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Competitiveness and symbiotic effectiveness of rhizobial inoculants on field pea (*Pisum sativum*) under greenhouse and field conditions

By:

Asrat Mekonnen

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Advisor

Dr Fassil Assefa (PhD)

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TABLE OF CONTENTS

LIST OF TABLE **PAGE**

LIST OF FIGURES

LIST OF APPENDEX

LIST OF ACRONYMS AND ABBREVIATIONS

BC	Before calendar
BNF	Biological Nitrogen Fixation
BTB	Bromo Thymol Blue
CEC	Cation Exchange Capacity
CR	Congo Red
DM	Dry Matter
E	East
EEPA	Ethiopian Export Promotion Agency
IBC	Institute of Biodiversity Conservation
KARC	Kulumsa Agricultural Research Center
Kgh^{-1}	Kilogram per hectar
MPN	Most probable number
Ndfa	Nitrogen derived from atmosphere
nif	nitrogen fixing
nm	nano metere
OC	Organic Carbon
RCBD	Randomized complete block design
SAS	Statistical Analysis Software
YEMA	Yeast Extract Manitol Agar
YEMB	Yeast extracts mannitol broth

ABSTRACT

*Field pea (*Pisum sativum*) is widely cultivated in Ethiopia as a source of protein and integrated in crop rotation to improve soil fertility for it fixes nitrogen with rhizobia. However, effectiveness in nitrogen fixation depends upon the selection of symbiotically effective rhizobia to enhance production. Thus, a study was conducted to evaluate the performance of three inoculants; FBR 11, FBR 15, FBR 23 on growth, nitrogen fixation and yield under greenhouse and field conditions in relation to a commercial rhizobial strain 1018 at Kulumsa Agricultural Research Center during 2015/16 growing seasons. Standard physiological and biochemical tests were conducted to look into the nodule occupancy (competitiveness) of the three inoculants against the local rhizobia in the soil. The study showed that Isolate FBR 15 was the most effective inoculants with nodule occupancy of 75%, and were highly effective under greenhouse conditions, whereas the other inoculants were effective and occupied 50-60% of the nodules of the inoculated plants. Inoculation of field pea showed a highly significant ($p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$) effect on all parameters compared to the un-inoculated plants in the field trial. Accordingly, plants inoculated with isolate FBR15 and Strain 1018 showed a significant increase in nodule number (84-112NN/plant (10 times), NDW (77-94mg/plant) (12 times) against the un-inoculated control plants, and they also showed a 20-25% increase in both parameters in comparison to FBR11 and FBR23 inoculated plants. The treatments with FBR15 and the reference strain 1018 also showed significant difference in grain total nitrogen and N uptake and straw N uptake, and seed protein with 50-100% and 12-20% difference from the un-inoculated control plants and other Rhizobium treatments, respectively. Positive correlations were observed with respect to the number of nodule and shoot dry weight ($r = 0.49$, $p \leq 0.05$), number of nodule and number of pod ($r = 0.59$, $p \leq 0.01$), number of nodules and total grain yield ($r = 0.56$, $p \leq 0.05$), and shoot dry weight and N content ($r = 0.73$, $p \leq 0.001$). Although The MPN count of rhizobia in the soil at the experimental site was 1.5×10^4 and contained sufficient number of indigenous rhizobia, they were not effective that was indicated by the nodulation and yield parameters of the un-inoculated treatments. The data in general, showed that the rhizobial inoculants, particularly FBR 15 was nutritionally versatile, ecologically competent, and symbiotically effective rhizobia compared to the commercial inoculant (strain 1018) that could be used as commercial inoculants for pea production after it is tested (validated) at different agro-ecological conditions.*

Key words: Biological yield**,** Grain yield, inoculation, nodule occupancy, N uptake.

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1. INTRODUCTION

Field pea (*Pisum sativum*) is one of the cool-season leguminous crops widely cultivated in Ethiopia at altitudes between 1800 and 3000 meters above sea level with annual average rainfall of 700-900 mm in the different regions of Oromia, Amhara, Tigray and Southern Ethiopia (EEPA, 2004). It is the second most important leguminous crops grown in the country after faba bean in terms of both area coverage and production. Field pea covers over 254,000 hectares with total production of 230,000 tons that accounts to 17% of the total grain legume production (IBC, 2008). It represents a useful complement to cereal-based diets as a relatively inexpensive source of high quality protein. It contains 21-25% protein, 33-50% starch and amino acid (Lazanyi, 2002). Consequently, it is an important pulse in the daily diet of the society in urban and rural areas. It is eaten whole, spilt or milled usually fresh, fried, boiled or mixed with other cereals to make various types of stews and soups (EEPA, 2004).

Field pea is integrated in different crop systems as a sole crop or an intercrop in crop rotation for its capability to fix nitrogen in symbiotic association with root nodule bacteria known as *Rhizobium leguminosarum biovar viciae* to the tune of 200-300 kg/ha/yr. Several studies have shown that field pea fulfills more than 80% of its nitrogen requirements through BNF and can subsequently transfers nitrogen to non-fixing plants in the agricultural system (Murat *et al*, 2008).

In Bangladesh *Rhizobium* inoculation has been reported to supplement up to 80 kg N ha^{-1} and increase the average yield of pea plants over uninoculated plants (Ahmed *et al*., 2007). *Rhizobium* inoculation positively affects plant height, the number of branches, root and shoot dry weight, the number of nodules, seed and biomass yield, the number of pods, the crude protein concentration, and seed P content (Murat *et al.,* 2009). In India, inoculation of pea with *Rhizobium leguminosarum* at 20 g kg^{-1} seed increased nodulation and activated the nitrogenase enzyme in the root nodules to fix more atmospheric N.

For many years various studies have been carried out nation-wide to improve field pea cultivars in Ethiopia, (Amare and Adamu, 1994). Many of these studies were restricted to soil plant nutrition and fertilizer trials in different agricultural research institutes (Tekalign and Asgelil, 1994). Recently few studies on taxonomic and symbiotic properties were undertaken on field pea rhizobia by collecting

root nodules from different parts of Ethiopia (Kassa *et al.*, 2015). Most of the studies were limited to laboratory and greenhouse based experiments and hence, further research must need to be worked on selected rhzobial strains under field conditions.

Although green house screening of isolates is a preliminary work for symbiotic effectiveness of rhizobia, field trials are essential to assess their adaptive capability to field conditions and their competitiveness against the most recalcitrant, but often ineffective indigenous rhizobia in the soil (Theis *et al.,* 1991; Evans *et al*., 1996). This necessitates a field trial in order to assess their field performance and select them for inoculant production to be used as biofertilizers.

1.1 General objective

- To evaluate the effect of symbiotically effective *Rhizobium leguminosarum* isolates on their competitiveness, nitrogen fixation and yield of field pea under green house and field conditions.

1.2 Specific objective

- To re-isolate and characterize the rhizobia from nodules in order to estimate nodule occupancy of the inoculated isolates in relation to the indigenous endosymbionts based on their physiological characters (markers).
- To re-evaluate the competitiveness and symbiotic properties of the selected rhizobia under greenhouse conditions.
- To determine the effect of rhizobial inoculants on different nodulation and symbio-agronomic characters of field pea plant under field conditions.

2. LITERATURE REVIEW

2.1. Legumes

Legumes (*Fabaceae*) are the most diverse and vast families of the flowering plants. They belong to the family *leguminoseae* which is classified into three major botanical subfamilies; *Ceasalpinioideae*, *Mimosoideae*, and *Papilionoidae* which contains the majority of the most important legumes (Subba Rao, 1999). There are nearly 750 genera and 16,000-19,000 species of leguminous plants (Giller, 2001). About 20% of the leguminous species has been so far examined for nodulation; of which 23% of sub family *Ceasalpinioideae*, 90% of *Mimosoideae* and the 97% of *Papilionoidae* are known for their ability to form N fixing root nodules with soil bacteria. Legumes such as soybean (*Glycine max* L.), field pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.), common bean (*Phaseolus vulgaris* L.), lentil (*Lens culinaris* L.) and chickpea (*Cicer arietinum* L.) form symbiotic association with rhizobia and variation have been reported in the amount of fixed N (Unkovich and Pate, 2000).

Legumes are an essential part of African farming systems, covering usually large parts of the farmlands in the region. For example, in the years 2006-2008 in Sub- Saharan Africa more than 20 million hectares of the farmlands (which represent about 28% of the global pulse areas) were used for food legume crop production (Akibode, 2011). Ethiopia has a long tradition of cultivation of food legumes and the country is considered as a center of genetic diversity for several cool season legume crops, such as field pea (*Pisum sativum*), faba bean (*Vicia faba*), lentil (*Lens culinaris*), chickpea (*Cicer arietinum* L.), fenugreek (*Trigonella foenum-graecum* L.) and grass pea (*Lathyrus sativus*) (Tilaye *et al*., 1994). These food legumes are extensively grown in the cooler highlands of the country, whereas the warm season pulse crops, such as common bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*) and groundnut (*Arachis hypogaea*) are common in warmer and lowland parts of Ethiopia.

They occupy 12-15% of the earth's arable land and account for a third of human dietary protein needs and for up to 2/3 of subsistence livelihood (Graham and Vance, 2003). Legume plants show a vast diversity in morphology, habitat and ecology. However, all legume species can be distinguished from non-leguminous plants as they produce pods as a common feature. With regard to economic and agricultural significance the family Leguminosae is second next to Poaceae (grasses). Legumes are

grown for production of food and oil and for fiber, fuel, timber, medicines, forages, biodiesel fuel, and chemicals.

2.2. Field Pea *(Pisum sativum)*

The Field Pea (*Pisum sativum L.),* which belongs to the family *Papilionaceae* (*Leguminosae*), has been traced to agriculture 7000-6000 B.C. and is now grown world-wide. However, pea is largely confined to temperate regions and the higher altitudes or cooler seasons of warmer regions (Steen, 1986). In Ethiopia Field Pea (*Pisum sativum)* is a highly consumed pulse in the daily diet of the society in urban and rural areas. It is eaten whole, spilt or milled usually fresh, fried, boiled or mixed with other cereals to make various types of stews and soups (EEPA, 2004). It represents a useful complement to cereal-based diets as a relatively inexpensive source of high quality protein. It contains 21-25% protein, 33-50% starch and amino acids, but it is limiting in fat, Tryptophan and in the Sulfur-contain amino acids, Methionine and Cysteine (Lazanyi, 2002).

Field Pea like other legumes is capable of fixing and utilizing atmospheric nitrogen through symbiotic relationship with *Rhizobium* bacteria at the root of the crop. *Rhizobium* inoculants significantly improves yield in many leguminous crops and can minimize the use of synthetic nitrogenous fertilizer, which is rather expensive and causes injury to soil properties (Lazanyi, 2002). This crop thus improves soil and economizes crop production not only for itself but also for the next cereals (non-legume crops) grown in the relation and thereby reducing the requirement of added nitrogen fertilizers (Newton *et al.*, 2006). Field Peas are used in crop rotation for improvement of soil fertility and yield of the succeeding crops. In crop rotation tests, spring wheat and durum wheat grown on pea stubble produced higher yields and a higher protein percentage compared to wheat grown on wheat stubble (Lazanyi, 2002).

2.3. Rhizobia

Rhizobia are a genetically diverse and physiologically heterogeneous group of bacteria (Somasegaran and Hoben, 1994) and they are able to bring out nodule formation on legumes are called rhizobia. Rhizobia are a ubiquitous part of the soil micro-flora in a free-living state in the rhizosphere of legumes until the point where nodulation becomes possible. Rhizobia are bacteria that selectively infect the roots of some legumes and have the following characteristics; gram negative, motile rod-

shaped (approximately 0.5-0.9 µm in width and 1.2-3.0 µm in length) and heterotrophic (Somasegaran and Hoben, 1994). Root nodule bacteria generally grow under the following conditions 25-30°C (optimum) in the pH range of 6-7. *Rhizobium* growth normally occurs under aerobic conditions. When fixing nitrogen, low levels of oxygen are required to protect the enzyme nitrogenase and hence, *Rhizobium* is able to grow in microaerophilic conditions (Somasegaran and Hoben, 1994).

Rhizobia are of great importance for nitrogen acquisition through symbiotic nitrogen fixation in a wide variety of leguminous plants. These bacteria differ from most of other soil microorganisms by taking dual forms, i.e., a free-living form in soils and a symbiotic form inside of host legumes. Therefore, they should have a versatile strategy for survival, whether inhabiting soils or root nodules formed through rhizobia-legume interactions.

2.4. Symbiotic association of legumes with rhizobia

The term symbiosis refers "the living together of differently named organisms". The word was used as synonymous to mutualism, in which both interacting organisms live together for mutual benefit in contrast to commensalism, where one organism benefits and the other partner is unaffected or parasitism, in cases when one of the interacting organism profits at the expense of the other partner (Martin and Schwab, 2013). The confusion, disagreements and turbulence of the use of the term "living together" have been continued among the biologists for more than 130 years (Martin and Schwab, 2013). Nevertheless, the "living together" usage has been accepted as a suitable concept to define the term "symbiosis" and recently the broader definition of symbiosis (i.e. mutualism, commensalism, and parasitism) is increasingly used in the textbooks (Martin and Schwab, 2013).

The nitrogen-fixing bacteria that form symbiotic associations with leguminous plants are commonly known as rhizobia. The rhizobia-legume symbiotic interaction induces specialized organs known as nodules on roots or stems of host legumes. Inside nodules rhizobia reduce atmospheric N_2 to NH_3. The symbioses between rhizobia and legume plants are mainly a mutualistic interaction (Lindström and Mousavi, 2010; Saffo, 2001). However, it seems that there are cases where these partnerships can also be considered as parasitic. The rhizobia can have two lifestyles: as endo-symbiont inside nodules or as free-living saprophytes in the soil or rhizosphere. As endo-symbiont, the rhizobia promote

growth of the host plant by supplying fixed nitrogen and in turn rhizobia get carbohydrates and energy from the host legumes. This phenomenon indicates the existence of mutualism between the two organisms. But the rhizobia can also be considered as parasitic when they form ineffective symbiosis with legumes, in which the rhizobia get a continuous nutrient supply while they fix little or do not fix nitrogen for the host plant. These types of situations may occur when multiple rhizobial strains compete for the same plant and when the strains infect non-specific hosts promiscuously. These rhizobia can also form effective symbioses (mutualistic) when they interact with their own specific host legumes (Denison and Kiers, 2004).

2.5. Nitrogen Fixation

Nitrogen (N) fixation is the process whereby legume crops and specific *Rhizobium* bacteria (rhizobia) work together to make nitrogen from the soil air surrounding the roots available for use by the plant. Soon after the crop germinates, rhizobia enter the root hairs (Long, 1996). Once inside, the bacteria penetrate further into the root through an infection thread. The rhizobia rapidly multiply within the root and the plant responds by forming specialized structures called nodules, in which the rhizobia are contained. The process of root infection and nodule formation is referred to as "nodulation." It may take three to four weeks after seed germination before nodulation is evident on the plant roots.

Air held in small pores in the soil contains approximately 78 percent nitrogen in a gaseous form (N_2). In this form, the nitrogen is not available for plant use. However, the *Rhizobium* bacteria in the root nodules can "fix" this form of nitrogen gas by binding it with hydrogen and forming ammonium (NH_4), which is available for use by plants. The nitrogen fixation process requires a considerable amount of energy which is provided by the plant. The plant also provides nutrients and water to the rhizobia in the nodules and, in return, the rhizobia provide fixed nitrogen to the plant (Vance, 2002). The amount of nitrogen fixed varies with the type of crop, crop health, the supply of nitrogen already available in the soil, and other environmental conditions. Under ideal conditions, pulse crops can fix as much as 50-80 percent of their total nitrogen requirement, with the remaining nitrogen coming from soil or fertilizer sources. Pulse crops can be ranked according to their estimated ability to fix nitrogen: faba bean > soybean > pea > chickling vetch > chickpea > lentil > lupin> dry bean (Vance, 2002).

