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ISOLATION AND CHARACTERIZATION OF RHIZOBIA FROM ROOT NODULE OF GRASS PEA (LATHYRUS SATIVUS) AND INVESTIGATING THE EFFECT OF GLYPHOSATE ON THE ISOLATES

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MSc. Thesis

By

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June, 2019

Bahir Dar, Ethiopia

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MSc Thesis Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the Award of Master of Science Degree in Biology (Applied Microbiology)

By

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June, 2019

Bahir Dar, Ethiopia

Declaration

I the under signed, MSc. student declare that this thesis is my original work for the partial fulfillment for the requirement of Master's degree in Applied Microbiology. All the sources of materials used for this thesis and everyone who support this work are fully acknowledged.

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As members of the board of examiners for the MSc. thesis open defense examination, we certify that we have read and evaluated the thesis prepared by Atrsaw Asrat and examined the candidate. We recommended the thesis to be accepted as fulfillment for the requirements of the degree of Master of Science in Biology (Applied Microbiology).

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List of Acronyms and Abbreviations

ANOVA	Analysis of Variance
BNF	Biological Nitrogen Fixation
BTB	Bromothymol Blue
CaCO3	Calcium Carbonate
CFU	Colony Forming Units
CSA	Central Statistical Authority
EFSA	European Food Safety Authority
EPS	Exo-polysaccharide
MRVPB	Methyl Red and Voges-Proskauer Broth
OD	Optical Density
ODAP	Oxalyl-Dimino-Propanoionic Acid
PI	Percentage of Inhibition
SIM	Sugar Indole Motility
SIM-YEMA	Sugar Indole Motility with Yeast Extract Manitol Agar
SPSS	Statistical Program for Social Sciences
SSU rRNA	Small subunit of ribosomal RNA
YE	Yeast Extract
UKNEA	United Kingdom National Ecosystem Assessment
YEM	Yeast Extract Mannitol
YEMA	Yeast Extract Mannitol Agar
YEMA-BTB	Yeast Extract Mannitol with Bromothymol Blue
YEMA-CR	Yeast extracts Mannitol with Congo red
YEMB	Yeast Extracts Mannitol Broth

Abstract

Grass pea (*Lathyrus sativus*) is an insurance crop grows extensively in the world and has symbiosis interaction with nitrogen fixing rhizobia. Farmers used glyphosate as weed control in agricultural process. However, the application this chemical herbicide affects soil fertility by decreasing nitrogen fixing rhizobia. The objective of this study was to assess the effects of glyphosate on the *Rhizobium* isolate. Grass pea with good growth and healthy appearance were selected purposefully form the field and rhizobia was isolated from root nodules. Isolates were characterized on the basis of their morphological, biochemical characteristics and resistance of abiotic condition. Isolates of rhizobia from grass pea were gram negative; circular, milky, 2-4.1mm diameter and most of them are smooth colony appearance on YEMA. Based on the morphological and biochemical characteristics of isolates were grouped under the genus *Rhizobium*. Isolates was grown best at the temperature between 28-38°C, pH 5-8 and at 0.5 and 1% salt concentration. Glyphosate was found to affect the survival of *Rhizobium* under laboratory. 5.52-47% of *Rhizobium* population was inhibited at 20ml L^{-1} concentration of glyphosate. At the concentration of 40mlL^{-1} percentage of inhibition was 17.1% - 53.38%. However, 87% of isolates were inhibited with higher concentration (60ml L^{-1}) of glyphosate. The number of colonies after the exposure of glyphosate was greatly dependent on concentration. Thus isolate from root nodules of grass pea was inhibited by glyphosate under laboratory condition. Future work needs to identify resistant *Rhizobium* from grass pea root nodule through evaluating their responses under greenhouse and field conditions and used as microbial inoculum.

Keywords: - *Glyphosate, Grass pea, Herbicide, Nitrogen fixation, Rhizobium*.

1. INTRODUCTION

1.1. Background

Grass pea *(Lathyrus sativus L)* belongs to the family Leguminosae which is a widely cultivated species of food crop in the genus *Lathyrus*. It is the main legume crop growing under different environmental conditions in the world (Diresse Tsegaye *et al.,* 2005). This legume crop spread over the world and adapted well in various harsh environments from the highland volcanic soil of Ethiopia to heavy clay in the paddy rice field (Fikre Asnake *et al*., 2011). In Ethiopia, the production of grass pea has increased both in area coverage and production volume of 185,490 tons in 2005 to 287,674 tons in 2015. The productivity per hectare was raised from 1260 kg to 1808 kg in the same period (Central Statistical Agency, 2010). Grass pea *(Lathyrus sativus L)* is largely considered as an insurance crop in areas that are susceptible to abiotic stresses, because it can produce reliable yields when all other crops fail due to drought (Vaz Patto and Rubiales, 2014). Grass pea is suitable for consumption that provides food and nutrition security to many low-income communities (Grela *et al*., 2010).

Nutrient enrichment of soil by nitrogen fixing symbiotic bacteria present in legumes has been known for centuries. The scientific demonstration of this symbiosis started in the nineteenth centuries and it established the fact that bacteria present in nodules on legume roots are responsible for fixing atmospheric nitrogen (Wolde-Meskel Endalkachew *et al*., 2005).

Rhizobia are gram negative motile symbiotic bacteria which very complex in soil environment and provide many key ecosystem services (Young and Crawford, 2004). The maintenance of soil quality is the critical role of rhizobia for ensuring the enhancement of agricultural process and sustainability of food production in the world (Bastida *et al*., 2008). Within the soil medium rhizobia perform an important task in the decomposition and transformation of organic soil

materials which is crucial for the functioning of nitrogen cycles (Bastida *et al.*, 2009). Interactions with legume crop in the agro-ecosystem were processed and enhance the productivity of the cropping system (Young and Crawford, 2004). Grass pea through its symbiotic relationship with *Rhizobium* helps to obtaining nutrients in the process nitrogen fixation (Adeleye *et al.*, 2004). Grass pea has strong penetrating root system that allows growing in a wide range of soil types including very poor soils and heavy clay effectively. *Rhizobium* living in the soil enriches nitrogen for the legume crop (Shiferaw Eleni *et al.*, 2012). The atmospheric nitrogen fixed by *Rhizobium* having a great role for higher yields not only for the grass pea but also for the succeeding crop. Therefore, the crop fits very well into a long-term sustainable farming system without requirement of chemical fertilizer (Singh and Wright, 2002).

Agricultural production has used herbicide to minimize and control weed problems in crop field (Berry *et al.*, 2008). Nowadays about one-third of agricultural products are produced by using herbicide application (Padgette *et al.*, 2018; Stalker *et al.*, 2018). Limited crop land and growing population forces to take all measures to increase crop production in order to ensure food safety for the global world (Zhang and Pang, 2009). However, the environmental fate of herbicides is a matter of recent concern given that only a small fraction of the chemicals reach the target organisms (Pimentel, 1995). The overall functioning of ecosystem is affected by herbicide either directly or indirectly (Aoki, 2003).

Glyphosate is non-selective weed killer of pre-emergent herbicide used to control all types of undesirable plants and herbs (Cedergreen and Streibig, 2005). Farmers used this herbicide in larger quantities as pre-emergent of weed controlling mechanism. The main problem is wrong application that commonly resulted from failure in sprayer. Application in the field should be properly carried out based on recommended concentration. Since it results in microbial damage in

soil medium (Badowski *et al.*, 2008). It is useful to keep in mind the concept that pollutant is a substance in the wrong place at the wrong time or in the wrong amount (Ayansina and Amusan, 2013). Therefore, the amount is calculated based on recommended rate and required for specific area (Badowski *et al.*, 2008). The toxic substance in glyphosate comes to be harmful for nitrogen fixing *Rhizobium* in the soil environment (Amakiri, 1982). Glyphosate herbicides enter into the soil environment from direct interception of spray by the soil surface and leaching into the soil environment and affect the *Rhizobium* population (Ayansina and Oso, 2006).

There are reports concerning glyphosate herbicides that inhibit the number of *Rhizobium* species, even though the effect varies from species to species (Drouin *et al.*, 2010). Many investigations showed that non-selective glyphosate adversely affects the nitrogen fixation process by decreasing rhizobia population (Mallik and Tesfai, 1983; Moorman, 1986; Zablotowicz and Reddy, 2004; Dos Santos *et al.*, 2005; Malty *et al.*, 2006; Berhan Aynalem and Fassil Assefa, 2017). Glyphosate reduced the growth rate of *Bradyrhizobium* in glyphosate-amended media and had negative effects on nodulation and N_2-fixation in greenhouse and field experiments (Zablotowicz and Reddy, 2004). The effects of glyphosate on rhizobia have been studied to a greater extent (Milosevia and Govedarica, 2002; Dos Santos *et al.*, 2005; Gricher, 2006; Adil *et al.*, 2012). However, the effects in related to different concentration glyphosate on *Rhizobium* isolated from grass pea (*Lathyrus sativus*) are limited.

1.2. Statement of the Problem

Birakat is the major grass pea producing area and farmers produce grass pea extensively and use it as food source. However, the productivity has progressively decreased from time to time (North Mecha Woreda Rural Land Administration and Use, 2008). Farmers in the study area and elsewhere used glyphosate herbicide to minimize the negative effects of weeds, but treatments

have a number of possible unfavorable side effects on nitrogen fixing rhizobia. The accumulations of high glyphosate concentration are more destructive to beneficial nitrogen fixing *Rhizobium* (Zablotowicz and Reddy, 2004). Many investigations have been done on the effect of glyphosate on *Rhizobium* population (Dos Santos *et al.*, 2005; Malty *et al.*, 2006; Berhan Aynalem and Assefa Fassil, 2017). However, the effects in related to different concentration of glyphosate on *Rhizobium* isolated from grass pea (*Lathyrus sativus*) are limited.

1.3. Significance of the Study

The results of this study are invaluable to both researchers and other stakeholders by serving as a stand point and go further study in the same area. In addition the knowledge on the effect of different concentration of glyphosate herbicides on *Rhizobium* has a vital role in the solution and as well as it is important in selecting resistant isolate as microbial inoculum for applying with the presence of toxic glyphosate. This study initiated that isolation and characterization of rhizobia from root nodules of grass pea and assesses concentration related effect of non-selective glyphosate herbicide at laboratory condition.

