the range of 30-40% of moisture content.

In the present study, the moisture contents of all carriers were in the range of 40.16 - 44.45% at the beginning of incubation. At the end of storage period, charcoal-based carrier kept the higher moisture content of 35.97% while Australia peat soil, Myanmar peat soil, fly ash, clay soil and saw dust-based carrier retained 34.42%, 33.82%, 30.86%, 28.82% and 26.03%, respectively (Table 4.9). A sharp decline in moisture content was observed in press mud-based inoculant to 19.35% (Table 4.9). A small moisture loss was to be expected especially during storage of the carrier. But the moisture loss should not be too high that it could decrease the viability of rhizobia except press mud and saw dust. Charcoal and Australia peat soil possessed high water holding capacity as a result of slow release of water. It can be concluded that higher water potential, higher survival of rhizobia and also vice versa.

Based on these results, it was noted that the initial population was not statistically different among tested carriers when all carriers received the same quantity of broth with similar cell counts. It was ranged from log 9.2 to 9.5 cfu g^{-1} (Table 4.10). However, there were statistically significant differences on rhizobial population at all the intervals among carriers except 0 day (Table 4.10 and Figure 4.4). These variations may be due to differences in physicochemical properties of carrier materials described in Table 4.9, storage temperature and storage period. Robert, Kremer and Harold (1983) stated that survival of rhizobia in inoculant is affected by the type of carrier, storage temperature, storage period and their interactions. Rebah et al. (2002) also recorded that WHC and pH are the factors that primilarly affect the survival of strains in carriers. Kaljeet et al. (2011) found that the viable cell count of rhizobia of both storage temperatures; 4 and 28°C (room temperature), decreased over time for all tested carriers. Argal, Rawat, Aher and Rajput (2015) also described that rhizobial count in all the carriers tested gradually decreased from the date of injection to 180 days. Singh, Khare, Rawat and Sengar (1992) revealed that longer storage time of inoculants reduce the inoculums survival.

Table 4.10 Survival of M-11 isolate in different carrier materials during 180 days of storage at room temperature

Carrier inoculants	Population of M-11 (cfu g^{-1}) ^{x} from 0 to 180 days												
	0	**15**	**30**	**45**	**60**	**75**	**90**	**105**	**120**	**135**	**150**	**165**	**180**
Charcoal	9.4 a^y	10.2 a	9.9 ab	8.8 a	8.6 a	8.6 a	8.5 a	8.4 a	8.2 a	7.9 a	7.7 a	7.5 a	7.1 b
Clay soil	9.4 a	9.3 b	10.2 ab	8.5 ab	8.2 a	8.1 b	8.0 ab	7.8 b	7.7 b	7.00 b	7.0 b	6.7 b	6.1 d
Fly ash	9.2 a	8.9 b	5.8 c	5.6 d	5.6 c	5.5 d	5.5 d	5.5 c	5.4 c	5.4 c	5.2 c	4.7 c	4.2 e
Australia peat soil	9.4 a	10.5 a	10.2 ab	8.8 a	8.5 a	8.5 ab	8.4 a	8.2 ab	8.1 a	8.0 a	7.8 a	7.6 a	7.6 a
Myanmar peat soil	9.5 a	10.7 a	9.7 b	8.5 ab	8.5 a	8.2 ab	7.9 b	7.7 b	7.3 b	7.3 b	6.9 b	6.7 b	6.5 c
Press mud	9.3 a	10.2 a	10.3 a	7.7 c	6.9 b	7.0 c	6.6 c	0.0 e	0.0 d	0.0 d	0.0 d	0.0 d	0.0 f
Saw dust	9.5 a	9.1 b	9.8 ab	7.9 bc	5.4 c	0.0 f	0.0 e	0.0 e	0.0 d	0.0 d	0.0 d	0.0 d	0.0 e
Pr > F	**ns**	****	****	****	****	****	****	****	****	****	****	****	****
LSD$_{0.05}$	**0.56**	**0.63**	**0.53**	**0.65**	**0.47**	**0.50**	**0.49**	**0.49**	**0.41**	**0.38**	**0.40**	**0.48**	**0.29**
CV (%)	**3.4**	**3.63**	**3.24**	**4.64**	**3.6**	**4.36**	**4.32**	**5.25**	**4.41**	**4.26**	**4.64**	**5.74**	**3.63**