Symbiotic nitrogen fixation is a process carried out by root nodule bacteria in association with legumes. The fixation of nitrogen provides the plant with available ammonium. The rhizobia-legume symbiotic interaction induces specialized organs known as nodules on roots or stems of host legumes. Inside nodules rhizobia reduce atmospheric N_2 to NH_3. The symbioses between rhizobia and legume plants are mainly a mutualistic interaction. The rhizobia can have two lifestyles: as endo-symbiont inside nodules or as free-living saprophytes in the soil or rhizosphere. As endo-symbiont, the rhizobia promote growth of the host plant by supplying fixed nitrogen and in turn rhizobia get carbohydrates and energy from the host legumes.

This phenomenon indicates the existence of mutualism between the two organisms. But the rhizobia can also be considered as parasitic when they form ineffective symbiosis with legumes, in which the rhizobia get a continuous nutrient supply while they fix little or do not fix nitrogen for the host plant. These types of situations may occur when multiple rhizobial strains compete for the same plant and when the strains infect non-specific hosts promiscuously. These rhizobia can also form effective symbioses (mutualistic) when they interact with their own specific host legumes (Denison and Kiers, 2004).

2.6. Biological Nitrogen Fixation

Biological nitrogen fixation (BNF) is a process by which N_2 in the atmosphere is reduced into a biologically useful, combined form of N-ammonia by living organisms (Giller, 2001). It is estimated that endosymbiotic biological nitrogen fixation globally represents approximately 90% of all the fixed nitrogen in the terrestrial environment. Chemically fixed in the form of N-fertilizer contributes an estimated amount of 140 tons of additional fixed nitrogen each year which is mainly used for agricultural fertilizers (Gage, 2004). The greatest proportion of N found on the earth is located in the atmosphere, as N_2. Nevertheless, the majority of organisms cannot utilize this free and abundant, but highly stable source of N because they can only use N which is combined with other atoms into plant usable forms, such as ammonium, nitrates and ammonia (Giller, 2001). The process of making N_2 available constitutes a specialized and intricately evolved interaction of soil microbe's bacteria (rhizobia) and higher plants and legumes via the formation of nodules (Sessitsch *et al.,* 2002). Nodules are formed on roots or, in some cases, on stems (Tamimi and Timko, 2003).

Biological nitrogen fixation is mediated by the nitrogenase enzyme system that catalyses the ATP dependent reduction of atmospheric dinitrogen to ammonia. Nitrogenase consists of two component metalloproteins, the MoFe-protein with the FeMo-cofactor that provides the active site for substrate reduction, and the Fe-protein that couples ATP hydrolysis to electron transfer (Rees *et al.,* 2005). It is a process that changes inert N_2 to biologically useful NH_3. This process is mediated in nature only by bacteria. Other plants benefit from nitrogen-fixing bacteria when the bacteria die and release nitrogen to the environment or when the bacteria live in close association with the plant (Lindemann and Gloves*,* 2003). Legume BNF involves a symbiosis between legume plants and the rhizobia that live in nodules on their roots.

2.7. Enumeration of Rhizoidal Population in the soil

It has been estimated that a gram of soil may contain a community of 10^9 microorganisms, with rhizobia representing around 0.1% of the soil microorganisms or 10^6 rhizobia per gram of soil (Thies *et al.,* 1991). These figures may vary depending on the presence of leguminous host plants or poorly defined soil conditions that require further investigation. The number of a particular rhizobial species is usually greater in soils where the host is present or has been recently grown, probably due to the release of bacteria from the senescent nodules; in the absence of the host, numbers are usually low. However, in most cases the natural rhizobial population in the soil is too low or the strain of the rhizobial species is not effective. Different studies have shown that pea shows problem in nodulation even in the soils with rich *Rhizobium* population. In such circumstances, special mixtures of appropriate rhizobia and bradyrhizobia inoculants may be applied (Brady and Weil, 2002).

Methods for rhizobial enumeration and measures of diversity do not usually give an accurate description. Numbers can be underestimated and diversity could also be masked due to discrepancies caused by choice of the host to trap them and the soil factors (Sadowsky and Graham, 1998). The most probable number (MPN) technique is an important technique in estimating microbial populations in soils, waters, and agricultural products. The important aspect of MPN methodology is the ability to estimate a microbial population size based on a process-related attribute. Many soils are heterogeneous, therefore exact cell numbers of an individual organism can be impossible to determine. This technique is used to estimate microbial population sizes in situations like this. The

technique does not rely on quantitative assessment of individual cells; instead it relies on specific qualitative attributes of the microorganism being counted. This method is based on four assumptions. First, inoculation of viable rhizobia on its specific host results in development of nodules. Second, nodulation on that inoculated plant becomes a proof of the presence of infective rhizobia. Third, absence of nodule is a proof of the absence of infective rhizobia. Finally, un- inoculated plants are used as control, with absence of nodule (Brady and Weil, 2002).

2.8. Inoculation

Products containing *Rhizobium* bacteria are called nitrogen inoculants. Inoculation is the process of introducing the appropriate *Rhizobium* bacteria to the soil in numbers sufficient to ensure successful nodulation (Brockwell *et al.*, 1995). This is done by coating the seed with a liquid or peat-based powder inoculant, or by treating the soil with a granular or liquid inoculant. Commercial inoculants, when properly applied, ensure that every plant is exposed to a sufficient number of bacteria to initiate nodulation and start the nitrogen fixation process. *Rhizobium* bacteria are not very mobile in the soil, and thus, the inoculant must come in contact with the developing seedling for infection of root hairs to occur. Specific pulse crops require specific *Rhizobium* species for nodulation (Brkić *et al.,* 2004). *Rhizobium* species capable of nodulation in lentil and pea crops is not capable of nodulation in chickpea (Table1). If the wrong *Rhizobium* species is used, inoculation will have no beneficial effect. Soils commonly lack sufficient numbers of the correct *Rhizobium* bacteria to optimize the nitrogen fixation process (Brockwell *et al.*, 1995)

Table 1 Rhizobium species required for some legume crops

pea, lentil, faba bean, chickling vetch	*Rhizobium leguminosarum var viciae*
Chickpea	*Rhizobium ciceri*
dry bean	*Rhizobium phaseoli*
Soybean	*Bradyrhizobium japonicum*
alfalfa, sweet clover	*Rhizobium meliloti*
Clover	*Rhizobium trifolii*
Fenugreek	*Rhizobium spp. Strain RGFU1*

(Source: Brockwell *et al.*, 1995)

Scientists select efficient strains of *Rhizobium* for each pulse crop. For example, a *Rhizobium* strain may be able to produce nodules in both lentil and pea, but may be much more effective in pea (Brkić *et al.,* 2004). Soils with a history of pulses may contain residual bacteria. Residual *Rhizobium* bacteria can produce nitrogen-fixing nodules. However, bacteria that survive in the soil for a number of years may not be present in sufficient numbers, may be inefficient fixers of nitrogen, or may be slow to colonize the roots (Sadowsky and Graham, 1998). For these reasons, most experienced pulse crop growers inoculate each time they plant a pulse crop.

2.9. Response of field peas to inoculation

In agricultural soils, where compatible and effective strains of Rhizobium are not present, or when efficient rhizobial soil populations are low, seed inoculation, with selected strains of Rhizobium can provide effective legume N fixation (Date, 2000; Chemining'wa and Vessey, 2006). Soil can also contain indigenous inefficient strains of Rhizobium. If these strains compete more successfully than the introduced strains for nodule formation, it can lead to a failure in legume production failure (Theis *et al*., 1991; Evans *et al*., 1996). Therefore the aim of inoculation is to achieve a high proportion of nodules formed on the target host legume occupied by an efficient strain of Rhizobium (Thies *et al*., 1991).

In Bangladesh *Rhizobium* inoculation has been reported to supplement up to 80 kg N ha-1 and increase the average yield of pea plants over uninoculated plants (Ahmed *et al*., 2007). *Rhizobium* inoculation positively affects plant height, the number of branches, root and shoot dry weight, the number of nodules, seed and biomass yield, the number of pods, the crude protein concentration, and seed P content (Murat *et al.*, 2009). In India, inoculation of pea with *Rhizobium leguminosarum* at 20 g kg-1 seed increased nodulation and activated the nitrogenase enzyme in the root nodules to fix more atmospheric N.

Treated plants grew taller and produced more biomass via branching. The harvest index (HI) was enhanced by an increased number of pods and number of seeds pod^{-1} (Murat *et al.*, 2009). Inoculation of peas may also transform root morphology compared with uninoculated plants. Inoculated peas had thicker primary and lateral roots, a greater stele root diameter but a lower root surface area and

shorter lateral roots than control plants. The higher stele: root diameter ratio is expected to increase transport from root to shoot through the xylem.

Inoculants can be applied in many ways such as inoculation on seed surface prior to sowing, pre-inoculation in conjunction with seed coating, soil implanting in the form of solid or liquid inoculant, irrigation water run inoculation, inoculant with furrow irrigation of flood irrigation and post emergence inoculation. Each of these methods has its own advantages. Inoculation responses are highly variable and are site specific (Date, 2000), due to; (1) a low effectiveness of the *Rhizobium* strain in the inoculants and, (2) a lower competitiveness of the bacterial strain in the inoculants than existing soil bacteria.

In addition, the response of plants to inoculation is affected by many factors such as the presence and quality of indigenous rhizobial populations, soil nutrients, soil chemical and physical properties, and the climatic conditions (Thies *et al*., 1991; Begum *et al*., 2001). High temperatures, before and during sowing, may negatively affect inoculum survival. Low rainfall and soil with pH < 5.0 or > 8.5 resulted in poorer persistence of introduced rhizobia than more moderate conditions (Howieson and Ballard, 2004).To have nodule occupancy of more than 50 % requires the number of rhizobia in the applied inoculant to be at least 1,000 times greater than the number of indigenous rhizobia (Thies *et al*., 1991). Under the same type of soil, an increase in the inoculation rate from 3.7×10^6 to 3.7×10^8 raised nodule occupancy by the inoculated strain from 74 to 90 %.

In the mid-1990's Brockwell *et al.*, (1995) concluded that most inoculants, in the world, were of relative low quality and about 90 % of all inoculants had no practical effect on legume productivity. Inoculation with rhizobia is only useful in locations where there are no indigenous strains of effective rhizobia in soil or the level of the indigenous population is low and the soil N concentration is lower than legume crop requirement (Thies *et al.,* 1991). However, technological development and improved quality control have given improved quality, reliability and efficacy of inoculation (Brockwell *et al.*, 1995). The trend to the use of sterile carriers, liquid inoculants or granule technology may avoid contaminants, increase inoculation rates, enhance inoculants shelf-life, improve survival and multiplication of rhizobia in the soil, and facilitate application by farmers.

Inoculation of legume seed is a simple, practical, widely used method to ensure effective N fixation (Date, 2000; Ahmed *et al*., 2007; Murat *et al*., 2009). Peat inoculants have become popular for inoculation because they are easy to produce and apply. Peat inoculants can support a high concentration of rhizobia, up to 10^9 to 10^{10} cell g^{-1} peat. Further it supports survival on inoculated seed (Date, 2000). However, some countries lack natural peat deposits or peat mines are located in forbidden exploitation areas. In addition, bacterial survival on seed is affected by factors which include desiccation, the toxic nature of seed coat exudates and temperature and these limit the availability of peat inoculants. Granular inoculants provide the potential to apply rhizobia with greater ease (Denton *et al*., 2009). Granular inoculants can be applied directly to the soil, adjacent to or under the seed.

This method of inoculation has the potential to replace peat slurry inoculation in situations where seed fungicides or insecticides need to be applied (Stephens and Rask, 2000). Granular inoculation enables higher application rates and the physical separation of rhizobia from the seed coat which may contain potentially toxic chemicals (Date, 2000). In addition, granular inoculants provide a major advance in the flexibility of inoculant application. For example, the requirement that seed is sown immediately after inoculation is unnecessary. Soil applied inoculants may result in higher pea biomass, pea seed yield and seed protein concentration, and greater yield stability compared with seed inoculants (Clayton *et al*., 2004).

2.10. Factors affecting biological nitrogen fixation

Biological nitrogen fixation affected by different ecological factors, such as soil pH, soil moisture, salt stress, soil temperature and Phosphorous deficiencies. These environmental restrictions influence plant growth, nodulation and nitrogen fixation in legumes (Giller, 2001).

2.10.1. Soil temperature

High temperatures strongly affect bacterial infection and N_2 fixation in several legume species. Critical temperatures for N_2 fixation are 30°C for clover and pea and range between 35 and 40°C for soybean, guar, peanut, and cowpea (Zaharan, 1999). *Rhizobium* strains differ in their ability to grow,

nodulate their host plants and expression of nitrogenase activity at extreme temperatures. Nodulation and symbiotic nitrogen fixation depend on the nodulating strain in addition to the plant cultivar.

Temperature affects root hair infection, bacteroid differentiation, nodule structure, and the functioning of the legume root nodule. High (not extreme) soil temperatures will delay nodulation or restrict it to the subsurface region. For most rhizobia, the optimum temperature range for growth in culture is 28 to 31°C, and many are not capable to grow at 37°C. Rhizobia have a poor growth at temperature below 10 ^{o}C (Graham, 1992). Nodulation in *sativum is* affected to greater extent by low temperature. In extreme temperature in addition of nodulation and physiological disorders, rhizobial plasmid also loss and genomic rearrangements can be affected.

2.10.2. Soil moisture

High and low moisture content of the soil can have a significant effect on growth, survival of soil rhizobia and production of crop. Symbiotic N_2 fixation of legumes is also highly sensitive to soil water deficiency. Soil moisture deficiency has a pronounced effect on N_2 fixation because nodule initiation, growth, and activity are all more sensitive to water stress than are general root and shoot metabolism (Giller 2001). The response of nodulation and N_2 fixation to water stress depends on the growth stage of the plants. It was found that water stress imposed during vegetative growth was more detrimental to nodulation and nitrogen fixation than that imposed during the reproduction stage. There was little chance for recovery from water stress in the reproductive stage. The wide range of moisture levels characteristic of ecosystems where legumes have been shown to fix nitrogen suggests that rhizobial strains with different sensitivity to soil moisture can be selected. Different strains of rhizobia and different legume cultivars display differences in tolerance of low water activity (Zahran, 1999).

Optimization of soil moisture for growth of the host plant is generally more sensitive to moisture stress than bacteria, results in maximal development of fixed-nitrogen inputs into the soil system by the *Rhizobium*-legume symbiosis. Drought also affects both the number of rhizobia and N2 fixation rates. It is the most harmful abiotic constraints to BNF, mainly due to its effect on soil physical and biological characteristics (Zahran, 1999).

2.10.3. Soil pH

Soil acidity is the major significant problem for agricultural production in many Legumes growing and production areas of the world. Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depend on symbiotic N_2 fixation. It reduces legumes productivity. Soil pH significantly influences rhizobia content of soils and their ability to nodulate legumes (Slattery *et al.,* 2004). Soil acidity reduces nitrogen fixation in legumes, particularly affecting *Rhizobium* survival in soil and reducing nodulation. The optimum pH for rhizobial growth is considered to be between 6.0 and 7.0, and relatively few rhizobia grow well at pH less than 5.0. The fast growing strains of rhizobia have generally been considered less tolerant to low pH than have slowly growing strains of *Bradyrhizobium* (Graham *et al.,* 1994). Vassilava *et al.* (1997) reported that, nodulation of legumes is reduced in acidic soil, mainly because of sensitivity of early nodulation events, such as attachment, root hair curling and initiation of infection thread formation.

The failure of legumes to nodulate under acid-soil conditions is common, especially in soils of pH less than 5.0. The incapability of some rhizobia to persevere under such conditions is one cause of nodulation failure (Zaharan, 1999), but poor nodulation can occur even where a viable *Rhizobium* population can be demonstrated. Zaharan (1999) found that nodulation of *P. sativum* was 10 times more susceptible to acidity than was either rhizobial multiplication or plant growth. Some legumes, e.g., *Trifolium subterranean*, *T. balansae*, *Medicago murex*, and *M. truncatula*, showed tolerance to soil acidity as indicated by dry-matter yield; however, the establishment of nodules was more sensitive to soil acidity in most of these plants than was indicated by the relative yields of dry matter. The growth, nodulation, and yield of *V. faba* were enhanced after inoculation with strains of *R. leguminosarum* bv. viciae in acid soils. It appears that the pH-sensitive stage in nodulation occurs early in the infection process and that *Rhizobium* attachment to root hairs is one of the stages affected by acidic conditions in soils. Acidity had more severe effects on rhizobial multiplication than did Al stress and low P conditions.