1.4. Objectives of the Study

General Objective

To isolate and characterize rhizobia from root nodules of grass pea (*Lathyrus sativus L*) and assess the effect of glyphosate on the isolate

Specific Objectives

1. To isolate and characterize rhizobia from root nodules of grass pea (*Lathyrus sativus L).*
2. To assess the responses of rhizobia isolate to different biochemical and abiotic conditions.
3. To investigate the effect of glyphosate on the isolate at different concentration under laboratory condition.

2. LITERATURE REVIEW

2.1. Grass Pea (*Lathyrus sativus L*)

Grass pea belongs to genus *Lathyrus* within the Fabaceae family (Schaefer *et al.*, 2012). It is annual pulse crop originating in south west and central Asia and subsequently spreading into the eastern Mediterranean and found in Israel (Mahler-Slasky and Kislev, 2010). Grass pea is an important crop of economic significance in India, Bangladesh, Pakistan and Ethiopia. It contains large number of species and subspecies being recognized. Production of grass pea (*Lathyrus sativus)* has an increased interest as a plant that is adapted to arid conditions and contains high levels of protein (Tadesse Wuletaw and Endeshaw Bekele, 2003).

2.1.1. Distribution of Grass pea (*Lathyrus sativus L*)

Grass pea (*Lathyrus sativus*) is a much-branched sprawling or climbing, herbaceous annual, with a well-developed taproot system, the rootlets of which are covered with small, cylindrical, branched nodules, usually clustered together in dense groups(Jackson and Yunus, 1984). Grass pea grows throughout the world and is used for human consumption (Smartt, 1984). This crop is grown towards the end of the crop harvest use residual moisture in the crop fields and growing to maturity during the dry season (Das, 2000). Grass pea is one of the cheapest sources of dietary protein in the developing world (Enneking, 2011). The genus *Lathyrus* is large with 187 species and subspecies being recognized (Allkin *et al.*, 1983). However, only one species *Lathyrus sativus* is widely cultivated as a food crop (Jackson and Yunus, 1984). Grass pea (*Lathyrus sativus*) is a popular food crop in some Asian and African countries such as India, Pakistan, Bangladesh and Ethiopia because of its resistance to drought, flood and moderate salinity. It becomes the only available source of food for the poor section of the population and sometimes a means of survival in times of drought induced famine (Mahler-Slasky and Kislev, 2010).

Nowadays people in drought-prone areas of Asia and Africa grass pea is a traditional popular crop because of its easy cultivation. Due to its resistance to drought, flood, insect attack and good yield of protein-rich tasty seeds with relatively little labor expenditure highly cultivated by farmers in different parts of the world (Lambein *et al.,* 2019). Grass pea can be produced without any fertilizers and hence its cost of production is very low compared to other crops (Getahun Haileyesus *et al.,* 2002). Grass pea (*L. sativus)* is also important in crop rotation systems because of its efficient nitrogen fixation, improving the fertility of the soil and increasing the yield of the subsequent cereal crop (Lambein *et al.,* 2019). Its extreme resistance to drought is a characteristic likely to become even more important in many regions as a result of climate change. In years when conditions are particularly harsh human consumption of the crop may increase through lack of any suitable alternative especially for the poorest rural people (Tadesse Wuletaw and Endeshaw Bekele, 2003).

2.1.2. Types of food prepared from grass pea (*Lathyrus sativus L*)

Different seed coat colour might be preferred in different regions according to traditional use of the crop. The seed coat colour can affect the nutritional value of the seed. Condensed tannin levels were found to be positively correlated with seed coat pigmentation (Deshpande and Campbell, 1992). In Nepal the dried grains are split either in a stone grinder on a home scale or milled to make dal which is consumed with rice. The grains are also ground and made into flour for use in a pancake-like preparation of badi or pakoda. In Ethiopia, particularly the northern regions, and in Eritrea tef, wheat, barley, maize and sorghum, either singly or in combination, are used to produce fermented, sour pancake-like unleavened bread called enjera. *Lathyrus* grain is ground into shiro and is used in the preparation of wott, a sauce that is eaten together with the enjera. For snacks, cereals, legumes or their mixture are most often consumed roasted or boiled. Boiled grass pea

(nifro) is consumed in most areas. Kitta, an unleavened bread made from grass pea, is consumed to a more limited extent, mainly at times of acute food shortages (Tekele-Haimanot *et al.* 1993). During the month of February in South East Asia the tender young vegetative parts are plucked (4-6 cm length) and cooked as a green vegetable. They are also rolled and dried for off-season use as vegetable (Bharati and Neupane, 1989).

2.1.3. Nutritional Composition of Grass Pea

The nutrient composition of grass pea is similar to that of field pea (*Pisum sativum*) and faba bean (*Viica faba)* with low fat and high starch content. The protein content is higher than field pea, but lower than soybean (Hungria *et al.*, 2000). The amino acid profile is also similar to other legumes, being rich in lysine (Yan *et al.,* 2006). Grass pea is highly suitable for human consumption as 58 % of the fatty acids are polyunsaturated (Grela *et al.*, 2010). The protein content of grass pea is 18-34 % (Rizvi *et al.,* 2016). These values are higher than those of field pea (*Pisum sativum)* (23 %) and faba bean (*Vicia faba*) (24%) (Petterson *et al.,* 1997) However, it is lower than those of soybean (42 %) (Ravindran and Blair, 1992). Grass pea protein is composed of albumins, globulins, glutelins and prolamins (Chandna and Matta, 1994).

2.1.4. Production of Grass Pea in Ethiopia

In Ethiopia grass pea cultivation has expanded relative to other pulses, for a total of 80,000 ha in 1990 to more than 1, 10,000 ha today. In north western Ethiopia, grass pea is the second most important food legume after faba bean (*Vicia faba*) (Central Statistical Agency, 2010). Planting is done in late August to early September and the crop is supposed to take advantage of the residual soil moisture. In years of severe flooding or drought, grass pea is the only crop that can remain green in the field (Tadesse Wuletaw *et al.*, 1997).

In Ethiopia, area and production of grass pea have increased steadily from 75, 950 ha and 80,430 ton in 1996 to 159,731 ha and 202,126 ton in 2009 respectively (Central Statistical Agency, 2010). These increases are attributed to the fact that grass pea cultivation has found preference in difficult areas where other crops have generally failed due to prevailing harsh climatic conditions (Tadesse Wuletaw *et al.*, 1997). Bangladesh and Ethiopia are the second and third largest producers of grass pea grain respectively (Tadesse Wuletaw and Endeshaw Bekele, 2003). In the north-eastern Ethiopia following the drought of1995/96 and the subsequent wide spread failure of other crops grass pea was an alternative food sources during periods of drought in Ethiopia (Haileyesus Getahun, 2000). Grass pea is the third most important pulse crop after faba bean and chickpea in Ethiopia. Central Shewa accounts for more than 90 % of the total area of grass pea cultivation, with an annual production of about 70,000 tons (Central Statistical Agency, 2009). The breeding station at Adet research Centre has identified some varieties with low ODAP content in multi-location trials conducted in the potential growing areas of north-west Ethiopia. However, genotype, environment and their interactions were found to be significant for ODAP content and grain yield (Tadesse Wuletaw *et al.*, 1995).

2.2. Rhizobia

Rhizobia are legume root-nodule bacteria that induce the formation of special structures on the roots of their host plant and fix nitrogen after becoming established inside the root nodules of legumes. Among the soil microorganism these are a unique group of bacteria that have a beneficial effect on the growth of legume that live either in the soil or within the root nodules of host legumes (Jordan, 1984).

The association between legumes and their symbiotic bacteria (rhizobia) was certainly studied at the earlier (Wilson, 1944). Eventually Jordan (1982) proposed that a new genus, *Bradyrhizobium*

and this opened the modern era which has seen an increasing pace of nomenclature change that still continues today. This has been provided the introduction of new and more trustworthy techniques for assessing the similarities and differences between bacteria. Then molecular techniques introduced new and abundant sources of data first DNA hybridization and protein electrophoresis and then DNA methods culminating in sequencing (Young and Haukka, 1996).

The most important single source of data for developing our current classification of rhizobia as of all other bacterial groups has been the sequencing of genes for the 16S or small subunit of ribosomal RNA (SSU rRNA). In the relatively short time since the first partial SSU rRNA sequences for rhizobia reported by Young *et al*. (1991). The SSU data support the well-established subdivision of rhizobia into three genera: *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (Yanagi and Yamasato, 1993). Further, rhizobia are divided into five genera (*Azorhizobium*, *Bradyrhizobium, Mesorhizobium*, *Rhizobium* and *Sinorhizobium)* (Young and Haukka, 1996).

Rhizobium is a gram-negative motile bacterium whose members are mostly having ability to establish a symbiotic relationship with leguminous crop such as peas, soybeans, check pea, grass pea and alfalfa (Jordan, 1984). They plays an essential role in maintaining soil fertility and nutrient cycling, improving soil structure; supporting healthy plant growth; degrading organic pollutants. It stabilize the ecological system in soil due to their ability to regenerate nutrients to support plant growth (Wang *et al*., 2008). Other genera, such as *Azorhizobium* and *Bradyrhizobium* can also nodulate leguminous plants as *Rhizobium* (Stougaard, 2000). *Rhizobium* has been characterized from different legumes based on their morphological, physiological and growth characteristics (Wolde-Meskel Endalkachew *et al*., 2005). The genus *Rhizobium* is non-spore forming, gram negative and motile by 1-6 peritrichous flagella. The temperature range for growth of *Rhizobium* is 25-30℃; some species can grow at temperatures

>40°C. Optimal pH for growth, 6-7 but it can grow up to pH 10. Colonies are usually white or beige, circular, convex, semi-translucent or opaque, raised and mucilaginous, usually 2-4 mm in diameter within 3-5 days on Yeast Mannitol mineral salts agar (YMA) (Jordan, 1984).

Rhizobia are chemoorganotrophic utilizing a wide range of carbohydrates and salt of organic acids as sole carbon sources, without gas formation (Yan *et al.*, 2007). Strains of some species grow in a simple mineral salts medium with vitamin free casein as the sole source of both carbon and nitrogen, but strains of many species require one or more growth factors such as biotin and peptone is poorly utilized. Casein, starch, chitin, and agar are not hydrolyzed. Cells of *Rhizobium* symbiotic species enter root hair cells of leguminous plants via invagination and elicit the production of root nodules where in the bacteria engage as intracellular symbiosis usually fix nitrogen (Jordan, 1984).