cfu = colony forming unit

^{x} = Mean of 3 replications

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

** Significant difference at 1% level
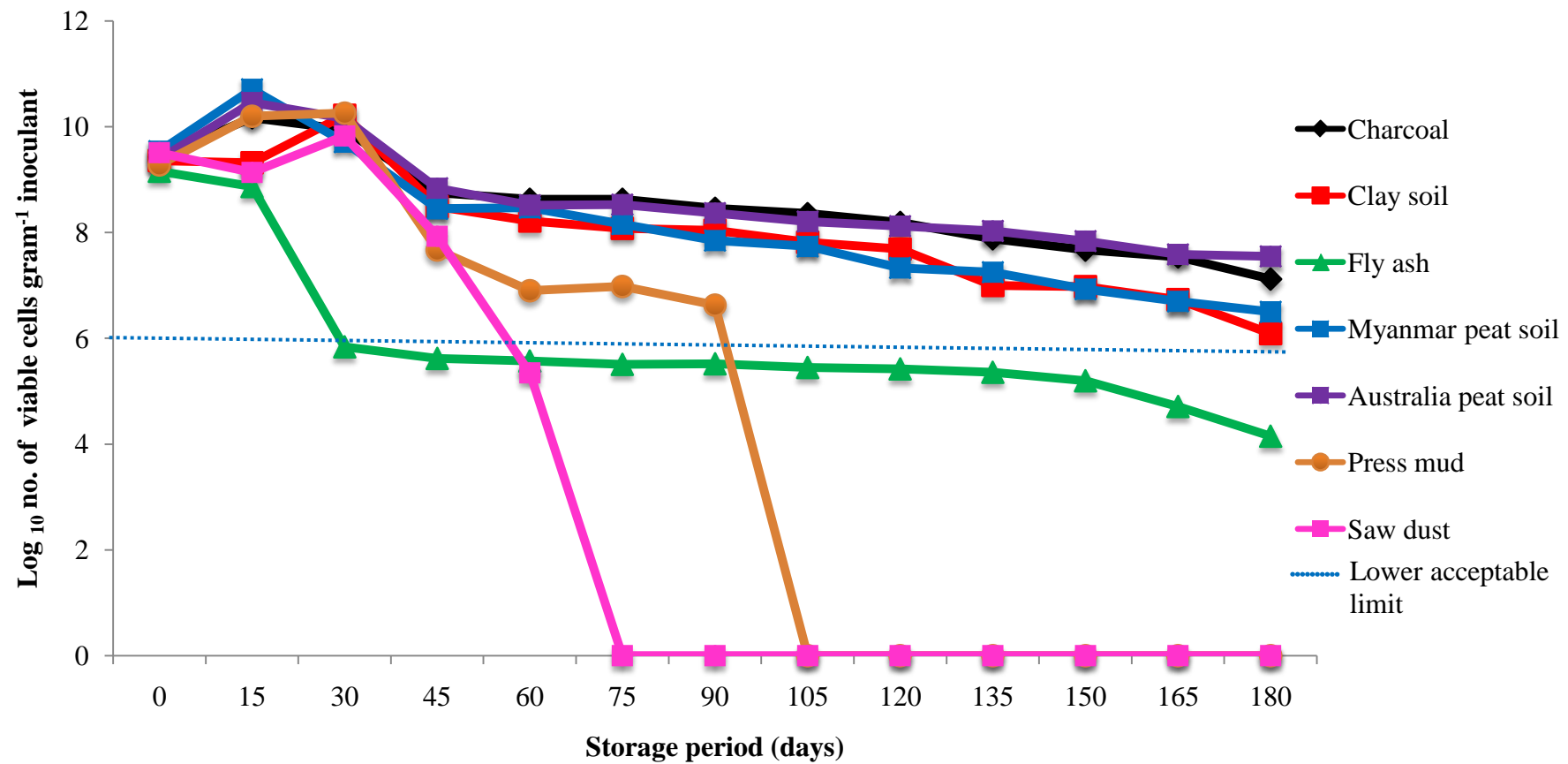
ns Non-significant difference

Figure 4.4 Survival of M-11 isolate in seven carrier materials at room temperature

Up to 30 days, all the carrier materials maintained the highest rhizobial population except in fly ash (Figure 4.4). Viable numbers of rhizobia declined sharply in fly ash after 15 days. It was observed that all carrier inoculants showed a significant reduction in rhizobial population after 30 days. Tabassam et al. (2015) also found that different carrier formulations as well as biozote carrier showed a significant decline in microbial population after 30 days. Neidhardt, Ingraham and Schaechter (1990) and Tate (2000) described that bacterial population dropped with the passage of time due to lack of moisture and nutrients (OM, N, P, K, etc.) of inoculum carriers, bacterial activities and storage conditions while transitioning from logarithmic to stationary phase during incubation period. In this study, acceptable rhizobial standard can be maintained in charcoal, clay soil, Australia peat soil and Myanmar peat soil for 180 days at room temperature (Table 4.10 and Figure 4.4).

One of the facts that they had high water holding capacity (Table 4.9). High WHC of charcoal has also been observed according to Sudhakar, Ghosh and Raje (2004). Somasegaran and Hoben (2012) and Valdiviezo et al. (2015) reported that WHC of peat was 282% and 120%. The origin of the peat might have resulted in a higher WHC (Tittabutr, Payakpong, Teaumroong & Boonkerd, 2007). Date (1974) revealed that high organic matter and high WHC are usually necessary properties of a suitable rhizobia carrier. Moreover, Rebah et al. (2007) and Valdiviezo et al. (2015) stated that high WHC of carriers favours the enzymatic processes involved in the degradation of the organic matter that provide important nutrients such as phosphorus for the rhizobial bacteria. Lal and Mishra (1998) observed that the higher population in charcoal may also be due to their ability to improve ventilation of inoculant in a better way than other carriers.