2.10.4. Salt stress

Salinity is a severe risk to agriculture and to the production of crop. Increases in the salinity of soils or water supplies used for irrigation result in decreased productivity of most crop plants and lead to noticeable changes in the growth pattern of plants (Zahran, 1999). Increasing salt concentrations may

have a negative effect on soil microbial populations as a result of direct toxicity as well as through osmotic stress. Salt tolerance in plants is a complex phenomenon that involves morphological and developmental changes as well as physiological and biochemical processes. Salinity decreases plant growth and yield, depending upon the plant species, salinity levels, and ionic composition of the salts (Delgado *et al.,* 1994).

The salinity response of legumes varies greatly and depends on such factors as climatic conditions, soil properties, and the stage of growth. The legume-*Rhizobium* symbioses and nodule formation on legumes are more sensitive to salt or osmotic stress than are the rhizobia (Rao *et al.,* 2002). Salt stress inhibits the initial steps of *Rhizobium*-legume symbioses. The reduction of N_2-fixing activity by salt stress is usually attributed to a reduction in respiration of the nodules and a reduction in cytosolic protein production, specifically leg hemoglobin, by nodules. The depressive effect of salt stress on N_2 fixation by legumes is directly related to the salt-induced decline in dry weight and N content in the shoot (Rao *et al.,* 2002). The salt-induced distortions in nodule structure could also be reasons for the decline in the N_2 fixation rate by legumes subject to salt stress. Although the root nodule-colonizing bacteria of the genera *Rhizobium* and *Bradyrhizobium* are more salt tolerant than their legume hosts, they show variation in salt tolerance. A growth of a number of rhizobia was inhibited by 100 Mm NaCl, whiles some rhizobia, and was tolerant to 300 to 700 mM NaCl. Strains of *Rhizobium leguminosarum* have been reported to be tolerant to NaCl concentrations up to 350 mM NaCl in broth culture (Rao *et al.,* 2002).

2.10.5. Soil mineral nitrogen

Depending on the accessible quantity present, the mineral nitrogen content of the soil can have both positive and negative effects on yield and growth response of field pea to inoculation. Usually a higher mineral nitrogen content in the rhizosphere leads to poor N_2 fixation through inhibition of nodulation of field pea. On the other hand, small amounts of soil or fertilizer N often have a stimulatory effect on nodulation and N_2 fixation which is principally due to the positive effect of N on growth and plant establishment during the period between root emergence and the onset of active N_2 fixation (Giller, 2001).

2.10.6. Soil Phosphorous

Phosphorous (P) is the major mineral nutrient yield determinant among legume crops. Its deficiency is usually affects plant growth, nodulation and nitrogen fixation. Plants dependent on symbiotic nitrogen by roots fixation have special ATP requirements for nodule development and function and need additional P for signal transduction and membrane biosynthesis. P is a building block of a plant energy source, P is important in N cycling because adenosine tri-phosphate is required in large quantities by legumes to develop nodules and undergo the fixation process (Sessitsch *et al*., 2002).

As N_2 fixation is energy demanding process, larger P quantities are needed by N_2 fixing plants than by mineral N supplied plants. In this regard, poor nodulation and poor plant vigour have been observed in beans grown in soils low in extractable P while acute deficiency of phosphorus can even prevent nodulation by legumes (Giller 2001), showing the sensitivity of the process of N_2 fixation to P deficiency. The increase of whole plant growth and plant nitrogen concentration in response to increased soil P supply has been noted for several leguminous species. Decreased specific-nitrogenase activity in nodules of leguminous plants was associated with decreased energy status of host plant cells of nodules. Nodules are strong sinks for P and range in P content from 0.72 to 1.2%; consequently, plants engaged in symbiotic N_2 fixation generally have a higher requirement for P than those grown with N fertilization (Panda and Panda, 2002)

3. MATERIAL AND METHODS

3.1. Description of Study Area

The experiment was conducted at Kulumsa Agricultural Research Centre (KARC), which is located in Tiyo wereda of Arsi zone in the Oromiya National Regional State, Asela, Ethiopia during the main cropping season of June-November, 2015. The experimental site is located within latitude $8^0$01' 10"N and longitude $39^0$09'11"E with altitude of 2200m above sea level. The site receive 832mm of rainfall with bimodal distribution (June to September and February-April) with average annual maximum temperatures of 22^0c and minimum temperatures of 10^0c.

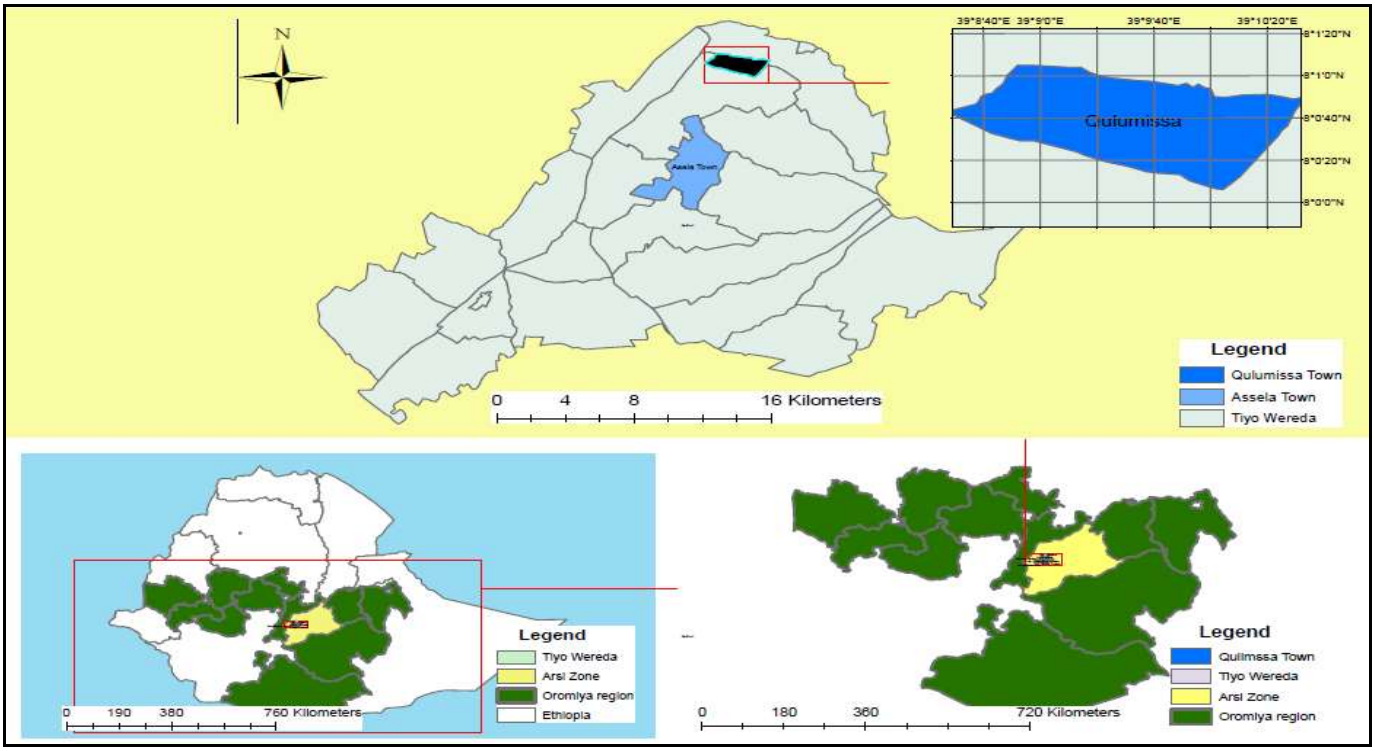

Figure 1 Location of the study area

3.2. Soil characteristics of the field site

The physico chemical characteristics of the soil are tabulated on Table 2. The soil textural class of the experimental site was clay with average proportions of 49% clay, 23% silt and 28% sand according to Tekalign (1991). The pH value of the soil was slightly acidic (pH 6.2) and within the optimum range for crop production, total nitrogen content (TN) of the soil was classified as very medium (0.15-0.25%) (Table 2). The available phosphorus has content was medium (15-25). As far as soil organic

carbon (OC) is concerned, the value was 2.5%, which was within the range of low organic carbon content (Landon, 1991), and the cation exchange capacity (CEC) was medium (15-25 cmol kg^{1}).

Table 2 Soil Physico-chemical properties of experimental site

Parameter	Unit	Status	Reference
Textural class	Clay	clay	Tekalign (1991)
Total N (%)	0.19	medium	Tekalign (1991)
Av.P (ppm)	21.8	medium	Olsen *et al.*(1954)
OC (%)	2.5%	low	Landon (1991)
C:N	13.16	Low	Tekalign (1991)
pH	6.2	Slightly acidic	Tekalign (1991)
CEC	16.18	medium	Landon (1991)

3.3. Enumeration of indigenous rhizobia in the soil

The rhizobial population from the soil (collected from the selected field site) was estimated using Most Probable Number (MPN) by inoculating soil dilutions on the host grown under greenhouse conditions after 28 days of growth (Somasegaran and Hoben, 1994). The MPN was calculated from the most likely number (m) found in MPN tables. The most likely numbers (m) were located from the table as MPN per gram of soils:

$$X = \frac{m \times d}{V}$$

Where X = rhizobia population per gram of soils, m = likely number from MPN table for the lowest dilution of series, d = lowest dilution (first unit used in tabulation) and V = volume of aliquot applied to plant.

3.4. Source of rhizobia, carrier, and Seed

The selected rhizobia were FBR 23, FBR 11 and FBR15 obtained from culture collections of the Department of Microbial Cellular and Molecular Biology at Addis Ababa University (App. 5 and 6). They were selected based on their nutritional versatility and eco-physiological tolerance under laboratory test and symbiotic effectiveness on sand culture under greenhouse conditions. The commercially released *Rhizobium leguminasorium* strain (strain 1018) obtained from Menagesha PLC was included as standard reference strain. Peat was obtained from Holeta Agricultural Research Center used as inoculants carrier. One of the seed of field pea improved varieties (tegegnech) was obtained from Kulumsa Agricultural Research Centre (KARC), EIAR.

3.5. Treatments and experimental design

A total of 6 treatments were arranged using the three selected rhizobia, a standard reference commercial inoculant, and 2 (1 positive and 1 negative control groups). A non nitrogen fixing reference crop (Barely variety, Direbe), released from KARC was also included to estimate the nitrogen derived from the atmosphere (Ndfa) using the N difference technique. The experiment was laid out in RCBD with factorial arrangement in three replications on one location. The plot size was 4m by 3.6m with a plot to plot spacing of 0.4m and block to block spacing of 1m.

3.6. Agronomic practices

3.6.1. Land preparation and sowing

Prior to sowing, the experimental plots were prepared three months before planting. A starter dose of P and N fertilizers with recommended rate 46 kg P_2O_5 and 20 kg urea ha^{-1} was used according to standard methods (Taye and Asfaw, 2010). One seed was planted per hill at a spacing of 20 cm between rows and 10cm between plants. All plots were isolated with ridges to minimize the movement of bacteria from one plot to another.

3.6.2. Seed inoculation and weeding

The seeds were surface sterilized with sodium hypochlorite and latter washed repeatedly with sterile distilled water (Somasegaren and Hoben, 1994). Inoculation was prepared from cool and clean water and sugar mixed with rhizobial isolates (10^8 ml^{-1} viable bacterial cell) applied at the rate of 7 ml kg^{-1}

seeds. All inoculants were applied just before planting under shade to maintain the viability of cells (Taye and Asfaw, 2010). The experimental field was weeded following prior recommendation.

3.6.3. Field pea harvest

All field pea plants were harvested at physiological maturity (120-150 days). The tops of the plants were cut and put in the cloth bag and sack, then sun dried up to attain constant weight then threshed manually to separate seed from straw.

3.7. Re isolation of root nodule bacteria from field pea nodules

Re isolation was used to differentiate introduced rhizobia from indigenous (soil) rhizobia using standard methods. After 30 days of planting, plants were randomly uprooted from the field to collect nodules for reisolation and determination of nodule occupancy of the original inoculants; FBR11, FBR 15 and FBR 23 compared to the indigenous rhizobia in the soil. Three representative nodules from each of 10 randomly picked individual plants of the three treatments were randomly collected to re-isolate rhizobia using standard methods (Somesegaran and Hoben, 1994). The nodules were surface sterilized with 95% ethanol for 10 seconds, and transferred to 3% (v/v) solution of sodium hypo chlorate for 2-minutes. The surface sterilized nodules were then rinsed with sterile distilled water six times to completely remove the sterilizing chemicals. The nodules were crushed with sterile glass rods in1 drop of sterilized 0.85% NaCl. The crushed nodules were then transferred to yeast extract manitol agar (YEMA) medium (DIFCO) and incubated at 28±2^{o}C for 3-5 days.

Table 3 YEMA Composition (Vincent, 1970)

Chemicals	Amount (g/l)
MgSO4.7H2O	0.2g
KH2PO4	0.5g
NaCl	0.2g
Yeast extract	0.5g
D-manitol	10g
Agar	15g
Distil water	1000ml

(Source: Vincent, 1970)

3.8. Purification and preservation

Colonies were picked with sterile inoculating loop and streaked repeatedly on sterile YEMA plates and incubated at 28±2^{0}C. A total of 30 selected pure rhizobial isolates from each inoculant treated (FBR11, FBR15, and FBR23) nodules were selected and were preserved on YEMA slant tubes containing 0.3% (W/V) $CaCO_3$ at 4^{0}c (Somesagaran and Hoben, 1994).

3.9. Preliminary screening of rhizobia from nodules (presumptive test)

3.9.1. Gram staining test

Gram staining was carried out to confirm that all isolates were gram negative and do not contain any gram positive bacteria or contaminants (Lupwayi and Haque, 1994).

3.9.2. Congo red absorption

Colonies were tested for congo-red absorption on Congo-Red (CR-YEMA) (Somasegaran and Hoben, 1994). Stock solution of Congo Red (CR) prepared by dissolving 0.25gm of CR in 100ml sterile distilled water from which, 10ml was added to one liter of YEMA. Culture suspensions were inoculated into YEMA-CR medium, and the plates were wrapped with aluminum foil and incubated at 28±2 ^{0}C for 3-5 days.

3.9.3. Acid and alkaline production on BTB

Isolates were tested for the production of acid or alkaline by incorporating (0.5%) bromothymol blue (BTB) as reaction indicator on yeast extract manitol agar (YEMA) according to Somassegaran and Hoben (1994). After 48 hours of growth, a loop full of *Rhizobium* culture (10^5cells/ml) was streaked on YEMA-BTB plate, and incubated at 28±2 ^{0}C for 3-5 days (Somasegaran and Hoben, 1994).

YEMA----------1 liter
BTB-(0.5 % w/v in 95% ethanol) ----------5ml
pH----------6.8

3.9.4 Growth on peptone glucose agar (PGA) medium

Isolates were inoculated on PGA containing bromocresol purple dye (10µ g/ml) in order to check a change in pH of the medium associated with the presence of contaminants (Somasegaran and Hoben, 1994). The PGA composition was glucose 5gm, peptone 10gm, agar 15gm and BCP 10ml then adjusted pH at 6.8. The BCP (Bromo cresol purple) was prepared as stock solution by dissolving 1gm/100ml of ethanol. The pH was adjusted to 6.8 by 1N NaOH and HCl. The bacterial culture suspension was inoculated on the medium and incubated at 28±2^{o}C; to detect the presence/absence of bacterial colonies.