2.3. Herbicide and Their Effect

Weed killers are a group of chemicals which prevent, inhibit, destroy, repel or kill weeds or undesirable plants. These are designed to be biologically active in the case of sensitive and it produces inhibition effects particularly if they are used at high concentrations (Reddy *et al.*, 2012; Ayansina and Amusan, 2013). Herbicides adversely affect nitrogen fixation by reducing symbiotic *Rhizobium* population (Singh and Wright, 2002). Herbicides are categorized in to two based on time of application as, pre-emergent herbicide and post emergent herbicide. Pre-emergent herbicides are applied in weed free ground before the sowing crop and act on the weed seeds preventing them to germinate and Post-emergent herbicides used after the emergence of weeds on crop fields (Ratcliff *et al.*, 2006).

2. 3.1. Glyphosate

Glyphosate (N-phosphonomethyl glycine) is one of the non-selective, broad spectrum type herbicide which is mostly applied in agricultural practice for cleaning up of the weeds from the farm land before sowing (Schluer and Aber, 1980). It is used by farmers, land managers and gardeners to effectively control unwanted vegetation. It directly kills all types of weeds on the ground (Tomlin, 2000).

This wide spread adoption of glyphosate is the result of ability to control a broad spectrum of weeds. It is often used by government agencies to control the spread of invasive, noxious, and non-native weed species and prevent them from crowding out native species, and also to control many poisonous weeds such as poison ivy and poison oak (European Food Safety Authority, 2011). The applications of glyphosate are done during winter when the main crop has been sown. Surface soils under no-till systems develop a mulch of crop residues (Franzluebbers *et al*., 2007). The extensive use of glyphosate in modern agriculture has toxicological effects on non-targeted microorganisms. The main important factor to keep in mind, when assessing the possible impacts of pesticides on the ecosystem, is the fact that pesticides differ from each other with regard to their environmental behavior and toxicological profile. Glyphosate is a foliar herbicide applied to crops, but most of the time due to application errors and it leaches into the soil (Alexander and Aging, 2000). The addition of glyphosate can cause qualitative and quantitative alterations in the soil microbial populations and their enzyme activities (Gricher, 2006; Wang *et al*., 2008). Glyphosate is effective against more than 100 annual broadleaf weed and grass species (Dill *et al*., 2010). A proportion of herbicides introduced as pre-emergence weed killer have greater ecologically destructive effects. An increase in doses of glyphosate herbicide tends to amplify its negative effect on microorganisms. These effects depend upon the concentration and possibly

moderated by environmental conditions (Gricher, 2006). Glyphosate is very sensitive to many microorganisms and protozoans which play key roles in soil nutrient cycling. It reduces the number of nodules by inhibiting symbiotic nitrogen-fixing microorganisms (Zablotowicz and Reddy, 2004). It is intercept into the soil medium during application and intercepted through flooding runoff and leaching of the deep soil during rainfall (Selim *et al.*, 2003). Glyphosate were affect the growth and survival of *Rhizobium* and its effect depends on the concentration, this can greatly affect the nitrogen fixation process (Konstantinovia *et al.*, 1999; Berhan Aynalem and Fassil Assefa, 2017).

2.3.2. Inhibition mechanism of glyphosate on the bacterial isolate

Glyphosate (N-phosphonomethyl glycine) is the active ingredient in roundup broad spectrum pre emergence herbicide sold worldwide use in large number of agricultural crops and largely used to control of mixed weed. Glyphosate: is Translocate systemic herbicide, glyphosate in Common name, round up in common trade name and chemical group is Glycine. Glyphosate inhibits aromatic amino acid biosynthetic pathway and It targets the enzyme 5-Enolpyruvylshikimate-3-phosphate Synthase (EPSPS) in the shikimate pathway and disrupts the formation of aromatic amino acids (Benbrook, 2016; Dutta *et al*., 2002).

2.3.3. Effect of Glyphosate on *Rhizobium* Population and Nitrogen Fixation

The application can affect the microbial composition and enzymatic activity in the plant rhizospher and surrounding soil (Arango *et al.*, 2014). There are many adverse effects of glyphosate on the biology and ecology of rhizospher micro-organisms. In addition, it has an effect on their interactions with plant roots when released into the rhizospher of crops (UK National Ecosystem Assessment, 2011). Schafer *et al.* (2014) investigated the taxonomic distribution of the microbial community diversity and genera abundance within the rhizospher susceptible to a

glyphosate herbicide. The application may be necessary because of weeds negatively affect crop production (Gricher, 2006). However, in agricultural systems may exert side effects on the soil micro-flora, including a possible shift in microorganism community structure. Glyphosate herbicide affected the growth and survival (Anderson *et al.*, 2004) and recognition of the host plant for nodule formation of *Rhizobium* (Fox *et al.*, 2001). This herbicide negatively affect nitrogen fixation either directly by affecting *Rhizobium* or indirectly by restricting root growth and the number of root sites available for infection (Anderson *et al.*, 2004). *Rhizobium* population was affected even at recommended rate or company specification of glyphosate. The initial studies by Jaworski (1972) demonstrated that glyphosate inhibited growth of *R. japonicum* strain USDA 71 by 69% and 92% at relatively low concentrations of 0.00497mL^{-1} and 0.0994mL^{-1} respectively. In addition, studies by Moorman *et al.* (1992) demonstrated differential growth inhibition sensitivity among *R. japonicum* in a defined mannitol glutamine broth. Hernandez *et al.* (1999) confirmed a differential growth inhibition by glyphosate among three *R. japonicum* strains at 2.982 mlL^{-1} is the most sensitive strain (ISJ-32), at 0.0994mL^{-1} is the most tolerant strain (ISJ-33) and strain ISJ-48 was intermediate. Santos *et al.* (2005) showed that the reduction of *Bradyrhizobium* population at concentration of 25.41mL^{-1} as compared to the control. Zablotowicz and Reddy (2004) indicates that *R. japonicum* strains were completely inhibited and caused rapid cell death at 0.497mL^{-1} of glyphosate. According to Berhan Aynalem and Fassil Assefa (2017) done on *Rhizobium* isolate from Nodules of *Vicia faba* showed only 19-22% survival rate at 5.9×10^{-5} mL^{-1} glyphosate concentrations as compared to the control. Therefore, the use of this glyphosate herbicide is not appropriate due to such interfere with N_2 fixation (Gricher, 2006). This poses a risk to the entire ecological system (Milosevia and Govedarica, 2002; Adil *et al.*, 2012)

3. MATERIALS AND METHODS

3.1. Description of Sampling Site

The study was conducted from December 07/ 2018 to March 30/ 2019 at Birakat keble found in west Gojjam zone of Mecha Woreda in Amhara region. It is located between north of tatek gebrie, south of felege birhan, west of Zemene Hiwot and east of Medre Gente. Birakat is the major grass pea producing area having clay soil at an altitude of 2147m above sea level and the mean annual rainfall and temperature are 1058 mm and 26℃ respectively (North Mecha Woreda Rural Land Administration and Use, 2008).

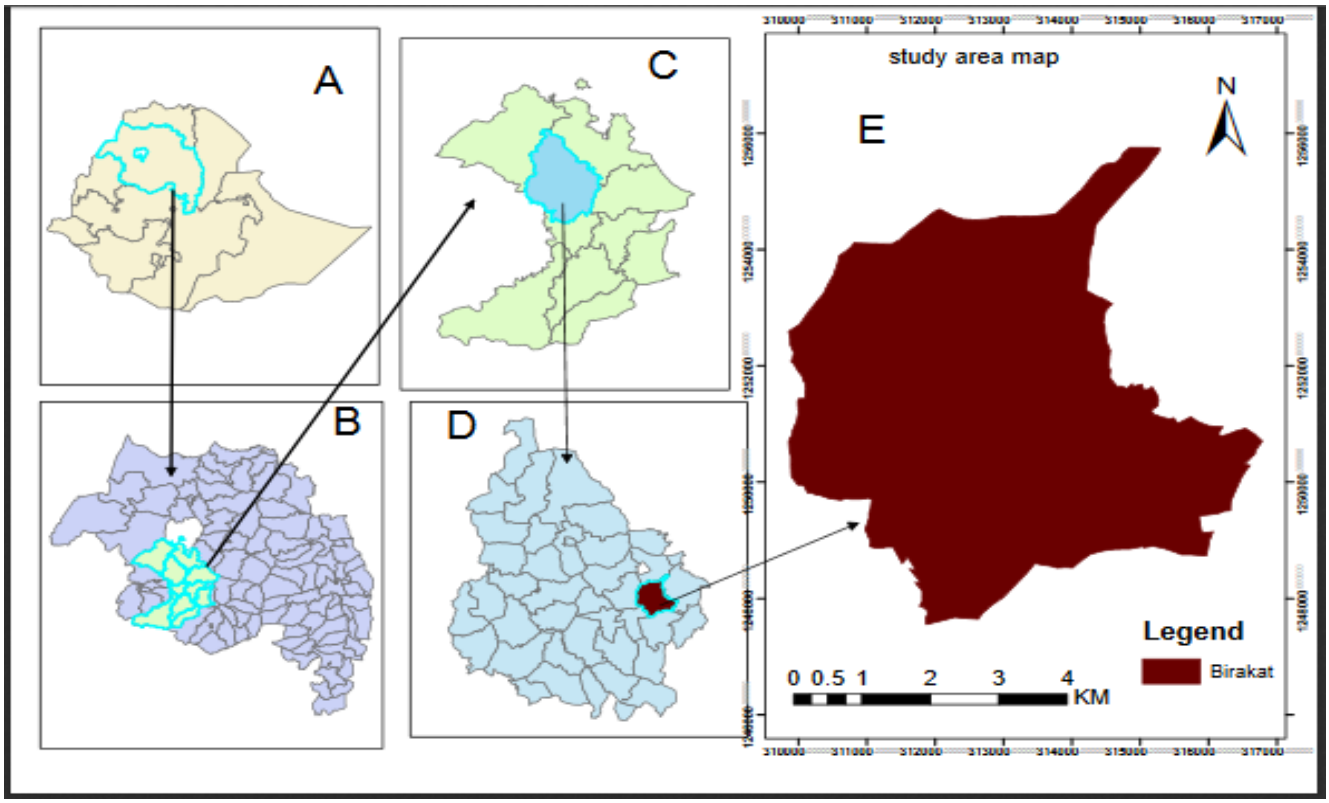

Figure 1: Description of study area

Where: A=Ethiopia, B=Amhara, C=West Gojjam zone, D= Mecha woreda, E= Birakat

3.2. Sample Collection

Thirty fields were selected by using simple random sampling without considering agro ecological climatic zone and one grass pea samples per field (thirty samples) with good growth and healthy appearance were selected purposefully at the Centre of fields. The selected grass pea was uprooted carefully wzith their rhizospher soil. The samples were brought to the microbiology laboratory of Bahir Dar University aseptically in an ice box.