Out of 7 carriers, Australia peat soil was found as the best carrier material to maintain optimum viable cell count up to 180 days which was statistically varied with other carrier materials. At 180 days, Australia peat soil gave maximum cfu (7.6 cfu g^{-1}) and was found significantly superior over all other carriers followed by charcoal (7.1 cfu g^{-1}), Myanmar peat soil (6.5 cfu g^{-1}) and clay soil (6.1 cfu g^{-1}). However, fly ash fell short of a suggested minimum standard of 10^6 cfu g^{-1} for inocula at planting. The rapid die-off in rhizobial population was observed in saw dust and press mud. Mahdi et al. (2010) reported that the suitability of various carrier materials for the development of biofertilizer were in the order of peat > lignite > charcoal > soil > rice husk. Therefore, based on these results, charcoal and clay soil can be considered as alternative carriers for the future rhizobium production as they supported an optimum population of rhizobia at the end of 180 days.

CHAPTER V
CONCLUSION

This study evaluated that inoculation of 45 indigenous rhizobium isolates showed positive effects on nodulation and plant growth of green gram variety 'Yezin-11' suggesting that all tested indigenous rhizobium isolates were highly effective. So, the soil in Myaing, Yesagyo and Pakokku Townships of Magway Region harboured green gram-nodulating rhizobia with good symbiotic properties. There were significant variations in symbiotic characteristics of root-nodulating rhizobium isolates in green gram variety 'Yezin-11'. The positive and significant correlation was observed between nodule dry weight and shoot dry weight. The patterns of nodule were not well distributed throughout the root system.