3.9.5. Authentication

Isolates were authenticated as root nodule bacteria according to Somasegaran and Hoben, (1994). Healthy seeds of field pea (Tegegnech) were surface sterilized as before and transferred into 0.75% water agar plates and incubated at 28±2^{o}c five seedlings were then transferred into surface sterilized 3kg capacity plastic pots filled with river sand soaked in H_2SO_4 for 24 hours and extensively washed with tap water several times. After 3-5 day, each seedling was inoculated with 1ml actively grown rhizobial culture (10^9cells/ml), and later were thinned down to three per pot.

The experiment was set up in randomized complete design in a greenhouse with a 12/12 light/ dark hour's cycle and average 25/18^{o}C day/night temperature. All experiments were done in triplicates by including positive control pots fertilized with nitrogen 0.05% KNO_3 (W/V once a week) but without inoculation and negative control pots neither fertilized nor inoculated. All treatments were fertilized

with full strength N-free nutrient at a rate of 100ml/pot once a week (Table 4) and washed with tap water every 2 days to control salt accumulation in pots.

Table 4 Nitrogen free solution

Stock solution	Chemical	Concentration g/l
Calcium chloride	$CaCl_2.2H_2O$	294.0
Potassium phosphate	KH_2PO_4	136.1
Ferric citrate	Fe $C_6H_5O_7$. $3H_2O$	6.700
	$MgSO_4$. $7H_2O$	123.3
	K_2SO_4. H_2O	87.00
	$MnSO_4$. H_2O	0.338
Trace elements	H_3BO_3	0.247
	$ZnSO_4$. $7H_2O$	0.228
	$CuSO_4$. $5H_2O$	0.100
	$CoSO_4$. $7H_2O$	0.056
	Na_2MoO_2. $2H_2O$	0.048

(Source: Somasegaran and Hoben, 1994)

After 6 weeks of planting, the whole plants were uprooted to count the number of nodules, and measure of nodule dry weight and shoot dry weight after drying at 70 ^oC for 48hrs until constant weight. Effectiveness of isolates in accumulating plant shoot dry weight was calculated according to the equation (Somasegaran and Hoben, 1994).

$$SE = \frac{\text{Inoculated plant DM}}{\text{N-fertilized plant DM}} \times 100\ \%$$

DM=dry matter

SE=symbiotic effectiveness

Nitrogen fixing effectiveness classified as ineffective, <35%; lowly-effective, 35-50%; effective, 50-80%; and highly effective, >80%.

3.10. Cultural and growth characteristics of authenticated rhizobial isolates

3.10.1 Cultural conditions

All experiments were done in triplicates by growing the isolate for 48 hours and adjusted to inoculum size of 10^6 /ml. They were inoculated on YEMA medium, incubated for 3-5 days at 30^oC unless stated otherwise.

3.10.2. Mean generation time

Each isolate was inoculated in to 10ml of YEMB test tube and shaken on orbital shaker at 120 rpm (revolution per minute) for 48hrs at room temperature. One ml of each culture was transferred into 250ml Erlenmeyer flasks containing 100ml of YEMB and placed on rotary shaker at 120 rpm (revolution per minute). After calibrating spectrophotometer to zero with sterile uninoculated YEMB (3.5 ml blank), 3.5ml of culture samples were was transferred into cuvette to read optical density (UV-7804C-Ultraviolat-Visible spectrophotometer) at 540 nm beginning from time of inoculation (0hr) and at every 6hrs interval for 72hours. The generation time (g) was calculated from the logarithmic phase according to White (1995).

The formula**; g = log2 (t)** / **logX-logXo**,
Generation time **(g) =t/n**

Where: g = generation time

t = time elapsed

XO = First OD,

X = second OD reading

OD=optical density

n=number of generations

3.10.3. Colony morphology

The cultural characteristics of the isolates were performed after having grown them for 3-5 days on YEMA medium according to (Lupwayi and Haque, 1994). Each single colonies of each isolate was characterized based on colony appearance (texture), diameter, color, shape and extra cellular polysaccharide production.

3.11. Nutritional characteristics

All isolates were checked for the following nutritional and eco-physiological characteristics as selective markers for the identification of the original inoculants (FBR11, FBR 15 and FBR 23) (provided by Genet G/Yohannes PhD thesis in progress) in order to determine their nodule occupancy in comparison to the local rhizobia in the soil**.**

3.11.1. Carbohydrate Utilization

Isolates were checked for their ability to utilize different carbohydrate sources i.e. fructose, galactose and Gluconate. The test was carried out according to Somasegaran and Hoben (1994). Each carbohydrate was prepared (10%) and mixed with basal medium containing $MgSO_4.7H_2O$ (0.2gm), KH_2PO_4 (0.5gm), NaCl (0.2gm), manitol (10gm), yeast extract (0.5gm) and agar (15gm). Heat stable carbohydrates (fructose) were autoclaved together with the medium, but heat labile carbohydrates (galactose and gluconate) were filter sterilized using disposable membrane filter of 0.22μm and added to the basal medium (YEMA). After sterilization when the medium temperature was reduced to 50 ^{O}C. Finally, a loop full of 72 hours old YEM broth culture was separately streaked on the plates and incubated at 28 ^{O}C for 3 to 5 days and growth was recorded as (+) for positive growth and (-) for no growth in relation to the positive control YEMA plates.

3.11.2. Amino Acid Utilization

The isolates were streaked on different nitrogen source including: Glutamine, DL-β-Phenylalanine, Peptone and Arginine in order to determine their ability to utilize them. Each nitrogen source was added to a basal medium at concentration of 0.5gm /l (Somasegran and Hoben, 1994). Finally 72hr old rhizobial culture was inoculated in to these medium and incubated at 28±2 C for 3-5 days.

Table.5 Basal Medium Composition

Chemicals	Amount (g/l)
$MgSO_4.7H_2O$	0.2g
KH_2PO_4	0.5g
NaCl	0.2g
Yeast extract	0.5g
D-manitol	10g
Agar	15g
Distil water	1000ml

(Source: Somasegaran and Hoben, 1994)

3.12. Eco-physiological characteristics

3.12.1. pH tolerance

The ability of isolates to grow at different pH was tested on YEMA adjusted to pH levels 4.0, 4.5, 5.0, 5.5, 8.0, 8.5, 9.0 and 9.5 with sterile 0.1N HCl and 1N NaOH (Bernal and Graham, 2001). The results were recorded qualitatively as + for presence or – for absence of growth after 3-5 days of incubation at $28 \pm 2^{\circ}C$.

3.12.2. Temperature tolerance

The ability of all isolates to grow at varying temperatures was assessed on YEMA plates incubated at the temperatures of, 4,10, 15, 35 and $40^{\circ}C$ (Lupiwayi and Haque, 1994). Growth was qualitatively recorded as (+) for growth and (-) for no growth.

3.12.3. Salt tolerance

Tolerance of all isolates to sodium chloride (NaCl) evaluated through determining growth on YEMA solid medium supplemented with 2, 3, 5 and 6 % (w/v), Nacl concentration. Growth was evaluated qualitatively as (+) for growth and (-) for no growth after 3-5 days (Lupiwayi and Haque, 1994).

3.12.4. Heavy metal resistance

The resistance of isolates to heavy metals was tested by streaking them on solid YEMA medium containing different concentrations of heavy metals as described in Lupiwayi and Haque (1994). The heavy metals $ZnCl_2$ 25; $HgCl_2$ 5; $CuCl_2$ 100; CrCl2 100; $CdCl_2$ 20; Ni Cl_2 60 and Pb $(CH_3CO)_2$ 100 and 500 were filter sterilized using sterile 0.22μm pore size membrane filter (μg ml^{-1}) and added to the media (YEMA) after autoclaving and cooling to approximately 50 ^oC and mixed thoroughly (Lupiwayi and Haque, 1994). The isolates were streaked on the plates and incubated at 28 $\pm$ 2^oC for 3-5 days. The result was recorded qualitatively either as +/- for growth and no growth, respectively.

3.12.5. Intrinsic antibiotic resistance (IAR)

The resistance of isolates to antibiotics was tested by streaking them on solid YEMA medium containing freshly prepared filter sterilized antibiotics using 0.22 μm sized membrane filters: Tetracyclin, Erythromycin, Streptomycin, Penicillin, Chloroamphenicol, Neomycin, Ampicillin and Nalidixic acid and five concentrations (5, 10, 20, 30 and 50). The stock solution of each antibiotic was first prepared by dissolving 2g of each antibiotic in 100ml of water as described in Lupiwayi and Haque (1994). Erythromycin was dissolved in ethanol and Nalidixic acid was dissolved in 1M NaOH, whereas the other was dissolved in sterilized distilled water. Each filter sterilized antibiotic solution was added to sterile YEMA cooled to 50^oC and mixed thoroughly. The isolates were then streaked on the plates and incubated at 28 $\pm$ 2^oC for 3-5 days. The result was recorded qualitatively either as +/- for growth and no growth, respectively.

3.13. Nodule occupancy of rhizobial inoculants from nodules in the field

Nodule occupancy of the three inoculants was determined by comparing the biochemical profiles of the three inoculants (FBR11, FBR15 and FBR 23) recorded previously by Genet G/Yohannes. The major biochemical markers were Inherent Antibiotic Resistance (IAR), pH tolerance, carbohydrate and amino acid utilization, and tolerance to heavy metals. Isolates with more than 80% similarity with the original characters (markers) were considered similar (identified as FBR 11, FBR 15 and FBR 23).

3.14. Performance of the selected inoculants on growth and yield of field pea under field conditions

3.14.1. Yield related parameters

Plant height, number pod of plant, number of seeds per pod, thousand grains weight, total biomass yield per hectare and grain yield per hectare were considered as parameters to study the effect of inoculation on growth and yield components of field pea.

At 60 days of growth five plants uprooted from each plot to determine nodule number, nodule dry weight, shoot dry weight and N content and also at physiological maturity (harvesting) five plants from each plot including the reference crop (barley) were harvested and separated into straw and grain. These samples were used to determine seed and straw N content, N uptake, N derived from the atmosphere (Ndfa), and seed protein content. Soil sampling was taken from all plots and reference crop after harvesting to determine N derived from the atmosphere (Ndfa).

3.14.2. Determination of grain and straw yield, total nitrogen content and N uptake

Total nitrogen in grain and straw sub samples was quantitatively determined by Kjeldhal procedure (Page *et al*., 1982). Nitrogen up take in the grain and straw was determined after multiplying their N contents with their respective yields (Taye and Asfaw, 2010).

3.14.3. Estimation of Biological nitrogen fixation (nitrogen derived from fixation) of pea plants using a reference crop

N- Difference Method: Field estimation of N_2 fixation (nitrogen derived from fixation) was determined by measuring the total amount of N in the legume crop and nitrogen content in a non-fixing reference crop (barely, variety name Direbe) (Beck *et al*., 1993). The amount of N_2 fixed was calculated by subtracting the N yield of the reference crop from the N yield of legume as follows. The quantity (Q) of N derived from N_2 fixation was calculated as:

$$\mathbf{Q} = \frac{\text{Total nitrogen of legume (Field pea)} - \text{total nitrogen of reference crop (Barley)}}{\text{Total nitrogen of legume (Field pea)}} \times 100$$

3.14.4. Determination of seed protein

In order to determine crude protein in seeds representative seed samples were taken from each treatment to determine total nitrogen in the seed through kjeldahl Method (Page *et al*., 1982). Then the percentage of protein in seeds was calculated by multiplying the factor 6.25 (Morrison, 1956).

3.15. Plant and seed analysis

At physiological maturity five plants from within each plot including the reference crop were harvested and separated into straw and grain. These samples were used to determine seed and straw N, total N, N derived from the atmosphere (Ndfa), and seed protein content. The sample materials were oven dried at 70 ^{o}C to a constant weight and ground to pass through a 2 mm sieve. Plant tissue N was determined using Kjeldhal method (Page *et al*., 1982).

3.16. Data Collection and analysis

Data on number of nodules, nodule dry weights and shoot dry weight was recorded after two months after planting. After having excavated selected plants from central rows, nodulation was scored as positive with at least one nodule, and counted as mean value of nodules plant^{-1}. At physiological maturity, five plants from central rows of each plot were randomly harvested to measure plant height, number of pods plant^{-1}, and number of seeds pod^{-1} (Birhanu and Pant, 2012). Thousand seeds weight**,** grain yield and above ground dry matter (biological yield) were recorded on net harvestable plot. Grain yields were adjusted to 10 % moisture content and the yield per plot was converted to kg hectare^{-1} for statistical analysis. The green house and field data were analyzed using SAS analytical software. Effects were considered significant if P values are $\leq$ 0.05 and means were separated using Duncan multiple range test (DMRT). Correlation analysis was carried out to study the nature and degree of relationship between numbers of nodule and selected parameters using the same software.

4. RESULTS AND DISCUTION

4.1. Estimation of indigenous Rhizobia (MPN) in the soil at the experimental site

The MPN count of rhizobia in the soil at the experimental site was 1.5 x 10^4, indicating that the soil contained sufficient number of indigenous rhizobia but not effective.

4.2. Authentication and characterization of rhizobia from nodules to estimate nodule occupancy of the test inoculants

Nodule bacteria were re-isolated from nodules to estimate the nodule occupancy (%) of the three rhizobial inoculants (FBR11, FBR15 and FBR 23) and the indigenous (local) rhizobia in the soil of the field site using different eco-physiological markers. All the isolates recovered from the nodules were gram negative and fast growing with doubling time between 2 and 4 h, colony diameters within the range of 2.5mm and 5.5mm and formed mucoid, convex and white colonies after 3-5 days incubation on YMA plates, and changed the YEMA-BTB medium into yellow (App. 4.1, 4.7 and 4.13). They did not absorb red color from CR-YEMA medium and failed to grow on peptone glucose agar (PGA) medium, and re-nodulated field pea host indicating that that they were fast growing rhizobia (Jordan, 1984) and *Rhizobium leguminosarum* var *viceae* (Kassa *et al*., 2015).

Isolates were utilized glucanate, galactose and fructose as the sole source of carbon, and almost all isolates grow on YEMA medium containing galactose (90%), fructose (88.9%) and glucanate (76.7%) (Table 6). Isolates were able to metabolize arginine (73.3%), glutamine (67.8%), peptone (63.3%), DL-B-phenylalanine (57.8%) and 65.5% of the isolates were able to utilize all amino acid tested this result greater than the findings of Fano Berhe (2010) where 48% of the isolates utilized all amino acids sources.

Table 6 Pattern of carbon and amino acid utilization by rhizobial isolates collected from nodules of host pea plants inoculated with the three inoculants FBR 11, FBR15 and FBR 23

Rhizobial isolates	Carbohydrate sources			Nitrogen sources			
	Gluconate	Galactose	Fructose	Glutamine	Phenylalanine	Peptone	Arginine
FBR 11	76.7	83.3	**86.7**	76.7	**56.7**	80.0	83.3
FBR15	**50.0**	96.7	90	80.0	90.0	**66.7**	86.7
FBR23	76.7	90	90	**46.7**	**56.7**	83.3	**50.0**
Average	67.8	90%	88.9	67.8	57.8	63.3	73.3

The different isolates displayed antibiotic resistance to different types of antibiotics (Table 7). The data on inherent antibiotic resistance of isolates showed that they were resistant to erythromycin, streptomycin and ampicillin, and relatively sensitive to Nalidixic acid, tetracycline and neomycin at concentration of 50µg/ml. This pattern of resistance of the isolates was higher than the report of Turco and Benzadicek (1987) which showed pea rhizobia from soils of eastern Washington grow well on the same type of antibiotics with concentration of 5-20µg/ml. Assefa Kenenie *et al. (*2010) also reported on resistant faba bean rhizobia, from Wollo, Northern Ethiopia. Aregu Amsalu (2007) reported that 90% and 88% of *Rhizobium leguminosarum biovar viciae* from field pea were resistant to chloramphenicol at concentrations of 5 and 10µg/ml respectively.