3.3. Isolation of Rhizobia from Nodules

Nodules were surface disinfected using 95% alcohol and 3% NaOCl and rinsed several times with sterile water. Each nodule was crushed in normal saline solution and a loop full of suspension was streaked on Yeast extract Mannitol (YEM) agar medium. Cultured plates were incubated at 28℃ for 24-48hr and re-streaked again to obtain a pure culture (Somesegaran and Hioben, 1994).

3.4. Characterization of the Isolate

Pure colonies were preserved at 4℃ (Short term) in YEM agar slants containing 0.3% (W/V) $CaCO_3$ (Somesegaran and Hioben, 1994).

The purified bacterial isolates were characterized on the basis of their morphological, biochemical characteristics and physiological features. Characterization of isolates was carried out by subjecting the isolated bacterial colonies to different characteristics such as morphological, biochemical and abiotic factors (pH tolerance, temperature tolerance, salt tolerance) (Dubey and Maheshwari, 2011).

3.5. Tolerance of abiotic factors

The bacterial isolates were activated in YEMB for 48hrs inoculated on to YEMA medium at 28℃ for 48 h and characterized based on their responses (Somasegaran and Hoben, 1994).

3.5.1 pH Tolerance

The ability of *Rhizobium* isolates to grow at different pH was tested in YEMA by adjusting the pH to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10 with NaOH and HCl (Bernal and Graham, 2001).

3.5.2. Salt Tolerance

The ability of *Rhizobium* isolate to grow in different salt concentrations medium was tested by streaking them on YEMA medium containing 0.5%, 1%, 2%, 3%, 4%, 5%, 6% and 7% (w/v) NaCl (Graham *et al.*, 1991)

3.5.3. Temperature Tolerance

Temperature tolerances of isolates were investigated by incubating bacterial cultures in YEM agar at 4, 10, 15, 20, 28, 38, 40, 45, and 50°C (Lindstorm, 1988).

3.6. Morphological Characterization

Morphological characteristics of the isolates were determined according to (Lupwayi and Haque, 1994). Loop full of 48hr broth culture from each isolate was inoculated on YEMA and incubated at 28°C 48h. Individual colonies were characterized based on their colony shape, colony diameter, colony texture and colony color (Jordan, 1984).

3.7. Biochemical Characteristics

3.7.1. Gram reaction

Gram staining was performed to check out whether the test organism was Gram positive or Gram negative (Jordan, 1984).

3.7.2. Congo red Absorption Test

Isolates were cultured for 48 hours in YEMB and streak onto YEMA-CR (0.5% v/v) medium to check Congo red absorption (Vincent, 1970).

3.7.3. Motility Test

Bacterial isolates were inoculated into a test tube containing a SIM medium with 0.5% of yeast extract mannitol ager using sterile straight wire and incubated at 28℃ for 48h. Migration of the isolates away from the line of inoculation was a positive result, while lack of migration away from the line of inoculation indicated a negative result (Dubey and Maheshwari, 2011).

3.7.4. Indole Test

Tryptophanase enzyme helps to hydrolyze the amino acid tryptophan. The sterile wire loops were used to inoculate bacterial isolate in a test tube containing DV tryptophan broth and incubated for 48 h at 28℃. After incubation, 0.5ml of kovac's reagent was added and mixed to stand for 10 min. The development of red ring color was indicated a positive result (Dubey and Maheshwari, 2011).

3.7.5. Methyl Red Test

To check the ability to isolate perform acid fermentation done through MR-VP broth with the addition of methyl red, since MR-VP broth contains glucose, peptone and phosphate buffers. Then 0.5 ml of methyl red (pH indicator) was added into the test tubes after 48h incubation at 28℃ and allowed to stand for 15 min. Red colour indicated a positive result (Dubey and Maheshwari, 2011).

3.7.6. Citrate Utilization Test

The test was carried out to test the bacteria that use citrate as the source of carbon. The Simmons citrate agar was prepared according to manufacturer's instruction. Then a loop full of a 48hr old bacteria culture from YEM broth were taken and at the butt part and of the slant t and incubated at 28℃ for 48hr then the result was recorded. A change in color from green to blue indicates a positive result (Koser, 1923).

3.7.7. Urease Test

The urease test used to identify the bacteria that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. In carrying out this test, a test isolate was inoculated in a test tube containing 5 ml of prepared urea broth and it was incubated for 48 h. at 28°C. After incubation the development of pink color recorded as a positive result and the non-development of pink color recorded as urease negative (Faddin, 1980).

3.7.8. Acid-Base Production Test

Loop full from a 48hrs old culture broth of each isolate were streaked onto the YEMA-BTB (0.25% v/v) medium and incubated for 48h and record the color changes of the medium characteristics of acid/ alkali production (Jordan, 1984).

3.8. Effects of Glyphosate on the Rhizobia Isolates

Rhizobium isolates were activated with YEM broth and stock solutions of glyphosate were prepared by adding 20ml L^{-1}, 40ml L^{-1} and 60ml L^{-1} of glyphosate through distilled Water (Mubeen *et al.,* 2006). In order to see the effect of different concentration of glyphosate on the isolates, three treatments, with controls such as Treatment-1, Treatment-2 and Treatment-3 were prepared in test tubes. Then l ml of each filtered glyphosate was separately added to sterilize test tube containing 10ml YEM broth. Then activated 0.1 ml (2.6×10^{12}-1×110^{13} cells) of each cultured isolates was inoculate into a test tube containing different concentration of glyphosate herbicide and incubated at 28^{o}c temperature for 72 hr. Growth of isolates in different treatments monitored through optical density measurement by using UV- spectrophotometer at 600 NM and compared each isolates grown on three different treatments with glyphosate-free control (Mubeen *et al.,* 2006).

Inhibition of glyphosate on *Rhizobium* isolate was determined using the formula:

$$\%PI = \frac{OD\ of\ control - OD\ of\ treated}{OD\ of\ control} \times 100$$

Where: - OD = optical density and % PI = the percentage of inhibition of glyphosate on *Rhizobium* (Mubeen *et al.*, 2006).

2.9. Cell Viability Test

The viability of the isolate was determined after chemical exposure by culturing serially diluted suspensions of 48h old culture on YEM agar plates. The 0.1ml from each treatment were transferred into the yeast extract agar medium in triplicate using Miles drop plate method and incubated at 28℃ for 72 h for direct plate counting (Somasegaran and Hoben, 2012).

3.10. Data Analysis

SPSS 23 version statistical software packages were used for determine the effect of glyphosate on the *Rhizobium* isolates at different concentration against *Rhizobium* population. The bacterial growth was evaluated by optical density (OD) spectrophotometer at 600 NM after 72 h. The value of OD for each of different concentration treatment and colony forming units (CFU) was analyzed through ANOVA and means were compared using Tukey.

4. RESULTS AND DISCUSSION

4.1. Tolerance of abiotic factors

4.1.1. pH tolerance of the isolates

In this study isolates of *Rhizobium* was tested by culturing in YEMA Medium by adjusting the pH through the addition of HCl and NaOH. The results of this study showed that resistance to grow at different pH was varying amongst the test isolates from grass pea. This finding indicated that all tested isolates (100%) grew at pH 5, 6 and 7. However, a small number of (26.6%) of isolates grew at the pH 4. In addition to this, 73.33% and 63.3% of the isolate grew at pH 9 and 10 respectively (Figure 1).

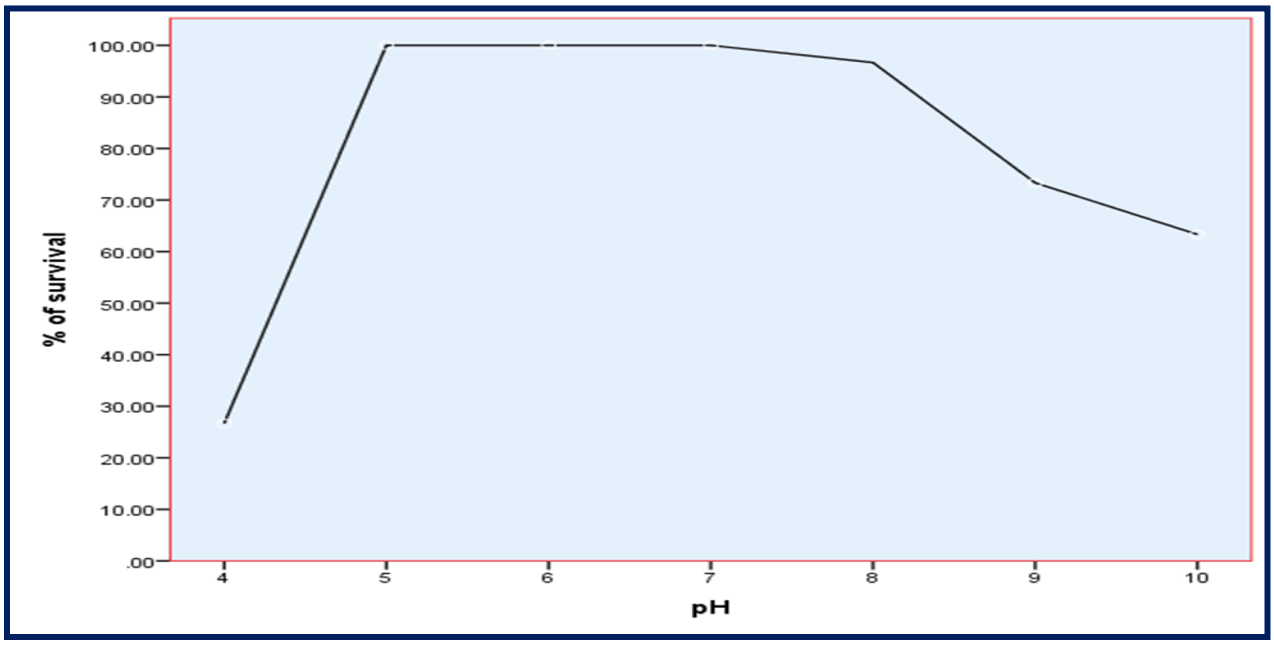

Figure 2: Tolerance *Rhizobium* to different of pH range

The variation of pH in the medium might have significant effects on the growth of *Rhizobium* bacteria (Singh *et al.*, 2008). The fact that different strains of the same species may vary widely in their pH tolerance has been demonstrated previously (Correa and Barneix, 1997). In this experiment small number of the isolates (11) was able to grow at pH 4. However, the results of

this study indicated that all of the isolates grew at pH of 5, 6 and 7. This indicated that the tested isolate cannot grow at the extreme low pH or high acidity. Results showed that most of the isolates are acid adapted, capable of surviving the range between 4 and 8 pH as reported for the genus *Rhizobium* by Jordan (1984). From all the isolate 23 and 19 isolates were grown at alkaline pH 9 and 10 respectively. This was contradicted with (Asrat Mekonen, 2015) on rhizobia isolated from field pea (*Pisum sativum*) were not grow at lower pH (4.5) and at higher pH (9.5) and corresponded with Girmaye Kenasa *et al.* (2014) showed that rhizobia isolates were tolerant to pH ranges 4.5-9. However, few number of *Rhizobium* isolate was to tolerate pH 4 as contrasting with Assefa Keneni *et al.* (2010) was reported that *Rhizobium* isolates was not grown at pH values lower than 4.5. The result was contrasted with Alemayehu Workalemahu (2006) shown that fast growing *Rhizobium* isolate appear to be more sensitive to low pH than slow growing isolate since, all isolates from grass pea root nodule were fast growing. However, it was agreed with Shetta *et al.* (2011) done on rhizobia isolated with woody legume trees grown in Saudi Arabia. In addition, the result was similar to (Kucuk *et al.*, 2006) isolates from the Turkey soil common bean.