It was observed that the SE% of all rhizobium isolates except M-1 and Y-1 were higher than that of N (+) control suggesting that all tested indigenous isolates were found to be highly effective. Among tested isolates, six rhizobium isolates; M-9, M-10, M-11, M-12, Y-6 and Y-9 isolates were found to be the most effective isolates to enhance the growth of 'Yezin-11'. Based on nodule dry weight and shoot dry weight, 14 rhizobium isolates; M-1, M-9, M-10, M-11, M-12, P-8, P-9, P-12, P-13, P-15, Y-1, Y-6, Y-9 and Y-15 were selected and their nitrogen (N) uptakes, morphological and biochemical properties were tested. The N uptakes (mg plant^{-1}) were highly significant among rhizobium inoculated plants. A significant and strong positive correlation was observed between nitrogen uptake and shoot dry weight. 78.57% of the indigenous rhizobium isolates were effective nitrogen fixers and others (21.43%) performed as good as N (+) control.

Morphological and biochemical characteristics generally varied among the isolates. Seven isolates; M-10, M-12, P-8, P-12, P-13, P-15 and Y-6 were fast-growing *Rhizobium* spp. while the rest; M-1, M-9, M-11, P-9, Y-1, Y-9 and Y-15 as slow-growing *Bradyrhizobium* spp. Morphologically, the rhizobium isolates showed typical characters of rhizobium but not identical in growth rate, colony size, colour and opacity on YMA. Biochemical studies revealed that all the isolates performed differently and showed diverse reactions towards the individual test. The fast growers were quite different biochemically from the slow growers. However, it was observed that some characteristics appeared to be related between the two groups of rhizobium. Based on N uptake results, all fast growers (*Rhizobium* spp.) were able to fix more

nitrogen than N (+) control. But only 57.14% of the slow growers (*Bradyrhizobium* spp.) were able to fix more nitrogen than N (+) control while the rest performed as effective as control. Therefore, *Rhizobium* spp. are higher in nitrogen fixation than *Bradyrhizobium* spp.

According to the results obtained in this experiment I, the highest symbiotic effectiveness of M-11 isolate collected from Myaing Township may be better compatible and thus may have potential as source of rhizobium inoculant. Thus, survival of M-11 in different carriers was studied at room temperature up to 180 days. It was observed that Australia peat soil still being a more superior carrier material as compared to others evaluated. However, charcoal, clay soil and Myanmar peat soil showed better results in survival of M-11 isolate as compared to the other carriers such as fly ash, press mud and saw dust. Therefore, charcoal and clay soil could be considered as the alternative carriers for the future rhizobium inoculant production.

Pot and field experiments should be carried out under different geographic locations and different environmental conditions to ensure the good performance of rhizobium isolate with the age of carrier inoculant. Moreover, in the present study, all measurement parameters were based on morphological characters and biochemical properties. Thus, the accurate identification of indigenous green gram-nodulating bacteria using molecular method should be investigated.

Rhizobial survival should be assessed at different storage temperatures and storage conditions. Fly ash, saw dust and press mud should not be used alone for inoculant production. Different combination of these with charcoal and clay soil should be formulated to maintain sufficient and acceptable population of bacteria at standard level for longer period of time. Some other indigenously available by-products need to be explored as carriers for microbial preparations.

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APPENDICES

Appendix 1 Number of samples collected from each Township

Sr. No.	Township	Sown area (acres)	No. of samples*
1	Myaing	95370	15
2	Pakokku	55397	15
3	Yesagyo	23894	15
	Total	174661	45

*Number of samples from each township was based on green gram growing areas of 2016-2017.

Appendix 2 Composition of N-free nutrient solution

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.