Table 7 Pattern of antibiotic of rhizobial isolates collected from the inoculants treated field pea plants grown on YEMA containing 50μg/ml

Rhizobial isolates	**Amp**	Nal	Tet	Chl	Pen	**Ery**	Neo	**Str**
FBR11	90	**43**	70	51	70	73	67	100
FBR 15	83	80	**45**	86	73	90	**43**	**57**
FBR 23	**67**	**50**	60	77	**55**	80	**53**	90
Average	83	57	58	71	66	81	54	82

Ecophysiological characteristics of isolates

Most of the rhizobial isolates were resistant to temperatures 35°C (95.5%) with optimum growth at 30°C (100%), but some of them were able to grow at 15°C (22.2%), but failed to form colonies at 4°C ,10°C and 40°C (Table 8). This was contrary to the report of Aregu Amsalu (2007) who showed 50% and 64% of field pea rhizobia were tolerant to 5 and 10°C respectively.

Table 8 Ecophysiological tolerance of the rhizibial isolates grown on YEMA medium

Rhizobial isolates	pH						T^{o}C					NaCl (%)		
	4	4.5	5	5.5-8.5	9	9.5	4	10	15	35	40	3	5	6
FBR11	-	-	60	100	50	-	-	-	22.4	94	-	96	46.7	23.3
FBR15	-	20	63.3	100	46.7	-	-	-	24	97	-	80	50.0	26.7
FBR23	-	-	56.7	100	50	-	-	-	20.2	95.5	-	79	53.3	26.7
Average	-	6.3	60	100	50	-	-	-	22.2	95.5	-	85.6	50	25.6

Almost all of the isolates were tolerant to pH 5.5, 8 and 8.5 (100%) and some of the isolates were grown at pH 4.5 (6.3%), pH 5 (60%) and pH 9 (50%) however no growth was observed at pH 4 and 9.5 this result was similar to the result of Kassa *et al.* (2015) that showed field pea rhizobial isolates did not grow at pH 4**.**

All isolates were tolerant to 2% NaCl concentration, but show diversity as the salt concentrations increased to 3 % (85.6%), 5 % (50%) and 6 % (25.6%). Field pea rhizobia from Ethiopia also showed tolerance up to 6% NaCl concentrations (Aregu Amsalu, 2007).

Evaluation of the intrinsic resistance to heavy metals showed that all tested isolates shown that high resistance to $ZnCl_2$, Pb $(CH_3CO)_2$, $CrCl_2$ and $CuCl_2$ at concentrations of 25, 100, 100 and 100 µg ml^{-1}, respectively and most of the isolates exhibited an intrinsic resistance to $CoCl_2$ 100 µg ml^{-1} (77.8%), $ZnCl_2$ 50 µgml^{-1} (74.4%), $NiCl_2$ 60 µgml^{-1} (74.4%) and Pb $(CH_3CO)_2$ 500 µgml^{-1} (54.3%). However, all isolates were sensitive to $HgCl_2$ 10 µgml^{-1} (table 9) but some of the isolates were tolerate $HgCl_2$ 5 µgml^{-1} (44.3%) this result was different with the finding of Aregu Amsalu (2007) who reported 54% tolerant field pea rhizobial isolates to $HgCl_2$ 10 µgml^{-1}.

Table 9 Pattern of heavy metals resistance of rhizobial isolates collected from the inoculants treated field pea plants grown on YEMA containing different concentration

Rhizobial isolates	Zn		Pb		Cu	Hg		Co	Ni	Cr
	25 µgml^{-1}	50 µgml^{-1}	100 µgml^{-1}	500 µgml^{-1}	100 µgml^{-1}	5 µgml^{-1}	10 µgml^{-1}	100 µgml^{-1}	60 µgml^{-1}	100 µgml^{-1}
FBR11	96.7	73.3	100	53.3	93.3	43.3	0	76.7	76.7	93.3
FBR15	100	76.7	100	56.7	100	46.7	0	80	73.3	100
FBR23	100	73.3	100	53.3	96.7	43.3	0	76.7	73.3	93.3
Average	98.9	74.4	100	54.3	96.7	44.3	0	77.8	74.4	95.5

4.3. Nodule occupancy of isolates FBR11, FBR15 and FBR23

Based on the original ecophysiological markers, the inoculants FBR11, FBR15 and FBR23 were identified from each nodule sample. Accordingly, FBR 11 was characterized by its resistance to Nalidixic acid, Chloramphenicol, heavy metal lead and chromium, utilization of fructose, and phenylalanine; whereas FBR15, was characterized on its resistance to neomycin, tetracycline, streptomycin, and heavy metals lead and nickel; utilization of gluconate and peptone. FBR23 was also detected based on its resistance to ampicillin and penicillin, mercury; utilization of phenylalanine, and tolerance to pH 4.5 (Table 10). Accordingly, FBR15 was the most competitive inoculant with nodule occupancy of 75%; followed by FBR11 andFBR23 with nodule occupancy of 60 and 54% respectively. It is known that high competitiveness is a precondition of successful inoculation of a plant seed by rhizobium. This is the way how to add selected rhizobia isolate or strains to legume seeds effectively and consequently prevent the formation of nodules by low effective indigenous soil rhizobia (Denton *et al*., 2009).

Table 10 Nodule occupancy of the inoculants on the basis of their specific ecophysiological characters originally identified during their prescreening activities.

Rhizobial Isolates	Markers (IAR)	Markers (pH) tolerance	Markers Carbon utilization	Markers Nitrogen utilization	Markers (HM) tolerance	Nodule occupancy (estimation)
FBR11	Nal, Chl		Fru	Phen	Pb, Cr	60%
FBR15	Neo, Tet Str,		Glu	Peptone	Pb, Ni	75%
FBR23	Amp, Pen	pH 4.5		Phen	$HgCl_2$	54%

4.4. Authentication and symbiotic effectiveness test of re-isolated rhizobial isolates on sand culture

All the three rhizobial isolates (FBR11, FBR15 and FBR23) and commercially released strain (strain 1018) nodulated pea plants on sand culture under greenhouse conditions (Table 11). Accordingly, they showed prolific nodulation (196-229 N/plant) with mean nodule number of 224; with nodule dry weight of 104-121mg/plant (mean weight of 113mg/plant), and shoot dry weight (2.3-2.9gm/plant) (mean 2.6g/plant) showing that the inoculants did not lose their nodulation capacity. The data showed that isolate FBR 15 and Standard strain 1018 showed significant variations in all parameters from the other two local isolates. Thus, isolates FBR15 and the standard isolate 1018 isolates displayed high effectiveness (HE) measured by percentage shoot dry matter accumulation (80-100%) in relation to the shoot dry weight obtained from the Nitrogen-fertilized control plants; whereas isolates FBR11 and FBR 23 were effective with SE of 50-80% (Table 11).

Table 11 Effect of rhizobia inoculants on degree of nodulation, shoot dry weight (symbiotic effectiveness), and N content of field pea on sand culture grown for 45 days under greenhouse conditions

Treatment	NN/p	NDW(mg plant^{-1})	SDW(g plant^{-1})	SE %	SE Rate	% N in shoot
Isolate FBR 11	198^{b}	105.0^{b}	2.3^{b}	77	E	1.7^{a}
Isolate FBR 15	230.^{a}	121.3^{a}	2.9^{a}	97	HE	2.0^{a}
Isolate FBR 23	197.^{b}	103.7^{b}	2.3^{b}	77	E	1.7^{a}
Strain 1018	232^{a}	121.3^{a}	3.0^{a}	100	HE	2.0^{a}
(+) control	0	0	3.0^{a}	-	-	1.9^{a}
(-) control	0	0	1.4^{c}	-	-	1.3^{b}
CV	8.3	4.2	8.1	-	-	4.3
F value	208.8***	812.6***	54.7**	-	-	41.7*

Means in the same column followed by the same letter are not significantly different at the 5% Probability level by Duncan test. ***, ** and * = significant at P= 0.001, 0.01 and 0.05 respectively; CV = coefficient of variation, SE = symbiotic effectiveness, HE = highly effective; E= effective; NN= nodule number; NDW=nodule dry weight and SDW= shoot dry weight

Field pea plants inoculated with isolate FBR 15 and strain 1018 produced the maximum nodule number of (232.3 and 229.7 per plant), nodule dry weight (121.3mg/plant) that were significantly different from plants treated with isolates FBR 11 and FBR 23 (103-105mg/plant). Similarly, the inoculated plants with the most effective inoculants (FBR 15 and strain 1018) accumulated the highest shoot dry weight of 2.9/3.0g plant^{-1} equivalent to N-fertilized plants that was statistically different from plants inoculated with the other inoculants (Table 11). The average nodule number counted from the inoculants was 151.2 nodules per plant were significantly higher than108 nodules per plant recorded from pea plants (Aregu Amsalu, 2007). The pattern of nodule number and nodule dry weight was similar and to the shoot dry weight displayed by the good performing inoculant and standard inoculum of FBR15 and strain 1018 was similar in that the plants displayed the higher nodule number also showed.

Based on dry matter accumulation of inoculated plants with nitrogen fertilized (N^+) control plants, FBR 15 and strain 1018 were rated as highly effective whereas the others were effective N-fixers. The fact the inoculated plants did not show significant variation in % N content (1.7%-2%) , indicated that shoot dry matter is a good indicator of relative effectiveness of isolates (Somasegaran and Hoben, 1994). The %N content of the inoculated plants was similar to % N content of PR3 inoculated field pea plants reported by Talukder *et al.* (2008).

4.5. Effects of rhizobial isolates on nodulation, shoot dry weight and plant height of field pea under field conditions

Number of nodules plant^{-1}

The field pea plants showed significant variation in nodulation and phenotypic characters ($P < 0.001$) with inoculation and fertilizer treatments under field conditions (Table 12). Accordingly, the FBR 15 and the commercial strain-1018 treated plants induced the highest number of nodules plant^{-1} (107nn/plant) and the (112nn/plant) that was significantly higher than the number of nodules produced(84/86nn/plant) by isolates FBR11 and FBR 23.

Table 12 Effects of inoculation of the rhizobia on nodulation and growth parameters of field pea under field conditions.

Treatment	No of nodule /plant	Nodule DW(mg)	Shoot DW(gm)	Plant height(cm)
Control	10.7c	8.7c	13.7c	169.3b
Recommended N	13.7c	10.7c	28a	200a
FBR 15	107a	94.3a	28.3a	200a
FBR 11	84b	77b	25b	198.5a
FBR 23	86b	73.7b	23.3b	198a
Strian 1018	112a	93.7a	28.7a	202.3a
F Value	***	***	***	***
CV	7.6	7.8	3.6	0.7

Means in the same column followed by the same letter are not significantly different at the 5% probability level by Duncan test. *** = significant at P= 0.001; CV = coefficient of variation

On the contrary, the lowest number of nodules plant^{-1} (13.7 and 10.7) was recorded from N-fertilized pea plants and uninoculated and non-fertilized plants respectively (Table 12). Number of nodule was higher in inoculated plants compared to the non-inoculated ones by the range of 20.3% -27.1%. Murat *et al.* (2009) also reported increase in nodule number by 11% compared to un-inoculated control plants.

Nodule dry weight plant^{-1}

The field pea plants also displayed differences in nodule dry weight ranging from 8.7mg/plant (uninoculated negative control) to 94.3mg/plant recorded from plants inoculated with rhizobial isolate FBR 15. The nodule dry weight of plants induced by the standard strain 1018 and isolate FBR15 (94.3 and 93.7mg/plant) was significantly higher than the nodule dry weight obtained from other treatments with isolates FBR 11 and FBR 23 (73.7 and 77mg/plant). The inoculated plants increased nodule dry weight by 8-10 times more than the un-inoculated control plants. This is much higher than the increase by 57% reported by Brkic *et al.* (2004).

Shoot dry weight

The inoculation of field pea with Strain 1018 and the indigenous isolate FBR15 significantly ($P \leq 0.001$) enhanced shoot dry weight of field pea comparable to the N-fertilized plants (28gm/plant) (Table 12). Thus, the field plants inoculated with the most effective isolates FBR15 increased shoot dry weight as much as N-fertilized plants which was 50% higher than the uninoculated (negative control) plants a (13.7gm/plant). This indicates inoculation by the most effective inoculants could increase shoot dry matter significantly. This finding similar to the finding of Murat *et al*. (2008) who reported the highest shoot dry weight from inoculated field pea plant was greater than 14.4% of non-inoculated field pea plant shoot dry weight.

Plant height

The different treatments showed a significant difference in plant height ranging from 169.3 cm (negative control) to that of 202.3 cm recorded from the inoculation of the standard strain-1018 (Table 12). The variation in plant height did not show significant difference amongst all treatments, except the uninoculated and non-fertilized treatments. The inoculated plant height was increased by

22% over the un-inoculated control plants. This result also obtained by Murat *et al*. (2008) which documented inoculated field pea plant height 11% greater than from non-inoculated field pea plant.

4.6. Effect of rhizobia isolates inoculation on yield and yield components of field pea

Number of pods Plant^{-1}

The inoculated plants with FBR15 and the standard strain 1018 increased the number of pods plant^{-1} by 14.7/plant compared to the number of 7.7 pods/plant (negative control).

Table 13 Effect of rhizobial inoculation on yield parameters of field pea under field conditions

Treatment	No of pod/plant	No of seed /pod	BY (kg ha^{-1})	1000 seed wt(gm)	TGY (kg ha^{-1})
Control	7.7^{c}	3.3^{c}	2656^{b}	157^{c}	1879.4^{b}
Recommended N	14^{a}	5.3^{b}	3584.3^{a}	292^{a}	2544.9^{a}
FBR 15	14.7^{a}	6.0^{a}	3577^{a}	291.7^{a}	2539.5^{a}
FBR 11	12^{b}	5.0^{b}	3491^{a}	207.5^{b}	2432.3^{a}
FBR 23	12.7^{b}	5.3^{b}	3498^{a}	218.7^{b}	2389.8^{a}
Strian 1018	14.7^{a}	6.3^{a}	3583^{a}	292.3^{a}	2537.7^{a}
F value	***	**	*	***	***
CV	*4.1*	*10.2*	*1.3*	*3.3*	*0.4*

Means in the same column followed by the same letter are not significantly different at the 5% Probability level by Duncan test. *, ** and *** = significant at P≤0.05, 0.01 and 0.001 respectively; CV = coefficient of variation

The inoculation gave the same pattern of pods counted from N fertilized plants. They showed significant increase of more than 50% pods per plant compared to the uninoculated treatments and produced 13% more pods than the other rhizobial treatments FBR 11 and FBR 23 (Table 13). Seed inoculation might have increased nitrogen supply to crop plants, which ultimately resulted in more number of pods plant^{-1}. This result was similar with the findings of Ahmed *et al.* (2007) who

conducted experiment with field pea and reported that *Rhizobium* inoculant significantly increased number of pods compared to inoculated (14.45pods/plant) and uninoculated control (9.25pods/plant).

Number of seeds pod^{-1}

Inoculation of different rhizobial strains showed significant difference in number of seeds pod^{-1}. The maximum number of seeds pod^{-1} was 6.3 and 6.0 obtained from the plant inoculated with strain-1018 and isolate FBR 15 respectively, showing significance difference from other treatments. Accordingly, these effective inoculants produced twice as much number of seeds per pod over the negative control (3.3 seed/pod) and more than 20% of number of pods produced with other treatments (table 13). Ahmed *et al.* (2007) reported that rhizobial inoculation produced 5.14 seeds pod $^{-1}$ in pea plants whereas Solaiman and Rabbani (2005) observed that r*hizobium* inoculant alone produced 6.3 seeds per pod of pea than the uninoculated control (3 seeds pod $^{-1}$).

1000 seed weight (gm)

The response of inoculation to rhizobial inoculants was significant on weight of 1000 seeds of field pea ($P \leq 0.001$) compared with the control. The maximum mean weight of 292 for 1000 seeds was recorded from plants inoculated with the isolate FBR 15, strain 1018 and the plants fertilized with Nitrogen (Table13). The treatments with the most effective inoculants significantly increased up to 20% and 50% of 1000seed weight/gm of the other inoculants (FBR11, 23) and the negative control plants, respectively. This finding was similar with 1000 seed weight obtained with rhizobial inoculation (47.2% increases from uninoculated control pea plants) (Rabbani *et al.,* 2005). However, it was more than twice higher than the seed weight of 161g and 131.9g obtained from the inoculated and uninoculated plants, respectively (Ahmed *et al.,* 2007).