4.1.2. Temperature Tolerance of the Isolates

In this study, all *Rhizobium* isolate (100%) grew at the temperature 20°C, 28°C and 38°C on YEMA medium. whereas 83.33% of isolate were able to survive at 45°C and 50% were tolerated 50°c. In addition, 66.66% of the isolates were found to tolerate and grow at a temperature of 15°C. However; all isolates were not grown at the temperature 4°C and 10°C (Figure 2)

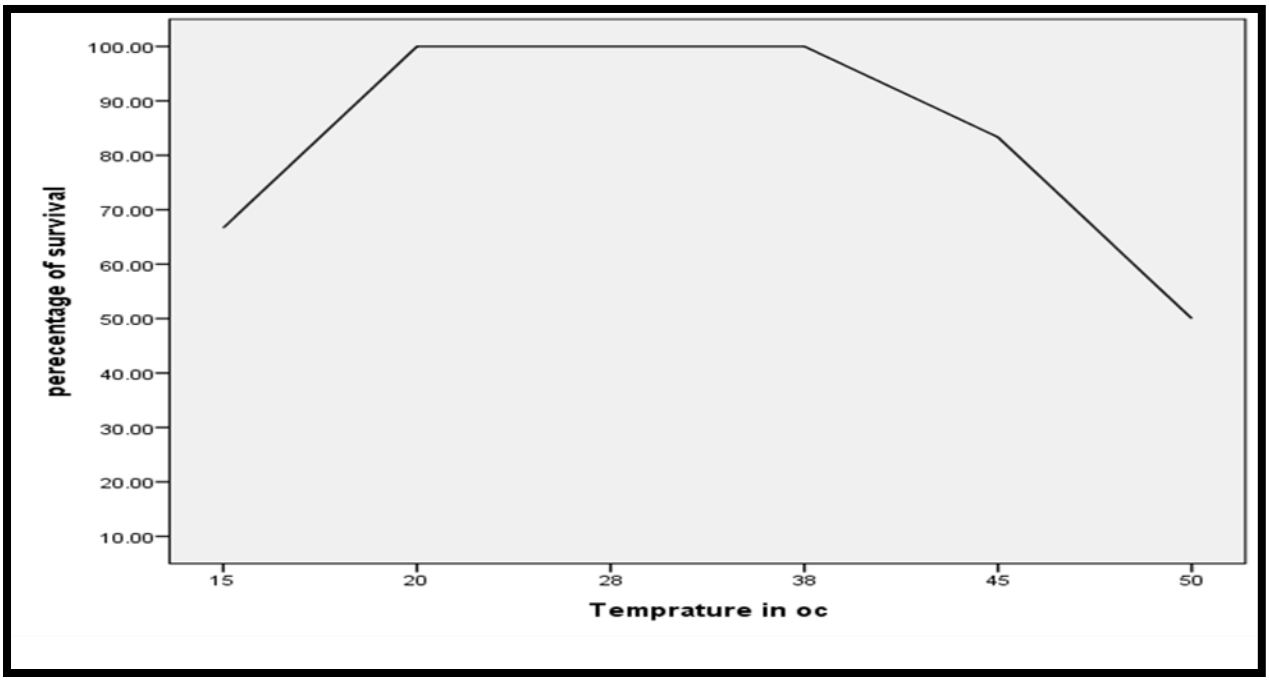

Figure 2: Tolerance of *Rhizobium* to different temperature range

According to Jordan (1984), the optimal temperature for the growth of *Rhizobium* was 20-30℃. However; temperature range was highly strain dependent for genus *Rhizobium*. The results of this study showed that *Rhizobium* isolates were not able to grow at 4 and 10℃. this, was disagreed with Drouin *et al.* (1996) who reported that isolates from *Lathyrus* species were capable to grow at lower temperature of 10℃. However, 50% of the isolate was survived to high temperature of 50℃ and all the isolate grew best at the temperature 20-38℃ as agreed with the results of Jordan (1984). The result was contrasted with Hungria *et al.* (2000) done on common bean and none of *Rhizobium* isolates grow above 38℃ . Since, the result indicates that *Rhizobium* isolate from grass pea were grow above 40℃ . While, the finding was corresponding to Kucuk *et al.* (2006) done on *Rhizobium* isolated from common bean in Turkey capable of growing up to the temperature of 42℃. In addition to this, results of this study was similar to Antenh Aragaw (2007) done on isolate of common bean 6% were survived to a temperature of 45℃. Likewise, this result was

similar with (Musa Adal, 2009) on rhizobia strains isolated from grass pea (*Lathyrus sativus*). Moreover, the finding was corresponding to Rasool *et al.* (2015) done on *Rhizobium* from wild legume and incubating at the temperature 30°C, 40°C and 50°C and bacteria incubated at 30°C and 40°C were able to grow well while the bacteria incubated at 50°C showed minimal growth.

4.1.3. Salt Tolerance of the Isolates

In this study, the growth of *Rhizobium* isolates form grass pea root nodule was related to the concentration of salt. Isolates was showed differences to grow concentration. *Rhizobium* isolates was varied in their response to salt stress on YEMA medium containing different NaCl from 0.5-7% of concentration. All isolates grew at 0.5 % NaCl concentration, whereas the concentration of salt increases the survival of the isolate were showed progressive reduction. Results showed that 96.66 % of the isolate grew at 1 % of salt concentration. However, most of the isolates of *Rhizobium* were not able to grow at the concentration of salt above 4% and few number of isolate was survive on YEMA medium containing 7 % of salt concentration (Figure 3).

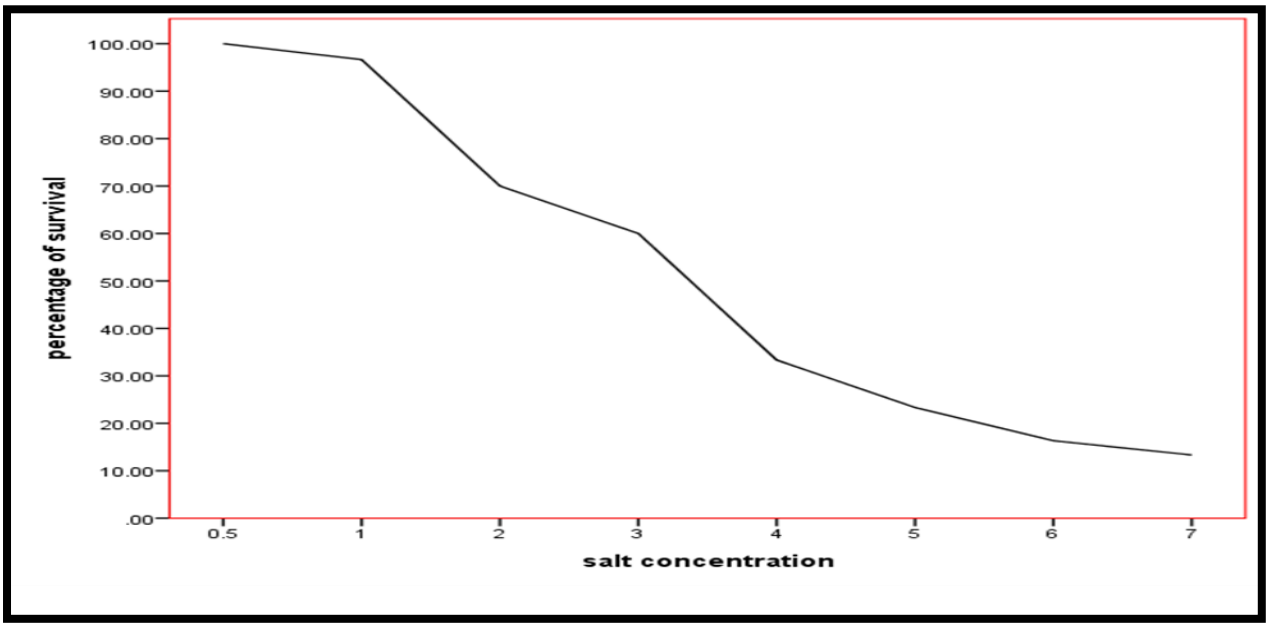

Figure 4: Tolerance of *Rhizobium* at different salt concentration (W/v)

Salinity was the major limiting factors restricting symbiotic nitrogen *Rhizobium*. Salt stress or salinity significantly reduces the *Rhizobium* population and affect nitrogen fixation and nodulation in legumes (Shetta, 2002). *Rhizobium* isolates were cultured on YEMA plates having 0.5%, 1%, 2%, 3%, 4%, 5%, 6% and 7% (w/v) NaCl and the percentage of survival was 100%, 99.66%, 70%, 60%, 33.33%, 23.33%, 16.33% and 13.33% respectively. In this study few numbers of isolates was highly tolerant to high salinity. Percentage survival of *Rhizobium* isolate was decreased with increasing the concentration of salt. Most of the isolates (96.66%) could grow at 1% NaCl. However, the percentage of survival was depends on the concentration of salt and only 4 isolates growth in 7% salt. This study was agreed with the result of (Musa Adal, 2009) on *Rhizobium* isolated from grass pea (*Lathyrus sativus*) and some isolates was surviving up to 7% of salt concentration. The finding was come to an agreement with previous work of (Kassem *et al.* (1985); Kucuk *et al. (*2006); Zerihun Belay and Fassile Assefa, (2011); Girmaye Kenasa *et al*., (2014). Salt tolerant *Rhizobium* has the potential to improve yield of legumes under salinity stress (Mokadem *et al.,* 1991). This finding was corresponding to Zahran (1997) showed that fast growing *Rhizobium* grew well at the concentration between 0.5-1% of NaCl. However, this result was diferent from that of the finding of Getahun Negash (2015) on *Rhizobium* strains isolated from faba bean (*Vicia faba*) were not grown at 0.5% NaCl concentration. In addition, this result was different from that of Hewedy *et al.* (2014) who did on *Rhizobium* isolate from Faba bean totally inhibited at 5% of NaCl and not revive in fresh medium.