Appendix 3 Composition of Yeast Extract Mannitol (YEM) Broth

(Hartley & Gemell, 2017)

Ingredients	Grams/Liter
Mannitol	10.000
K_2HPO_4	0.500
$MgSO_4.7H_2O$	0.200
NaCl	0.100
Yeast	1.000

Appendix 4 Chemical reagents for determination of shoot N by Kjeldahl's technique

(Bremner, 1960)

Sr. No.	Chemical reagents
1	Concentrated sulphuric acid
2	Potassium sulphate + copper sulphate + titanium oxide
3	Four percent boric acid: four grams of boric acid was dissolved in 100 ml of distilled water at the temperature of 50°C
4	35 percent sodium hydroxide solution
5	Mixed indicator: A 0.1 percent of methyl red and 0.1 percent of bromocresol green dissolved in 100 ml of 95% alcohol separately
6	0.1 N hydrochloric acid

Appendix 5 Composition of Yeast Extract Mannitol Agar (YMA) Medium

(Kapembwa, 2014; Hartley & Gemell, 2017)

Ingredients	Grams/Liter
Mannitol	10.000
K_2HPO_4	0.500
$MgSO_4.7H_2O$	0.200
NaCl	0.100
Yeast	1.000
Agar	15.000
Congo-red	0.025

Appendix   6 Composition of Bromothymol Blue (BTB) Agar Medium

(Kapembwa, 2014)

Ingredients	Grams/Liter
Mannitol	10.000
K_2HPO_4	0.500
$MgSO_4.7H_2O$	0.200
NaCl	0.100
Yeast	1.000
Agar	15.000
BTB	0.025

Adjust the reaction to pH 6.8 and autoclave for 20 mins at 121°C

Appendix   7 Composition of YMA containing 2% NaCl

(Pervin et al., 2017)

Ingredients	Grams/Liter
Mannitol	10.000
K_2HPO_4	0.500
$MgSO_4.7H_2O$	0.200
NaCl	20
Yeast	1.000
Agar	15.000
Congo-red	0.025

Appendix   8 Composition of Glucose Peptone Agar (GPA) Medium

(Hartley & Gemell, 2017)

Ingredients	Grams/Liter
Glucose	5
Peptone	10
Agar	15
Bromocresol purple 1% in ethanol	10 ml

Appendix   9 Composition of Nutrient Agar (NA) Medium

(Deora & Singhal, 2010)

Ingredients	Grams/Liter
Peptone	5.000
Potato starch	2.000
Beef extract	3.000
Agar	15.000

Appendix 10 Composition of Urease Hydrolysis Test

(Gauri et al., 2011)

Ingredients	Grams/Liter
Mannitol	10.000
K_2HPO_4	0.500
$MgSO_4.7H_2O$	0.200
NaCl	0.100
Yeast	1.000
Urea	20.000
Phenol red	0.1

Appendix 11 Composition of Liquid Culture for Methyl Red Test

(Bhatt et al., 2013)

Ingredients	Grams/Liter
Glucose	5.000
Peptone	5.000
K_2HPO_4	5.000

** 5 drops of the indicator (0.1 g methyl red in 300ml of ethanol + 200 ml distilled water) was added to each tube containing 2 ml of liquid culture**.

Appendix 12 Summary of the biochemical tests for different strains of *Rhizobium*

<div align="right">(Datta et al., 2015)</div>

Characteristics	Strains			
	Rhizobium phaseoli	*Rhizobium trifolii*	*Bradyrhizobium japonicum*	*Rhizobium leguminosarum*
Gram Reaction	-	-	-	-
Bromothymol Blue	+	+	-	+
Starch Hydrolysis	+	-	+	-
Caesinase	-	+	-	-
Urease	+	+	+	-
Catalase	+	+	+	+
Lysine De Carboxylase	+	+	+	-
Lipase	+	+	+	+
Citrate	+	+	-	-

"+"indicates positive reaction, "-" indicates negative reaction