Total biomass or biological yield (BY) production (kg ha^{-1})

Analysis of variance indicated that total biomass (biological yield) of the inoculated and N-fertilized treatments were within the range of 3491 and 3584kg ha^{-1} without showing any significant difference among the treatments. However, the inoculated plants showed a 35% increase in BY compared to the 2656kg ha^{-1}BY obtained from uninoculated negative control plants (Table 13) indicating that the different treatments significantly ($P \leq 0.001$) improved pea production. Interestingly, Murat *et al*.

(2008) showed a significant difference (33% in pea production of BY) of 3616.7kg ha^{-1} compared to the 1866.7kg ha^{-1} obtained from un-inoculated pea plants.

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Total Grain Yield (kg ha^{-1})

The different treatments also significantly increased grain yield of field pea (P=0.01) (2389.8-2544.9kg/ha compared to the negative control (1879.4) (Table 13). Although the treatments increased total grain yield by 35% compared to the un-inoculated control plants, the different treatments did not show significant difference amongst one another. The increase in yield may be due to effective nodulation and nitrogen fixation of different rhizobial isolate inoculation on total grain yield of field pea. These results have similarity with those of Murat *et al*. (2009) find significant difference among the rhizobial treatments although they increased total grain yield (GY) of 2583.7kg/ha in comparison with total grain yield obtained 2269.7kg/ha obtained from un-inoculated control.

4.7. Effect of rhizobia isolates inoculation on total N and N up take from grain and straw

Grain total N and N up take

The field pea plants inoculated with the most effective rhizobial inoculants; strain-1018 and isolate FBR15 significantly increased total N content and N uptake of grain up to 4% and 102 kg ha^{-1} , respectively ($P \leq 0.001$) which was more than 32% and 46% of the total N content and N uptake recorded from the uninoculated negative control plants (Table 14). The data also showed that these inoculants were more effective (12-14% increases) in accumulation of grain nitrogen content and grain N uptake than the other inoculants (FBR 11 and FBR 23). Shabir *et al*. (2010) reported that rhizobial inoculation significantly increased total N content (3.8%) compared to control (2.6%) in field pea plants.

Table 14 Effects of rhizobial inoculation on Ndfa (BNF), N contents of grain and straw and seed protein of field pea under field conditions

Treatment	Grain N		Straw N		Seed protein	%Ndfa
	Total N (%)	Uptake (Kg ha^{-1})	Total N (%)	Uptake (Kg ha-1)	**%**	**%**
Control	2.9c	55.1c	2.2c	61.6c	18.3c	44.8c
Recommended N	3.4b	88.4b	2.8b	100.4a	21.7b	70.2b
FBR 15	4.0a	100.4a	3.3a	108.4a	25a	74.3a
FBR 11	3.5b	85.7b	2.7b	92b	22b	71b
FBR 23	3.5b	84.4b	2.7b	92.5b	22.1b	70.8b
Strian 1018	4.1a	102.1a	3.3a	108.6a	25.1a	74.4a
CV	3.4	3.5	3.7	4.1	3.3	6.6
F value	32.4***	96.7***	19.6***	61.9***	34.3***	21.5*

*and *** = significant at P= 0.05 and 0.001 respectively; CV = coefficient of variation,

Ndfa= nitrogen derived from fixation

Straw total N and N uptake

The highest straw N (3.3%) and N uptake (108.6 and 108.4kg ha^{-1}) of the field pea plants was recorded from plants inoculated with strain 1018 and isolate FBR 15 (Table 14). This was significantly different in N content (33%) and N uptake (45%) compared to the uninoculated plants (negative control plants) and 12 % more than the other inoculated plants (FBR 11 and FBR 23) in both parameters. The lowest straw N (2.2%) and N uptake (61.6 kg ha^{-1}) was recorded from the non inoculated control plant. This result is similar to the finding of Shabir *et al*. (2010) who recorded (30-35%) of straw N content and (47%) of N uptake compared to the un-inoculated field pea plants.

The result in general indicated positive correlations between number of nodule and shoot dry weight ($r = 0.49$, $p \leq 0.05$), number of nodule and number of pod ($r = 0.59$, $p \leq 0.01$), number of nodules and total grain yield ($r = 0.56$, $p \leq 0.05$), and shoot dry weight and N content ($r = 0.73$, $p \leq 0.001$). Khondaker *et al*. (2003) also showed strong positive correlation between nodule dry weight and N content ($r=0.563$, $p \leq 0.01$) for pea plant.

4.8. Seed protein

The inoculated field pea plants with the effective inoculants (FBR 15 and standard reference strain) showed the highest seed protein content (25%) which was higher by 28% compared to the negative control plants, and indicating a significant difference of 12-16-% ($p < 0.001$) from other inoculated treatments (Table 14). The result was similar to the finding of Solaiman and Rabbani (2005) that showed a difference between the highest seed protein content (24.6%) of inoculated plants and the lowest protein content (17.4%) recorded from uninoculated pea plants

4.9. Nitrogen Derived from Fixation (Biological nitrogen fixation)

Rhizobium inoculation of field pea showed significance variation ($p \leq 0.05$) in %Ndfa (Table 14). The highest Ndfa (74.4%) was recorded in plants inoculated with strain 1018 followed by plants inoculated with isolate FBR 15, whereas non inoculants resulted in the lowest Ndfa (44.8%). Seed inoculation significantly influenced the amount of N fixed, which increased and significantly difference between inoculated and the control. The best criteria for a rhizobium used as bio-fertilizer is that it must be highly effective in nitrogen fixing ability forming symbiotic association with the host plants (O' Hara *et al*., 2002). This result similar to the result of Clayton *et al.* (2004) documented that the highest nitrogen fixation 76.2% from rhizobial inoculation and the lowest nitrogen fixation 59% from the control field pea plant.

5. CONCLUSION AND RECOMMENDATION

The tolerance of rhizobial isolates to different pH levels, temperature, salinity, carbon and nitrogen utilization, phosphate solublization and antibiotics and heavy metal resistance is an important quality of rhizobial strains to screen and develop inoculants that are capable with ecological competitiveness. In the presence of different environmental stresses, the tolerant isolates would survive, occupy nodules, and fix nitrogen and provide the host to boost plant production.

The result of this experiment indicated that inoculation of selected rhizobial isolate FBR 11, FBR 15 FBR 23 and commercially released rhizobial strain 1018 improved number of nodule, nodule dry weight, shoot dry weight, number of pod plant^{-1}, number of seed pod^{-1}, grain yield, N content, seed protein and nitrogen fixation as compared to recommended N and the control. The study showed that isolate FBR 15 and strain 1018 were the most effective inoculants with nodule occupancy of 70-75% and were highly effective under greenhouse conditions, whereas the other inoculants were effective and occupied 50-60% of the nodules of the inoculated plants. Also from the results of the correlation analysis showed that the nodule number was directly and highly significantly ($p \leq 0.01$) correlated with shoot dry weight ($r=0.49$), number of pod ($r=0.59$), number of seed ($r=0.63$), total grain yield ($r=0.56$) and N up take ($r=0.80$). The result indicated that there was no significant difference between rhizobial isolate FBR15 and commercial rhizobial strain 1018 but there was significant difference with rhizobial isolate FBR 11 and FBR 23. Also selected rhizobial isolates was shown that highly effective and competitive. Based on the findings of this study, inoculation with selected rhizobial isolates improved nitrogen uptake and symbiotic nitrogen fixation efficiencies in field pea.

The data in general, showed that the rhizobial inoculant, particularly FBR 15 was nutritionally versatile, ecologically competent, and symbiotically effective rhizobia comparable to the commercial inoculant Strain 1018.

Based on the findings of this study the following recommendations are forwarded

- Rhizobial isolate FBR 15 can be used as commercial inoculants for pea production after it is tested (validated) at different agro-ecological conditions.
- Selected rhizobial isolate FBR 15 can be recommended as bio-fertilizer for better field pea production in the future.

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7. APPENDIX

Appendix1: ANOVA for nodulation parameters

Source of variation	Degree of freedom	Nodule number		Nodule dry weigh(mg plant^{1})		Shoot dry weight (g plant^{-1})	
		Mean square	F-value	Mean square	F-value	Mean square	F-value
Replication	2	30.39	1.13^{ns}	13.94	0.67^{ns}	1.88	2.46^{ns}
Treatment	5	6128.25	228.19*	4650.1	223.26*	97.91	127.75*
Error	9	26.85		4.56		0.76	
Means		68.12		58.64		24.47	
CV (%)		7.61		7.78		3.57	

^{ns} = Non-significant; * = Significant at P $\leq$ 0.05, CV= Coefficient of variation

Appendix 2: Pearson Correlation coefficient of same parameters

	TGY	NS	NP	SDW	NN	SN
TGY	1.00000					
NS	0.81960	1.00000				
	<.0001					
NP	0.95485	0.82734	1.00000			
	<.0001	<.0001				
SDW	0.87015	0.74527	0.89715	1.00000		
	<.0001	0.0004	<.0001			
NN	0.56521	0.63180	0.59617	0.49362	1.00000	
	0.0145	0.0049	0.0090	0.0374		
SN	0.80791	0.89454	0.85961	0.73468	0.80466	1.00000
	<.0001	<.0001	<.0001	0.0005	<.0001	

Appendix 3 Different parameters result on tested treatments

Parameter	Best performing tests	Negative control	From otrhers	Note
NN	15, 1018	10X	25%	
NDW	"	12X	21%	
SDW	15, 1018, +N control	2X	10-20%	
PH	NSD	20%	NSD	
No pod/plant	15, 1018, +N control	2X	>20%	
No seed/pod	15, 1018	2X	12%	
1000 seed wt	15, 1018, +C	2X	20%	
BY	NSD	35%	NSD	
TGY	NSD	35%	NSD	
Grain N	15, 1018	50%	18%	
Grain Nuptake	15, 1018	77%	15%	
StrawN	15, 1018	41%	12%	
N uptake	15, 1018	2X	21%	
Seed protein	15, 1018	28%	12-16%	

Appendix 4: Different laboratory test result of isolate FBR11, FBR15 and FBR23

Appendix 4.1: Presumptive Test and Colony Morphology of Isolates from strain FBR 11

isolates	size of the colony (mm)	colony type	colony shape	colony texture	Colony color	Gram rxn test	YEMA-BTB	YEMA-CR	PGA
FBR11-1	4	MM	Convex	buttery	W	-	Yellow	+	-
FBR11-2	2.5	MM	Convex	buttery	W	-	Yellow	+	-
FBR11-3	3	LM	Convex	elastic	W	-	Yellow	+	+
FBR11-4	4	LM	Convex	elastic	W	-	Yellow	+	+
FBR 11-5	5	LM	Convex	elastic	W	-	Yellow	+	+
FBR 11-6	4	LM	Convex	elastic	W	-	Yellow	+	+
FBR 11-7	4.5	LM	Convex	buttery	W	-	Yellow	+	-
FBR 11-8	3	MM	Convex	buttery	W	-	Yellow	+	-
FBR 11-9	3.8	LM	Convex	elastic	W	-	Yellow	+	+
FBR 11-10	2.5	MM	Convex	elastic	W	-	Yellow	+	+
FBR 11-11	2.8	MM	Convex	buttery	W	-	Yellow	+	-
FBR 11-12	4.2	LM	Convex	elastic	W	-	Yellow	+	+
FBR 11-13	4	LM	Convex	elastic	W	-	Yellow	+	+
FBR 11-14	2.5	MM	Convex	elastic	W	-	Yellow	+	+
FBR 11-15	3.5	LM	Convex	elastic	W	-	Yellow	-	-
FBR 11-16	3	MM	Convex	buttery	W	-	Yellow	+	-
FBR11-17	3	MM	convex	elastic	W	-	Yellow	+	+
FBR11-18	5	LM	convex	elastic	W	-	Yellow	+	+
FBR11-19	3.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 11-20	4	LM	convex	elastic	W	-	Yellow	+	+
FBR 11-21	4	LM	convex	elastic	W	-	Yellow	+	+
FBR 11-22	4.5	LM	convex	elastic	w	-	Yellow	+	+
FBR 11-23	4.5	LM	Convex	buttery	W	-	Yellow	+	-
FBR 11-24	4	LW	Convex	buttery	W	-	Yellow	+	-
FBR 11-25	3.8	LM	convex	elastic	W	-	Yellow	+	+
FBR 11-26	3.8	LM	convex	elastic	W	-	Yellow	+	+
FBR 11-27	5.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 11-28	4.5	LM	Convex	buttery	W	-	Yellow	+	-
FBR 11-29	4	LM	convex	elastic	W	-	Yellow	+	+
FBR 11-30	2.5	MM	convex	elastic	w	-	Yellow	+	+

+ = Positive rxn, - = Negative rxn, MM =medium mucoid, LM=large mucoid

Appendix 4.2: Amino acid and Carbon source test of isolates from strain FBR 11

	Amino acids				Carbon sources		
isolates	Glutamine	Phenylalnine	peptone	Arginine	Glucanate	galactose	Fructose
FBR11-1	+	+	+	+	+	+	+
FBR11-2	+	+	+	+	+	+	+
FBR11-3	+	+	+	+	+	+	-
FBR11-4	-	+	+	-	-	+	+
FBR 11-5	+	+	+	+	+	+	+
FBR 11-6	+	-	-	+	+	+	+
FBR 11-7	-	-	-	-	-	+	-
FBR 11-8	+	+	+	+	+	+	+
FBR 11-9	+	+	-	+	+	+	+
FBR 11-10	-	+	+	-	-	-	-
FBR 11-11	+	+	+	+	+	+	+
FBR 11-12	-	+	+	-	-	+	+
FBR 11-13	+	-	-	+	+	+	+
FBR 11-14	+	+	+	+	+	+	+
FBR 11-15	+	+	-	+	+	+	+
FBR 11-16	+	+	+	+	+	+	+
FBR11-17	-	-	-	-	-	+	+
FBR11-18	+	+	-	+	+	+	+
FBR11-19	+	+	-	+	+	+	+
FBR 11-20	+	+	+	+	+	+	+
FBR 11-21	-	-	-	-	-	+	+
FBR 11-22	+	+	+	+	+	+	+
FBR 11-23	+	+	-	+	+	+	+
FBR 11-24	+	+	-	+	+	+	+
FBR 11-25	+	-	+	+	+	+	+
FBR 11-26	+	+	+	+	+	+	+
FBR 11-27	-	+	+	-	-	-	-
FBR 11-28	+	+	+	+	+	+	+
FBR 11-29	+	+	+	+	+	+	+
FBR 11-30	+	-	-	+	+	+	+
Total growth	23	17	24	25	23	25	26
percentage	76.7	56.7	80	83.3	76.7	83.3	86.7

+ = growth and - = no growth

Appendix 4.3: PH tolerance test of Isolates from strain FBR 11

Isolates	4	4.5	5	5.5	8	8.5	9	9.5
FBR11-1	-	-	+	+	+	+	+	-
FBR11-2	-	+	+	+	+	-	-	-
FBR11-3	-	-	+	+	+	+	-	-
FBR11-4	-	-	-	+	+	+	+	-
FBR 11-5	-	-	+	+	+	+	-	-
FBR 11-6	-	-	-	+	+	+	+	-
FBR 11-7	-	+	+	+	+	+	-	-
FBR 11-8	-	-	+	+	+	+	+	-
FBR 11-9	-	+	+	+	+	+	+	-
FBR 11-10	-	-	-	+	+	+	-	-
FBR 11-11	-	-	+	+	+	+	-	-
FBR 11-12	-	-	+	+	+	+	+	-
FBR 11-13	-	-	+	+	+	+	-	-
FBR 11-14	-	-	-	+	+	+	+	-
FBR 11-15	-	-	+	+	+	+	-	-
FBR 11-16	-	-	-	+	+	+	+	-
FBR11-17	-	-	-	+	+	+	-	-
FBR11-18	-	-	+	+	+	+	+	-
FBR11-19	-	-	+	+	+	+	+	-
FBR 11-20	-	-	-	+	+	+	-	-
FBR 11-21	-	-	+	+	+	+	+	-
FBR 11-22	-	-	-	+	+	+	-	-
FBR 11-23	-	-	+	+	+	+	+	-
FBR 11-24	-	-	-	+	+	+	-	-
FBR 11-25	-	-	-	+	+	+	+	-
FBR 11-26	-	-	+	+	+	+	+	-
FBR 11-27	-	-	-	+	+	+	-	-
FBR 11-28	-	-	-	+	+	+	+	-
FBR 11-29	-	-	+	+	+	+	-	-
FBR 11-30	-	-	+	+	+	+	-	-
Total growth	0	3	18	30	30	29	15	0
percentage	0	10	60	100	100	96.7	50	0