4.2. Morphological Characteristics of Rhizobia Isolates

The diversity of rhizobia isolates were tested based on their phenotypic characteristics, gram reaction, colony shape, colony diameter, colony texture and colony colour. Result of this finding indicated that colonies of rhizobia isolated from grass pea root nodule were gram negative,

circular in shape, milky, 83.33% of them were smooth and 16.66% were rough colony appearance having 2-4.5 colony diameters as showed in (Table 1).

Table 1: morphological characteristics of *Rhizobium* isolate

Morphological character	Result
Colony shape	Circular
Colony diameter	2-4.5mm
Colony colour	Milky
Colony texture	83.33% smooth and 16.66% rough

According to Bergey's classification of systematic bacteriology rhizobia were classified as *Rhizobium* and *Bradyrhizobium*. The genus *Rhizobium* was gram negative, motile, white or milky, circular and raised in 3-5 days on Yeast Extract Mannitol Agar (YEMA) (Jordan, 1984). Most of rhizobia isolates from root nodules of grass pea was not varied in their morphological characteristics. The characteristics of native rhizobia isolates that nodulating grass pea were correspond to the finding reported by Kawake *et al.* (2014) in Kenya that isolates were smooth, gram negative and circular in shape. Microscopic examination of this study revealed that the rhizobia isolates were circular shaped and gram negative in nature as similar to findings of (Jordan, 1984; Alemayehu Workalemahu, 2006; Singh *et al.*, 2008; Hewedy *et al.* 2014; Niste *et al*., 2015; Berhan Aynalem and Fassil Assefa, 2017). Most of the isolates (86.6%) formed a colony diameter greater than 2.5-4.5mm on YEMA medium and 13.33% was formed the colony diameter 2 mm this result was agree with Jordan (1984) the colony diameter of *Rhizobium* was 2-4 mm after 5-6 days of incubation at 28℃. 83.33% of the isolate were showed smooth colony appearance however, few number of isolates ($BDUs_{3,}$ $BDUs_{13,}$ $BDUs_{28}$, $BDUs_{30}$ and $BDUs_{18}$) showed rough colony appearance. This was agreed with the results of Silva *et al.* (2003); Hewedy *et al.* (2014); Kawake

et al. (2014). Colonies of rhizobia were observed on YEMA medium was milky as similar to the results of Hewedy *et al.* (2014) isolated from common bean.

4.3. Biochemical Characterization of the Isolate

Biochemical characteristics indicate that rhizobia isolates were motile, acid producer, and they use urea as a source of nitrogen and 60% of the isolate oxidize DEV-tryptophan. The results of this study showed that 73.33% of the isolates were used citrate as a carbon source (Table 1).

Table 2: Biochemical characteristics of *Rhizobium* isolate

Biochemical character	Result
Gram reaction	Gram negative
Methyl red test	Positive
Motility test	Positive
Acid- Base production test	Acid producer
Citrate test	73.33% positive
Indole test	60% positive
Urease test	Positive

The isolate from grass pea root nodules were tested by Congo red technique to ensure that all isolates were rhizobia and did not contaminate with agrobacterium as similar to Solomon Legesse and Fassil Assefa (2014) done on the isolates of rhizobia from faba bean. Results of this study indicated that rhizobia isolates are not so different biochemically since they are not varied regarding most tests. However, rhizobia isolates were varied in their carbon and nitrogen source. This result was similar to the result of Jordan (1984) that showed rhizobia isolates vary in their carbon and nitrogen source. On the bases of biochemical and morphological characteristics of rhizobia isolates from grass pea was classified under the genus *Rhizobium*. The YEM medium

enriched with BTB selectively identifies the genus *Rhizobium* (Vincent, 1970). The colour change on YEMA-BTB from deep green to yellow and all the biochemical characteristics of rhizobia isolates suggested that all isolates could be under the genus *Rhizobium*. This made an agreement with Ayneabeba Adamu *et al*. (2001); Alemayehu Workalemahu (2006); Antenh Aragaw (2007); Asrat Mekonen (2017); Musa Adal (2009); Getahun Negash (2015).

4.4. Effects of Glyphosate on the *Rhizobium* Isolates

The survival of *Rhizobium* isolate varied at different concentration of glyphosate. However, all isolates of *Rhizobium* from grass pea root nodule were grown on YEM broth containing different concentrations of glyphosate. The highest and lowest percentage of inhibition was obtained at 20ml L^{-1} showed the isolate $BDUs_{27}$ and $BDUs_{7}$ (45.7% and 5.5%) respectively. At the concentration of 40mlL^{-1} $BDUs_{1}$ and $BDUs_{12}$ showed less percentage of inhibition (17.1%) and the isolate $BDUs_{7}$ indicated that 53.38% of inhibition. The survival rate of *Rhizobium* population at the higher concentration (60 mL^{-1}) was 14.6-24.8% and the percentage of inhibition was 75.1%-85.4% respectively. *Rhizobium* isolates from grass pea root nodule was not totally inhibited even at the higher concentration as indicated (Table 1) the OD of glyphosate herbicide treated culture of *Rhizobiam* in YEMB medium.

Table 3: Inhibition effects of glyphosate against *Rhizobium* at different concentration (n=30)

Isolate	Inhibition effects of glyphosate against *Rhizobium* at different concentration						
	Control OD	OD 20ml L^{-1}	PI (%)	OD 40ml L^{-1}	PI (%)	OD 60ml L^{-1}	PI (%)
$BDUs_1$	0.601	0.529	11.98	0.498	**17.13**	0.147	75.54
$BDUs_2$	0.882	0.622	29.47	0.493	44.10	0.144	83.67
$BDUs_3$	0.893	0.716	19.82	0.517	42.10	0.15	83.20
$BDUs_4$	0.943	0.713	24.39	0.484	48.67	0.138	**85.36**
$BDUs_5$	0.802	0.682	14.96	0.504	37.15	0.149	81.42
$BDUs_6$	0.989	0.776	21.53	0.517	47.72	0.157	84.12
$BDUs_7$	1.034	0.977	**5.51**	0.482	**53.38**	0.139	86.55
$BDUs_8$	0.913	0.619	32.20	0.548	39.97	0.166	81.81
$BDUs_9$	0.825	0.562	31.87	0.553	32.96	0.154	81.33
$BDUs_{10}$	0.779	0.693	11.04	0.539	30.80	0.158	79.71
$BDUs_{11}$	0.863	0.538	37.66	0.484	43.91	0.136	84.24
$BDUs_{12}$	0.601	0.529	12.00	0.498	**17.13**	0.147	75.54
$BDUs_{13}$	0.906	0.635	29.91	0.563	37.85	0.174	80.79
$BDUs_{14}$	0.904	0.680	24.77	0.529	41.48	0.143	84.18
$BDUs_{15}$	0.971	0.597	34.89	0.481	47.54	0.146	84.07
$BDUs_{16}$	0.643	0.534	16.95	0.406	36.85	0.146	77.29
$BDUs_{17}$	0.793	0.563	29.00	0.466	41.23	0.136	82.84
$BDUs_{18}$	0.861	0.486	43.55	0.485	43.67	0.138	83.97
$BDUs_{19}$	0.823	0.481	41.55	0.465	43.49	0.137	83.35
$BDUs_{20}$	0.867	0.541	37.60	0.532	38.63	0.168	80.62
$BDUs_{21}$	0.907	0.512	43.55	0.508	43.99	0.169	81.36
$BDUs_{22}$	0.827	0.501	39.41	0.494	40.26	0.157	81.01
$BDUs_{23}$	0.607	0.403	33.60	0.367	39.53	0.151	**75.12**
$BDUs_{24}$	0.793	0.514	35.18	0.507	36.06	0.179	77.42
$BDUs_{25}$	0.871	0.544	37.54	0.540	38.00	0.210	75.88
$BDUs_{26}$	0.835	0.521	37.60	0.518	37.96	0.163	80.47
$BDUs_{27}$	0.861	0.467	**45.76**	0.462	46.34	0.133	84.55
$BDUs_{28}$	0.823	0.495	39.85	0.482	41.43	0.155	81.16
$BDUs_{29}$	0.864	0.544	37.03	0.536	37.96	0.202	76.62
$BDUs_{30}$	0.835	0.493	40.95	0.472	43.47	0.147	82.39

The mean difference between treatments was significant at $p < 0.05$.

Where: OD=optical density, PI= percentage of inhibition, BDUs= Bahir Dar University sample.

This finding indicated that about (5.51-45.75%) of *Rhizobium* isolates was inhibited at the recommended concentration of glyphosate (20ml L^{-1}) and this indicates that the percentage of survival at the specification of the company was 54.25%-94.49%. The result was disagreed with

Moorman (1986) showed that growth of *Rhizobium* was not affected by exposure 20 ml L^{-1} glyphosate. In addition this was contrast to Mallik and Tesfai (1983) showed that glyphosate had no effect on the growth of *Rhizobium* at concentrations up to 0.147mlL^{-1} in YEMB. However, with the initial studies by Jaworski (1972) demonstrated that 69% and 92% *R. japonicum was* inhibited by glyphosate at 0.00497mlL^{-1} and 0.0994mlL^{-1} concentrations respectively. The percentage of survival was decreased with increasing the concentration of glyphosate. About 17.13-53.38% and 7.12-85.36% of *Rhizobium* isolate were inhibited at the concentration 40ml L^{-1} and 60ml L^{-1} liquid glyphosate respectively (Table 1). The percentage survival of *Rhizobium* isolates were 14.64-24.88% at 60mlL^{-1}. This was contrasted to the result of Berhan Aynalem and Fassil Assefa (2017) reported that the percentage survival of *Rhizobium* was 19-22% at the concentration of 5.9×10^{-5}mlL^{-1}. Likewise, the result of this study opposes Zablotowicz and Reddy (2004) reported that *R. japonicum* strains were completely inhibited at 0.497mlL^{-1}. Since, *Rhizobium* culture at (60 mlL^{-1}) after 72hr revives on YEMA medium. The finding indicates that sensitivity of *Rhizobium* isolate for glyphosate was different at the same concentration. This come to an agreement to Dos Santos *et al.* (2005) indicated commercial formulations of glyphosate herbicide were inhibited *Bradyrhizobium* and differential response was observed in same species.