Appendix 4.4: Temperature and salt tolerance test of Isolates from strain FBR 11

isolates	4^0c	10^0c	15^0c	35^0c	40^0c	Salt concentration				
						2	3	4	5	6
FBR11-1	-	-	-	+	-	+	+	+	-	-
FBR11-2	-	-	+	+	-	+	+	-	-	-
FBR11-3	-	-	-	+	-	+	+	-	-	-
FBR11-4	-	-	-	+	-	+	+	+	+	-
FBR 11-5	-	-	-	-	-	+	+	+	+	+
FBR 11-6	-	-	-	+	-	+	+	-	-	-
FBR 11-7	-	-	-	+	-	+	+	+	+	-
FBR 11-8	-	-	-	+	-	+	+	-	-	-
FBR 11-9	-	-	-	+	-	+	+	+	+	+
FBR 11-10	-	-	-	+	-	+	+	+	-	-
FBR 11-11	-	-	-	+	-	+	+	+	-	+
FBR 11-12	-	-	-	+	-	+	+	+	+	-
FBR 11-13	-	-	-	+	-	+	-	-	-	-
FBR 11-14	-	-	+	-	-	+	+	+	+	-
FBR 11-15	-	-	-	+	-	+	+	-	-	-
FBR 11-16	-	-	-	+	-	+	+	-	-	-
FBR11-17	-	-	-	+	-	+	+	+	+	+
FBR11-18	-	-	+	+	-	+	+	+	+	-
FBR11-19	-	-	+	+	-	+	+	-	-	-
FBR 11-20	-	-	-	+	-	+	+	+	+	+
FBR 11-21	-	-	-	+	-	+	+	+	+	-
FBR 11-22	-	-	+	+	-	+	+	+	-	-
FBR 11-23	-	-	-	-	-	+	+	+	+	-
FBR 11-24	-	-	-	+	-	+	+	+	+	-
FBR 11-25	-	-	-	+	-	+	+	+	-	-
FBR 11-26	-	-	+	+	-	+	+	+	+	+
FBR 11-27	-	-	-	+	-	-	-	-	-	-
FBR 11-28	-	-	-	+	-	+	+	+	+	+
FBR 11-29	-	-	-	+	-	+	+	-	-	-
FBR 11-30	-	-	+	+	-	+	+	+	-	-
Total growth	0	0	7	27	0	30	29	20	14	7
percentage	0	0	23.3	90	0	100	96.7	66.7	46.7	23.3

Appendix 4.5: Heavy metal test of Isolates from strain FBR 11

isolates	Zn		Pb		Cu	Hg		Ni	Co	Cr
	25	50	100	500	100	5	10	60	100	100
FBR11-1	+	+	+	+	+	-	-	+	+	+
FBR11-2	+	+	+	+	+	+	-	+	+	+
FBR11-3	+	+	+	-	+	+	-	+	+	+
FBR11-4	+	-	+	-	-	-	-	+	+	+
FBR 11-5	+	+	+	+	+	+	-	+	+	+
FBR 11-6	+	+	+	-	+	-	-	+	-	+
FBR 11-7	+	+	+	-	+	+	-	-	+	+
FBR 11-8	+	+	+	+	+	+	-	+	+	+
FBR 11-9	+	+	+	+	-	-	-	-	-	-
FBR 11-10	+	+	+	-	+	-	-	+	+	+
FBR 11-11	+	-	+	-	+	-	-	+	+	+
FBR 11-12	+	+	+	+	+	+	-	+	+	+
FBR 11-13	-	-	+	-	+	+	-	+	+	+
FBR 11-14	+	+	+	+	+	-	-	+	-	+
FBR 11-15	+	+	+	-	+	-	-	+	-	+
FBR 11-16	+	-	+	+	+	-	-	+	+	+
FBR11-17	+	-	+	-	+	+	-	+	-	+
FBR11-18	+	+	+	-	+	-	-	-	+	+
FBR11-19	+	+	+	+	+	-	-	-	+	+
FBR 11-20	+	-	+	-	+	+	-	+	+	+
FBR 11-21	+	+	+	+	+	-	-	-	+	+
FBR 11-22	+	+	+	+	+	+	-	+	+	+
FBR 11-23	+	+	+	-	+	-	-	+	+	+
FBR 11-24	+	-	+	-	+	-	-	+	-	+
FBR 11-25	+	+	+	+	+	+	-	-	+	+
FBR 11-26	+	+	+	+	+	-	-	+	+	+
FBR 11-27	+	+	+	+	+	-	-	+	+	+
FBR 11-28	+	+	+	-	+	+	-	+	+	+
FBR 11-29	+	-	+	+	+	+	-	-	-	-
FBR 11-30	+	+	+	+	+	-	-	+	+	+
Total growth	29	22	30	16	28	13	0	23	23	28
percentage	96.7	73.3	100	53.3	93.3	43.3	0	76.7	76.7	93.3

Appendix 4.6: Intrinsic Antibiotics resistance of Isolates from strain FBR 11

Isolates	Tetracycline					Erythromycin					Streptomycin				
	5	10	20	30	50	5	10	20	30	50	5	10	20	30	50
FBR11-1	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+
FBR11-2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR11-3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR11-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-5	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+
FBR 11-6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-8	+	+	-	-	-	+	+	+	-	-	+	+	+	+	+
FBR 11-9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-10	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+
FBR 11-11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-12	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-14	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+
FBR 11-15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-16	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
FBR11-17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR11-18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR11-19	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-20	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
FBR 11-21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-23	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+
FBR 11-24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-25	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+
FBR 11-26	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-29	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+
FBR 11-30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Total growth	30	30	25	22	21	30	30	30	22	22	30	30	30	30	30
percentage	100	100	83.33	73.3	70	100	100	100	73.3	73.3	100	100	100	100	100

Isolates	Penicillin					Chloroamphenicol					Neomycin				
	5	10	20	30	50	5	10	20	30	50	5	10	20	30	50
FBR11-1	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+
FBR11-2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR11-3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR11-4	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-
FBR 11-5	+	-	-	-	-	+	+	+	-	-	+	+	+	-	+
FBR 11-6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
FBR 11-7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-8	+	+	-	-	-	+	+	+	+	-	+	+	+	-	+
FBR 11-9	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
FBR 11-10	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-
FBR 11-11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
FBR 11-13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-14	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-
FBR 11-15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
FBR 11-16	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
FBR11-17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR11-18	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-
FBR11-19	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
FBR 11-20	+	+	-	-	-	+	+	+	+	-	+	+	+	+	-
FBR 11-21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-22	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-23	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-
FBR 11-24	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
FBR 11-25	+	+	-	-	-	+	+	+	+	-	+	+	+	-	+
FBR 11-26	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 11-27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
FBR 11-28	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
FBR 11-29	+	+	-	-	-	+	+	+	+	-	+	+	+	-	+
FBR 11-30	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Total growth	30	25	23	21	21	30	30	30	26	15	30	30	30	22	20
percentage	100	83.3	76.7	70	70	100	100	100	86.7	51	100	100	100	73.3	66.7

isolates	Ampicillin					Nalidixic acid				
	5	10	20	30	50	5	10	20	30	50
FBR11-1	+	+	+	+	+	+	+	+	-	-
FBR11-2	+	+	+	+	+	+	+	+	+	+
FBR11-3	+	+	+	+	+	+	+	+	+	+
FBR11-4	+	+	+	+	+	+	+	-	-	-
FBR 11-5	+	+	+	-	-	+	+	+	+	+
FBR 11-6	+	+	+	+	+	+	+	+	-	-
FBR 11-7	+	+	+	+	+	+	+	+	+	+
FBR 11-8	+	+	+	+	+	+	+	+	+	+
FBR 11-9	+	+	+	+	+	+	+	+	-	-
FBR 11-10	+	+	+	+	+	+	+	-	-	-
FBR 11-11	+	+	+	+	+	+	+	+	+	+
FBR 11-12	+	+	+	+	+	+	+	+	+	+
FBR 11-13	+	+	+	+	+	+	+	+	-	-
FBR 11-14	+	+	+	+	+	+	+	+	+	+
FBR 11-15	+	+	+	-	-	+	+	-	-	-
FBR 11-16	+	+	+	+	+	+	+	+	-	-
FBR11-17	+	+	+	+	+	+	+	+	-	-
FBR11-18	+	+	+	+	+	+	+	+	+	+
FBR11-19	+	+	+	+	+	+	+	+	+	-
FBR 11-20	+	+	+	+	+	+	+	+	-	-
FBR 11-21	+	+	+	+	+	+	+	+	+	-
FBR 11-22	+	+	+	+	+	+	+	+	+	+
FBR 11-23	+	+	+	+	+	+	+	+	-	-
FBR 11-24	+	+	+	+	+	+	+	+	+	+
FBR 11-25	+	+	+	+	+	+	+	+	+	+
FBR 11-26	+	+	+	+	+	+	+	+	+	-
FBR 11-27	+	+	+	+	+	+	+	-	-	-
FBR 11-28	+	+	+	-	-	+	+	+	-	-
FBR 11-29	+	+	+	+	+	+	+	-	-	-
FBR 11-30	+	+	+	+	+	+	+	+	+	+
Total growth	30	30	30	27	27	30	30	25	16	13
percentage	100	100	100	90	90	100	100	83.3	53.3	43

Appendix 4.7: Presumptive Test and Colony Morphology of Isolates from strain FBR 15

isolates	size of the colony (mm)	colony type	colony shape	colony texture	Colony color	Gram rxn test	YEMA-BTB	YEMA-CR	PGA
FBR15-1	2.5	MM	convex	buttery	W	-	Yellow	+	-
FBR15-2	2.5	MM	convex	elastic	W	-	Yellow	+	+
FBR15-3	3.5	LM	convex	elastic	W	-	Yellow	+	+
FBR15-4	4.8	LM	convex	buttery	W	-	Yellow	+	-
FBR 15-5	5.2	LM	convex	buttery	W	-	Yellow	+	-
FBR 15-6	4.2	LM	convex	buttery	W	-	Yellow	+	-
FBR 15-7	4.2	LM	convex	elastic	W	-	Yellow	+	+
FBR 15-8	3.2	MM	convex	elastic	W	-	Yellow	+	+
FBR 15-9	3.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 15-10	4.5	MM	convex	buttery	W	-	Yellow	+	-
FBR 15-11	4.8	MM	convex	buttery	W	-	Y ellow	+	-
FBR 15-12	4	LM	convex	elastic	W	-	Yellow	+	+
FBR 15-13	4.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 15-14	3.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 15-15	3.5	LM	convex	buttery	W	-	Yellow	+	-
FBR 15-16	3.8	LM	convex	elastic	W	-	Yellow	+	+
FBR15-17	3	MM	convex	elastic	W	-	Yellow	+	+
FBR15-18	5	LM	convex	buttery	W	-	Yellow	+	-
FBR15-19	3.2	LM	convex	buttery	W	-	Yellow	+	-
FBR 15-20	4.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 15-21	2.4	MM	convex	elastic	W	-	Yellow	+	+
FBR 15-22	2.5	MM	convex	elastic	W	-	Yellow	+	+
FBR 15-23	5.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 15-24	4.2	LM	convex	buttery	W	-	Yellow	+	-
FBR 15-25	4.8	LM	convex	buttery	W	-	Yellow	+	-
FBR 15-26	3.8	LM	convex	elastic	W	-	Yellow	+	+
FBR 15-27	5.2	LM	convex	buttery	W	-	Yellow	+	-
FBR 15-28	4.2	LM	convex	elastic	W	-	Yellow	+	+
FBR 15-29	3.2	MM	convex	elastic	W	-	Yellow	+	+
FBR 15-30	3.5	LM	convex	elastic	W	-	Yellow	+	+

+ = Positive rxn, - = Negative rxn, MM =medium mucoid, LM=large mucoid

Appendix 4.8: Amino acid and Carbon source test of isolates from strain FBR 15

	Amino acids				Carbon sources		
isolates	Glutamine	Phenylalnine	peptone	Arginine	Glucanate	galactose	Fructose
FBR15-1	-	+	+	+	-	+	+
FBR15-2	+	+	+	+	+	+	+
FBR15-3	+	+	+	+	-	+	-
FBR15-4	-	+	-	-	-	+	+
FBR 15-5	+	+	+	+	+	+	+
FBR 15-6	+	+	-	+	+	+	+
FBR 15-7	-	-	+	-	-	+	-
FBR 15-8	+	+	+	+	+	+	+
FBR 15-9	+	+	+	+	+	+	+
FBR 15-10	-	+	-	+	-	-	-
FBR 15-11	+	+	+	+	+	+	+
FBR 15-12	-	+	-	-	-	+	+
FBR 15-13	+	-	+	+	+	+	+
FBR 15-14	+	+	-	+	+	+	+
FBR 15-15	+	+	-	+	-	+	+
FBR 15-16	+	+	+	+	-	+	+
FBR15-17	-	-	+	+	-	+	+
FBR15-18	+	+	-	+	+	+	+
FBR15-19	+	+	+	+	-	+	+
FBR 15-20	+	+	-	+	+	+	+
FBR 15-21	-	+	+	+	-	+	+
FBR 15-22	+	+	+	+	+	+	+
FBR 15-23	+	+	-	+	+	+	+
FBR 15-24	+	+	+	+	-	+	+
FBR 15-25	+	+	+	+	+	+	+
FBR 15-26	+	+	+	+	+	+	+
FBR 15-27	-	+	-	-	-	-	-
FBR 15-28	+	+	+	+	+	+	+
FBR 15-29	+	+	+	+	+	+	+
FBR 15-30	+	+	+	+	-	+	+
Total growth	24	27	20	26	15	29	27
Percentage	80	90	66.7	86.7	50	96.7	90

Appendix 4.9: PH tolerance test of Isolates from strain FBR 15

isolates	4	4.5	5	5.5	8	8.5	9	9.5
FBR15-1	-	-	-	+	+	+	+	-
FBR15-2	-	-	+	+	+	+	-	-
FBR15-3	-	+	+	+	+	+	+	-
FBR15-4	-	+	-	+	+	+	+	-
FBR 15-5	-	-	+	+	+	+	-	-
FBR 15-6	-	-	-	+	+	+	+	-
FBR 15-7	-	-	+	+	+	+	+	-
FBR 15-8	-	-	-	+	+	+	-	-
FBR 15-9	-	-	-	+	+	+	-	-
FBR 15-10	-	+	+	+	+	+	+	-
FBR 15-11	-	-	-	+	+	+	+	-
FBR 15-12	-	-	+	+	+	+	-	-
FBR 15-13	-	-	+	+	+	+	+	-
FBR 15-14	-	-	-	+	+	+	+	-
FBR 15-15	-	-	+	+	+	+	-	-
FBR 15-16	-	-	+	+	+	+	-	-
FBR15-17	-	+	-	+	+	+	-	-
FBR15-18	-	-	-	+	+	+	+	-
FBR15-19	-	-	+	+	+	+	+	-
FBR 15-20	-	+	+	+	+	+	+	-
FBR 15-21	-	-	-	+	+	+	-	-
FBR 15-22	-	+	+	+	+	+	+	-
FBR 15-23	-	-	-	+	+	+	+	-
FBR 15-24	-	-	+	+	+	+	+	-
FBR 15-25	-	-	-	+	+	+	-	-
FBR 15-26	-	-	+	+	+	+	+	-
FBR 15-27	-	+	+	+	+	+		-
FBR 15-28	-	-	-	+	+	+	-	-
FBR 15-29	-	-	+	+	+	+	+	-
FBR 15-30	-	-	-	+	+	+	-	-
Total growth	0	7	19	30	30	30	14	0
Percentage	0	20	63.3	100	100	100	46.7	0