4.5. Viability Test

The viability of isolates was determined by culturing serially diluted suspensions of 72hr old culture onto YEMA plates. The sample was taken from 10^{-8}, 10^{-9} and 10^{-10} dilution from each treatment. The % of CFU was decreased when increasing concentration of glyphosate as compared to glyphosate free control. $BDUs_{30}$ and $BDUs_1$ showed the highest and lowest % of CFU under laboratory at the lowest recommended concentration of glyphosate. At the concentration 40mlL^{-1} the lowest % of CFU showed BDU_{20} (6.6%). The number of colony forming unit was

depends on the concentration of glyphosate. The minimum % of CFU (BDU_{14} and BDU_{20}) and maximum % of CFU (BDU30) was 2 and 47.39% at higher concentration of 60mlL^{-1} respectively (Table 2).

Table 4: Viability of *Rhizobium* after exposure to glyphosate (n=30)

Isolate	Control CFU	CFU 20ml L^{-1}	%of CFU	CFU 40ml L^{-1}	%of CFU	CFU 60ml L^{-1}	%of CFU
$BDUs_1$	169.72	49.72	**29.29**	20.60	12.14	5.16	3.04
$BDUs_2$	259.99	188.27	72.41	140.16	54.08	88.66	34.10
$BDUs_3$	290.26	174.52	60.13	148.61	51.20	86.61	29.84
$BDUs_4$	322.88	179.82	55.69	142.16	44.03	81.60	25.27
$BDUs_5$	499.83	313.49	62.72	190.60	38.13	113.71	22.75
$BDUs_6$	431.44	221.88	51.42	137.27	31.81	99.88	23.15
$BDUs_7$	530.89	374.22	70.48	219.94	41.42	73.44	13.83
$BDUs_8$	386.75	163.55	42.28	123.21	31.85	62.55	16.17
$BDUs_9$	291.77	230.82	79.11	168.05	57.59	100.83	34.55
$BDUs_{10}$	223.71	186.44	83.33	120.77	53.98	55.88	24.98
$BDUs_{11}$	296.88	241.05	81.19	68.99	23.23	22.61	17.61
$BDUs_{12}$	79.27	43.33	54.66	27.60	34.82	11.27	14.22
$BDUs_{13}$	154.83	109.77	70.89	47.77	30.85	5.05	3.26
$BDUs_{14}$	249.71	80.21	53.57	20.93	8.38	4.74	**2.00**
$BDUs_{15}$	459.22	282.45	61.50	229.49	49.47	151.16	32.91
$BDUs_{16}$	174.55	124.22	71.16	66.80	38.28	21.21	12.15
$BDUs_{17}$	200.78	105.27	52.42	39.22	19.53	12.10	6.02
$BDUs_{18}$	287.61	103.88	36.11	65.44	22.75	25.27	8.78
$BDUs_{19}$	311.27	137.05	44.02	75.94	24.39	33.32	10.70
$BDUs_{20}$	249.21	104.11	41.77	16.44	**6.59**	4.77	**2.00**
$BDUs_{21}$	359.38	170.72	47.50	66.27	18.43	9.94	2.76
$BDUs_{22}$	303.99	99.61	32.76	75.43	24.81	30.60	10.06
$BDUs_{23}$	169.28	137.66	81.32	51.61	30.48	23.77	14.04
$BDUs_{24}$	229.44	145.27	63.31	94.66	41.25	51.10	22.27
$BDUs_{25}$	244.99	117.72	48.04	70.05	28.59	38.38	15.66
$BDUs_{26}$	290.05	129.83	44.76	117.66	40.56	66.33	22.86
$BDUs_{27}$	290.99	212.94	73.17	139.55	47.95	66.38	22.81
$BDUs_{28}$	357.49	183.72	51.39	127.49	35.66	82.05	22.95
$BDUs_{29}$	235.16	153.38	65.22	115.32	49.04	64.04	27.23
$BDUs_{30}$	279.38	259.11	**92.74**	193.16	**69.13**	118.49	**47.39**

The mean difference between each treatment was significant at $p < 0.05$.

Where: - CFU = colony forming unit, BDUs= Bahir Dar University sample.

Rhizobium isolate treated with different concentration of glyphosate herbicide was cultured on YEMA to check their viability. The growth of treated *Rhizobium* culture on YEMA medium decreased with increasing the concentration of glyphosate. At the recommended concentration of liquid glyphosate (20 ml L^{-1}) 60% of the isolate grew 51-83 % CFU and 40% of the isolate was 29-48% CFU growth at the same concentration. In the medium containing 20mlL^{-1} the maximum % of CFU 83.33% and the minimum % of CFU was 29.29%. Whereas, at the concentration of 40ml L^{-1} of glyphosate 56.66 % of the isolate grew 30-57% and 43.33% of the isolate grew 6-28% of CFU as compared to the glyphosate free control. Maximum and minimum % of CFU at 40mlL^{-1} was 57.59 and 6.59 respectively. In addition, 76.66% CFU at 60mlL^{-1} grew 10-47.39% and 23.33% of the isolate was growing 2-8% of CFU. The lowest % colony at 60ml L^{-1} was 2% and the maximum in the same concentration was 34% of colony as compared to the control. Moreover, the % of CFU was reduced with concentration increase, but it was not totally eliminated up to the higher concentration of liquid glyphosate (60ml L^{-1}). The result of this study was disagreed with the results of Berhan Aynalem and Fassil Assefa (2007) where no growth of bacteria after the exposure at 5.9×10^{-5}mlL^{-1} and 2.2×10^{-5} mlL^{-1} concentration of liquid glyphosate. In addition, this result was diferent from Zablotowicz and Reddy (2004) that showed there is complete elimination of *Rhizobium* threated with 0.497 mlL^{-1} glyphosate.

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

The low and high pH affected the growth and survival of *Rhizobium* isolate and pH in the range 5-8 was the best growing condition for the isolate. *Rhizobium* isolates best grew at the temperature between 20-30℃ and some isolated survive up to 50℃. However, all isolate were not grow below10℃. High salt concentration (4-7%) affected the growth of *Rhizobium* isolate and they can grow at 0.5 % and 1% NaCl. Glyphosate reduce *Rhizobium* population in laboratory condition and the effect was varied between isolates of *Rhizobium* from grass pea root nodule. High concentration of this herbicide concentration is hazardous and causes a reduction of *Rhizobium* population. Even at the recommended concentration *Rhizobium* isolates were inhibited. The inhibitory effects of glyphosate were dependent on the concentration of herbicide and the number of CFU after the exposure was decreased when the concentration increased. Due to this considering the considerable behavior of glyphosate herbicide is very important. Since, it used all over the world in the agricultural process as a pre-treatment of weeds. Based on the result of the study can be concluded that glyphosate herbicide can reduce nitrogen-fixing *Rhizobium* population from root nodules of grass pea at laboratory condition and the effects were greatly depends on its concentration.

5.2. Recommendations

The isolates of *Rhizobium* from grass pea root nodule showed reduction of population treated with glyphosate at the recommended level and the effect was respect to concentration. This is the evident that accumulations of high glyphosate herbicide are more destructive to beneficial

Rhizobium population. Farmers used an eco-friendly and safe physical control of weeds through making association in groups.

In addition to this any interested group for this area were carry out research on the effects of glyphosate herbicides using the data to formulate alternative use which are safe for *Rhizobium* population.

Furthermore, Future research needs for identification of resistant strain from grass pea and used as microbial inoculum by evaluating *Rhizobium* responses to glyphosate herbicide applications at different concentration in greenhouse and filed condition.

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APPENDEX

Table 1 Morphological and biochemical characteristics of *Rhizobium*

Isolate	Colony diameter(mm)	Colony Texture	Indole test	Citrate test
$BDUs_1$	2.5	Smooth	+	-
$BDUs_2$	3.0	Smooth	+	+
$BDUs_3$	4.5	Rough	-	-
$BDUs_4$	3.5	Smooth	-	+
$BDUs_5$	2.0	Smooth	+	+
$BDUs_6$	2.0	Smooth	+	+
$BDUs_7$	2.5	Smooth	-	+
$BDUs_8$	3.0	Smooth	+	-
$BDUs_9$	4.0	Smooth	+	+
$BDUs_{10}$	2.6	Smooth	-	+
$BDUs_{11}$	3.2	Smooth	-	-
$BDUs_{12}$	4.1	Smooth	-	+
$BDUs_{13}$	3.5	Rough	+	-
$BDUs_{14}$	2.8	Smooth	+	+
$BDUs_{15}$	2.6	Smooth	+	+
$BDUs_{16}$	2.0	Smooth	+	+
$BDUs_{17}$	4.0	Smooth	+	+
$BDUs_{18}$	3.7	Rough	-	+
$BDUs_{19}$	2.0	Smooth	+	+
$BDUs_{20}$	4.0	Smooth	+	+
$BDUs_{21}$	4.2	Smooth	-	+
$BDUs_{22}$	4.1	Smooth	+	+
$BDUs_{23}$	4.5	Smooth	+	+
$BDUs_{24}$	3.3	Smooth	+	+
$BDUs_{25}$	2.6	Smooth	+	+
$BDUs_{26}$	4.2	Smooth	+	+
$BDUs_{27}$	2.9	Smooth	-	+
$BDUs_{28}$	3.4	Rough	-	-
$BDUs_{29}$	4.3	Smooth	+	-
$BDUs_{30}$	3.9	Rough	-	-

Key: (+): positive result; (-): negative result, (%G): % of positive result

Table 2 Physiological characterization of Rhizobium isolate

Isolate	Medium in different PH						
	4	5	6	7	8	9	10
$BDUs_1$	-	+	+	+	-	-	-
$BDUs_2$	-	+	+	+	+	+	-
$BDUs_3$	-	+	+	+	+	+	+
$BDUs_4$	-	+	+	+	+	+	-
$BDUs_5$	-	+	+	+	+	+	+
$BDUs_6$	-	+	+	+	+	+	+
$BDUs_7$	-	+	+	+	+	+	+
$BDUs_8$	+	+	+	+	+	+	+
$BDUs_9$	-	+	+	+	+	-	-
$BDUs_{10}$	-	+	+	+	+	+	+
$BDUs_{11}$	+	+	+	+	+	-	-
$BDUs_{12}$	-	+	+	+	+	-	-
$BDUs_{13}$	-	+	+	+	+	+	+
$BDUs_{14}$	-	+	+	+	+	-	-
$BDUs_{15}$	+	+	+	+	+	+	+
$BDUs_{16}$	-	+	+	+	+	+	+
$BDUs_{17}$	-	+	+	+	+	+	+
$BDUs_{18}$	+	+	+	+	+	+	+
$BDUs_{19}$	-	+	+	+	+	+	+
$BDUs_{20}$	+	+	+	+	+	+	+
$BDUs_{21}$	+	+	+	+	+	+	+
$BDUs_{22}$	-	+	+	+	+	+	+
$BDUs_{23}$	+	+	+	+	+	+	+
$BDUs_{24}$	+	+	+	+	+	-	-
$BDUs_{25}$	-	+	+	+	+	+	+
$BDUs_{26}$	-	+	+	+	+	-	-
$BDUs_{27}$	-	+	+	+	+	+	+
$BDUs_{28}$	-	+	+	+	+	+	+
$BDUs_{29}$	+	+	+	+	+	-	-
$BDUs_{30}$	-	+	+	+	+	+	-

(+) = presence of growth (-) = absence of growth

Table 3 Temperature tolerance of the isolate

Isolate	different temperature value							
	(4^oc)	(10^oc)	(15^oc)	(20^oc)	(28^oc)	(38^oc)	(45^oc)	(50^oc)
$BDUs_1$	-	-	+	+	+	+	+	-
$BDUs_2$	-	-	+	+	+	+	+	+
$BDUs_3$	-	-	-	+	+	+	-	-
$BDUs_4$	-	-	-	+	+	+	+	+
$BDUs_5$	-	-	+	+	+	+	-	-
$BDUs_6$	-	-	+	+	+	+	+	-
$BDUs_7$	-	-	-	+	+	+	-	-
$BDUs_8$	-	-	+	+	+	+	-	-
$BDUs_9$	-	-	+	+	+	+	+	+
$BDUs_{10}$	-	-	-	+	+	+	+	+
$BDUs_{11}$	-	-	-	+	+	+	+	-
$BDUs_{12}$	-	-	-	+	+	+	+	-
$BDUs_{13}$	-	-	+	+	+	+	+	+
$BDUs_{14}$	-	-	+	+	+	+	+	-
$BDUs_{15}$	-	-	+	+	+	+	+	+
$BDUs_{16}$	-	-	+	+	+	+	+	-
$BDUs_{17}$	-	-	+	+	+	+	+	+
$BDUs_{18}$	-	-	-	+	+	+	+	+
$BDUs_{19}$	-	-	+	+	+	+	+	+
$BDUs_{20}$	-	-	+	+	+	+	+	-
$BDUs_{21}$	-	-	-	+	+	+	+	+
$BDUs_{22}$	-	-	+	+	+	+	+	+
$BDUs_{23}$	-	-	+	+	+	+	+	-
$BDUs_{24}$	-	-	+	+	+	+	+	+
$BDUs_{25}$	-	-	+	+	+	+	+	-
$BDUs_{26}$	-	-	+	+	+	+	+	+
$BDUs_{27}$	-	-	-	+	+	+	-	-
$BDUs_{28}$	-	-	-	+	+	+	+	-
$BDUs_{29}$	-	-	+	+	+	+	+	+
$BDUs_{30}$	-	-	+	+	+	+	+	+

(+) = presence of growth (-) = absence of growth

Table 4 Salt tolerance of the isolate

Isolate	Growth of *Rhizobium* in the medium containing salt in different concentration (%)							
	0.5	1	2	3	4	5	6	7
$BDUs_1$	+	+	+	+	-	-	-	-
$BDUs_2$	+	+	+	+	+	+	+	-
$BDUs_3$	+	+	+	+	-	-	-	-
$BDUs_4$	+	+	-	-	-	-	-	-
$BDUs_5$	+	+	+	+	+	+	+	+
$BDUs_6$	+	+	+	-	-	-	-	-
$BDUs_7$	+	-	-	-	-	-	-	-
$BDUs_8$	+	+	+	+	+	+	+	+
$BDUs_9$	+	+	+	+	+	+	+	+
$BDUs_{10}$	+	+	-	-	-	-	-	-
$BDUs_{11}$	+	+	-	-	-	-	-	-
$BDUs_{12}$	+	-	-	-	-	-	-	-
$BDUs_{13}$	+	+	+	+	+	+	+	+
$BDUs_{14}$	+	+	+	+	-	-	-	-
$BDUs_{15}$	+	+	+	+	-	-	-	-
$BDUs_{16}$	+	+	+	+	+	+	-	-
$BDUs_{17}$	+	+	+	+	+	+	-	-
$BDUs_{18}$	+	+	-	-	-	-	-	-
$BDUs_{19}$	+	+	+	+	-	-	-	-
$BDUs_{20}$	+	+	+	+	-	-	-	-
$BDUs_{21}$	+	+	-	-	-	-	-	-
$BDUs_{22}$	+	+	+	+	+	-	-	-
$BDUs_{23}$	+	+	+	+	+	-	-	-
$BDUs_{24}$	+	+	+	+	+	-	-	-
$BDUs_{25}$	+	+	+	+	-	-	-	-
$BDUs_{26}$	+	+	+	-	-	-	-	-
$BDUs_{27}$	+	+	-	-	-	-	-	-
$BDUs_{28}$	+	-	-	-	-	-	-	-
$BDUs_{29}$	+	+	+	+	-	-	-	-
$BDUs_{30}$	+	+	+	-	-	-	-	-

(+) = presence of growth (-) = absence of growth

ANOVA

optical density

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	7.119	3	2.373	361.708	.000
Within Groups	.761	116	.007		
Total	7.881	119			

Multiple Comparisons

Dependent Variable: optical density measured after glyphosate treatment of each concentration

Tukey HSD

(I) concentration	(J) concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control OD	OD at 20ml L^{-1}	.253167*	.020914	.000	.19865	.30768
	OD at 40ml L^{-1}	.337733*	.020914	.000	.28322	.39225
	OD at 60ml L^{-1}	.680767*	.020914	.000	.62625	.73528
OD at 20ml L^{-1}	Control OD	-.253167*	.020914	.000	-.30768	-.19865
	OD at 40ml L^{-1}	.084567*	.020914	.001	.03005	.13908
	OD at 60ml L^{-1}	.427600*	.020914	.000	.37308	.48212
OD at 40ml L^{-1}	Control OD	-.337733*	.020914	.000	-.39225	-.28322
	OD at 20ml L^{-1}	-.084567*	.020914	.001	-.13908	-.03005
	OD at 60ml L^{-1}	.343033*	.020914	.000	.28852	.39755
OD at 60ml L^{-1}	Control OD	-.680767*	.020914	.000	-.73528	-.62625
	OD at 20ml L^{-1}	-.427600*	.020914	.000	-.48212	-.37308
	OD at 40ml L^{-1}	-.343033*	.020914	.000	-.39755	-.28852

The mean difference is significant at the 0.05 level.

ANOVA

Number of colony after the exposure of glyphosate

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	918237.208	3	306079.069	57.505	.000
Within Groups	617427.319	116	5322.649		
Total	1535664.527	119			

Multiple Comparisons

Dependent Variable: number of colony after exposure of Glyphosate

Tukey HSD

(I) concentration	(J) concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control CFU	CFU at 20mlL^{-1}	120.2240833*	18.8372845	.000	71.121594	169.326573
	CFU at 40mlL^{-1}	183.6486500*	18.8372845	.000	134.546161	232.751139
	CFU at 60mlL^{-1}	233.9900500*	18.8372845	.000	184.887561	283.092539
CFU at 20mlL^{-1}	Control CFU	-120.2240833*	18.8372845	.000	-169.326573	-71.121594
	CFU at 40mlL^{-1}	63.4245667*	18.8372845	.006	14.322077	112.527056
	CFU at 60mlL^{-1}	113.7659667*	18.8372845	.000	64.663477	162.868456
CFU at 400mlL^{-1}	Control CFU	-183.6486500*	18.8372845	.000	-232.751139	-134.546161
	CFU at 20mlL^{-1}	-63.4245667*	18.8372845	.006	-112.527056	-14.322077
	CFU at 60mlL^{-1}	50.3414000*	18.8372845	.042	1.238911	99.443889
CFU at 60mlL^{-1}	Control CFU	-233.9900500*	18.8372845	.000	-283.092539	-184.887561
	CFU at 20mlL^{-1}	-113.7659667*	18.8372845	.000	-162.868456	-64.663477
	CFU at 40mlL^{-1}	-50.3414000*	18.8372845	.042	-99.443889	-1.238911

The mean difference is significant at the 0.05 level.

Figure: 5 microscopic observation of *Rhizobium* isolate

Figure: 2 measure of bacterial growth through spectrophotometer

Figure: 3 colony counting after exposure of glyphosate herbicide

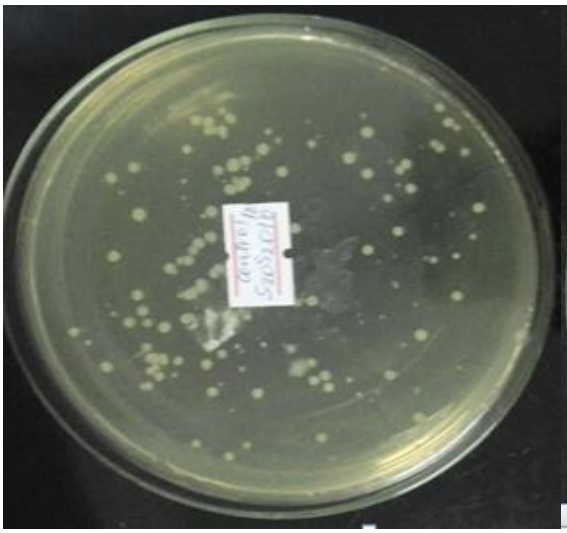

Figure 4 control (no herbicide added)

Figure 5 CFU at 60mlL-1 glyphosate concentration