Appendix 4.10: Temperature and salt tolerance test of Isolates from strain FBR 15

isolates	4^0c	10^0c	15^0c	35^0c	40^0c	Salt concentration				
						2	3	4	5	6
FBR15-1	-	-	-	+	-	+	-	+	+	-
FBR15-2	-	-	-	+	-	+	+	-	-	-
FBR15-3	-	-	+	+	-	+	+	+	+	-
FBR15-4	-	-	-	+	-	+	+	+	-	-
FBR 15-5	-	-	-	-	-	+	+	+	-	
FBR 15-6	-	-	-	+	-	+	+	+	+	+
FBR 15-7	-	-	-	+	-	+	+	+	+	+
FBR 15-8	-	-	+	+	-	+	-	-	-	-
FBR 15-9	-	-	-	+	-	+	-	-	-	-
FBR 15-10	-	-	-	+	-	+	+	+	+	+
FBR 15-11	-	-	-	+	-	+	+	+	+	-
FBR 15-12	-	-	+	+	-	+	+	-	-	-
FBR 15-13	-	-	-	+	-	+	+	+	+	+
FBR 15-14	-	-	-	+	-	+	+	+	+	-
FBR 15-15	-	-	-	+	-	+	+	+	-	-
FBR 15-16	-	-	-	+	-	+	+	+	-	-
FBR15-17	-	-	+	+	-	+	+	+	+	+
FBR15-18	-	-	-	+	-	+	-	-	-	-
FBR15-19	-	-	-	+	-	+	+	-	-	-
FBR 15-20	-	-	-	+	-	+	+	+	+	+
FBR 15-21	-	-	+	+	-	+	-	+	-	-
FBR 15-22	-	-	-	+	-	+	+	+	+	-
FBR 15-23	-	-	-	+	-	+	+	+	-	-
FBR 15-24	-	-	-	+	-	+	+	+	+	+
FBR 15-25	-	-	+	+	-	+	+	-	-	-
FBR 15-26	-	-	-	+	-	+	+	+	-	-
FBR 15-27	-	-	-	+	-	+	-	-	-	-
FBR 15-28	-	-	-	+	-	+	+	+	+	+
FBR 15-29	-	-	+	+	-	+	+	+	-	-
FBR 15-30	-	-	-	+	-	+	+	+	+	-
Total growth	0	0	7	29	0	30	24	23	15	8
Percentage	0	0	24	96.7	0	100	80	76.7	50	26.7

Appendix 4.11: Heavy metals test of Isolates from strain FBR 15

Isolates	Zn		Pb		Cu	Hg		Co	Ni	Cr
	25	50	100	500	100	5	10	100	60	100
FBR15-1	+	+	+	-	+	-	-	+	-	+
FBR15-2	+	-	+	+	+	+	-	+	+	+
FBR15-3	+	-	+	+	-	-	-	+	+	+
FBR15-4	+	+	+	+	+	+	-	+	+	+
FBR 15-5	+	+	+	-	+	-	-	+	-	+
FBR 15-6	+	-	+	-	+	-	-	+	+	+
FBR 15-7	+	+	+	+	+	+	-	+	+	+
FBR 15-8	+	+	+	-	+	+	-	+	+	+
FBR 15-9	+	+	+	+	-	-	-	+	+	+
FBR 15-10	+	+	+	-	+	-	-	+	+	+
FBR 15-11	+	+	+	+	+	+	-	+	-	+
FBR 15-12	+	+	+	+	+	-	-	+	+	+
FBR 15-13	+	-	+	+	+	+	-	+	+	+
FBR 15-14	+	+	+	-	-	-	-	+	-	+
FBR 15-15	+	+	+	+	+	+	-	+	+	+
FBR 15-16	+	-	+	-	+	-	-	+	-	+
FBR15-17	+	+	+	-	+	+	-	+	+	+
FBR15-18	+	+	+	+	+	-	-	+	+	+
FBR15-19	+	+	+	-	+	-	-	+	+	+
FBR 15-20	+	-	+	+	+	+	-	+	-	+
FBR 15-21	+	+	+	+	+	-	-	+	+	+
FBR 15-22	+	+	+	+	+	+	-	+	+	+
FBR 15-23	+	+	+	-	+	+	-	+	+	+
FBR 15-24	+	+	+	+	+	+	-	+	-	+
FBR 15-25	+	+	+	+	+	-	-	+	+	+
FBR 15-26	+	+	+	-	+	-	-	+	+	+
FBR 15-27	-	-	+	+	+	-	-	+	+	+
FBR 15-28	+	+	+	-	+	+	-	+	+	+
FBR 15-29	+	+	+	+	+	+	-	+	-	+
FBR 15-30	+	+	+	-	+	-	-	+	+	+
Total growth	30	23	30	17	30	14	0	24	22	30
Percentage	100	76.7	100	56.7	100	46.7	0	80	73.3	100

Appendix 4.12: Intrinsic Antibiotics resistance of Isolates from strain FBR 15

isolates	Tetracycline					Erythromycin					Streptomycin				
	5	10	20	30	50	5	10	20	30	50	5	10	20	30	50
FBR15-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+
FBR15-2	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
FBR15-3	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
FBR15-4	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+
FBR 15-5	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-
FBR 15-6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 15-7	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-
FBR 15-8	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
FBR 15-9	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
FBR 15-10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 15-11	+	+	+	+	-	+	+	-	-	-	-	-	+	+	+
FBR 15-12	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-
FBR 15-13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 15-14	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
FBR 15-15	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-
FBR 15-16	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
FBR15-17	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
FBR15-18	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-
FBR15-19	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
FBR 15-20	+	+	+	+	+	+	+	-	-	-	+	+	-	-	-
FBR 15-21	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
FBR 15-22	+	+	+	-	-	+	+	+	+	+	+	+	+	-	-
FBR 15-23	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-
FBR 15-24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 15-25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 15-26	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
FBR 15-27	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
FBR 15-28	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-
FBR 15-29	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
FBR 15-30	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Total growth	30	30	30	22	14	30	30	27	27	27	27	27	20	17	17
percentage	100	100	100	73.3	45	100	100	90	90	90	90	90	68	56.7	56.7

isolates	Penicillin					Chloroamphenicol					Neomycin				
	5	10	20	30	50	5	10	20	30	50	5	10	20	30	50
FBR15-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR15-2	-	-	-	-	-	+	+	-	-	-	+	+	+	+	+
FBR15-3	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
FBR15-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
FBR 15-5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 15-6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 15-7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
FBR 15-8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 15-9	+	+	-	-	-	+	-	-	-	-	+	+	+	+	-
FBR 15-10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
FBR 15-11	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
FBR 15-12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 15-13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
FBR 15-14	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-
FBR 15-15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 15-16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR15-17	+	+	-	+	+	+	+	+	+	+	+	-	-	-	-
FBR15-18	+	+	+	-	-	+	+	+	+	+	+	+	+	-	-
FBR15-19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
FBR 15-20	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
FBR 15-21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 15-22	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+
FBR 15-23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 15-24	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
FBR 15-25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
FBR 15-26	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-
FBR 15-27	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
FBR 15-28	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
FBR 15-29	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+
FBR 15-30	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
Total growth	27	27	24	22	22	30	27	26	26	26	29	27	25	22	14
percentage	90	90	80	73.3	73.3	100	90	86.7	86.7	86.7	96.7	90	83.3	73.3	43

isolates	Ampicillin					Nalidixic acid				
	5	10	20	30	50	5	10	20	30	50
FBR15-1	+	+	-	-	-	+	+	+	-	-
FBR15-2	+	+	+	+	+	+	+	+	+	+
FBR15-3	+	+	+	+	+	+	+	+	+	+
FBR15-4	+	+	+	+	+	+	+	+	+	+
FBR 15-5	+	+	+	+	+	+	+	+	+	+
FBR 15-6	+	+	+	+	+	+	+	+	+	+
FBR 15-7	+	+	+	-	-	+	+	-	-	-
FBR 15-8	+	+	+	+	+	+	+	+	+	+
FBR 15-9	+	+	+	-	-	+	+	+	-	-
FBR 15-10	+	+	+	+	+	+	+	+	+	+
FBR 15-11	+	+	+	+	+	+	+	+	+	+
FBR 15-12	+	+	+	+	+	+	+	+	+	+
FBR 15-13	+	+	+	+	+	+	+	+	+	+
FBR 15-14	+	+	+	+	+	+	+	+	+	+
FBR 15-15	+	+	-	-	-	+	+	-	-	-
FBR 15-16	+	+	+	+	+	+	+	+	+	+
FBR15-17	+	+	+	+	+	+	+	+	+	+
FBR15-18	+	+	+	+	+	+	+	+	+	+
FBR15-19	+	+	+	+	+	+	+	+	+	+
FBR 15-20	+	+	+	+	+	+	+	+	+	+
FBR 15-21	+	+	+	+	+	+	+	+	+	+
FBR 15-22	+	+	+	+	+	+	+	+	+	+
FBR 15-23	+	+	+	+	+	+	+	+	+	+
FBR 15-24	+	+	+	+	+	+	+	+	+	+
FBR 15-25	+	+	+	+	+	+	+	+	+	+
FBR 15-26	+	+	+	+	+	+	+	+	+	+
FBR 15-27	-	-	-	-	-	+	+	-	-	-
FBR 15-28	+	+	+	+	+	+	+	+	+	+
FBR 15-29	+	+	+	+	+	+	+	+	+	+
FBR 15-30	+	+	+	+	+	+	+	+	+	+
Total growth	29	29	27	25	25	30	30	27	25	24
percentage	96.7	96.7	90	83.3	83.3	100	100	90	83.3	80

Appendix 4.13: Presumptive Test and Colony Morphology of Isolates from strain FBR 23

Isolates	size of the colony (mm)	colony type	colony shape	colony texture	Colony color	Gram rxn test	YEMA-BTB	YEMA-CR	PGA
FBR23-1	3.5	LM	Convex	elastic	w	-	Yellow	+	+
FBR23-2	3	MM	Convex	elastic	w	-	Yellow	+	+
FBR23-3	3.8	LM	convex	elastic	W	-	Yellow	+	+
FBR23-4	4	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-5	4.5	LM	convex	buttery	W	-	Yellow	+	-
FBR 23-6	4.2	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-7	4	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-8	3.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-9	3.8	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-10	2.8	MM	convex	elastic	W	-	Yellow	+	+
FBR 23-11	2.8	MM	convex	elastic	W	-	Yellow	+	+
FBR 23-12	3.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-13	3.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-14	3	MM	convex	buttery	W	-	Yellow	+	-
FBR 23-15	4.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-16	3	MM	convex	elastic	W	-	Yellow	+	+
FBR23-17	3	MM	convex	elastic	W	-	Yellow	+	+
FBR23-18	5.2	LM	convex	buttery	W	-	Yellow	+	-
FBR23-19	3.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-20	4.5	LM	convex	buttery	W	-	Yellow	+	-
FBR 23-21	4.2	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-22	4.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-23	3.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-24	4	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-25	3.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-26	3	MM	convex	elastic	W	-	Yellow	+	+
FBR 23-27	4.5	LM	convex	buttery	W	-	Yellow	+	-
FBR 23-28	5.5	LM	convex	buttery	W	-	Yellow	+	-
FBR 23-29	3.5	LM	convex	elastic	W	-	Yellow	+	+
FBR 23-30	2.8	MM	convex	buttery	w	-	Yellow	+	-

+ = Positive rxn, - = Negative rxn, MM =medium mucoid, LM=large mucoid and MW=medium watery

Appendix 4.14: Amino acid and Carbon source test of isolates from strain FBR 23

	Amino acids				Carbon sources		
isolates	Glutamine	Phenylalnine	peptone	Arginine	Glucanate	galactose	Fructose
FBR15-1	+	-	+	-	+	+	+
FBR15-2	-	+	+	+	+	+	+
FBR15-3	-	+	-	-	-	-	-
FBR15-4	+	-	-	-	+	+	+
FBR 15-5	+	+	+	+	+	+	+
FBR 15-6	+	+	+	+	+	+	+
FBR 15-7	-	-	-	-	-	-	-
FBR 15-8	+	+	+	+	+	+	+
FBR 15-9	-	+	+	+	+	+	+
FBR 15-10	+	-	+	-	+	-	-
FBR 15-11	-	+	+	+	-	+	+
FBR 15-12	+	-	-	-	+	+	+
FBR 15-13	+	+	+	+	+	+	+
FBR 15-14	+	+	+	+	+	+	+
FBR 15-15	+	-	+	-	+	+	+
FBR 15-16	-	-	+	-	-	+	+
FBR15-17	+	-	+	-	+	+	+
FBR15-18	+	+	+	+	+	+	+
FBR15-19	+	-	+	-	+	+	+
FBR 15-20	-	+	+	+	+	+	+
FBR 15-21	+	-	+	-	-	+	+
FBR 15-22	-	+	+	+	-	+	+
FBR 15-23	-	+	+	+	+	+	+
FBR 15-24	+	-	+	-	+	+	+
FBR 15-25	+	+	+	+	+	+	+
FBR 15-26	-	+	+	+	+	+	+
FBR 15-27	+	-	-	-	-	-	-
FBR 15-28	+	+	+	+	+	+	+
FBR 15-29	+	+	+	+	+	+	+
FBR 15-30	+	+	+	-	+	+	+
Total growth	14	17	25	15	23	27	27
Percentage	46.7	56.7	83.3	50	76.7	90	90

Appendix 4.15: PH tolerance test of Isolates from strain FBR 23

isolates	4	4.5	5	5.5	8	8.5	9	9.5
FBR23-1	-	-	-	+	+	+	+	-
FBR23-2	-	-	+	+	+	+	+	-
FBR23-3	-	-	+	+	+	+	-	-
FBR23-4	-	-	-	+	+	+	+	-
FBR 23-5	-	-	+	+	+	+	-	-
FBR 23-6	-	-	-	+	+	+	+	-
FBR 23-7	-	-	-	+	+	+	+	-
FBR 23-8	-	-	-	+	+	+	+	-
FBR 23-9	-	-	+	+	+	+	-	-
FBR 23-10	-	-	+	+	+	+	-	-
FBR 23-11	-	-	-	+	+	+	+	-
FBR 23-12	-	-	+	+	+	+	-	-
FBR 23-13	-	-	+	+	+	+	+	-
FBR 23-14	-	-	-	+	+	+	-	-
FBR 23-15	-	-	-	+	+	+	+	-
FBR 23-16	-	-	+	+	+	+	-	-
FBR23-17	-	-	+	+	+	+	+	-
FBR23-18	-	-	+	+	+	+	-	-
FBR23-19	-	-	+	+	+	+	-	-
FBR 23-20	-	-	-	+	+	+	+	-
FBR 23-21	-	-	+	+	+	+	+	-
FBR 23-22	-	-	+	+	+	+	-	-
FBR 23-23	-	-	-	+	+	+	+	-
FBR 23-24	-	-	-	+	+	+	+	-
FBR 23-25	-	-	-	+	+	+	+	-
FBR 23-26	-	-	+	+	+	+	-	-
FBR 23-27	-	-	+	+	+	+	-	-
FBR 23-28	-	-	+	+	+	+	-	-
FBR 23-29	-	-	-	+	+	+	+	-
FBR 23-30	-	-	+	+	+	+	-	-
Total growth	0	-	17	30	30	30	15	0
Percentage	0	-	56.7	100	100	100	50	0

Appendix 4.16: Temperature and salt tolerance test of Isolates from strain FBR 23

Isolates	4^0c	10^0c	15^0c	35^0c	40^0c	Salt concentration				
						2	3	4	5	6
FBR23-1	-	-	-	+	-	+	+	-	-	-
FBR23-2	-	-	-	+	-	+	+	+	+	-
FBR23-3	-	-	+	+	-	+	+	+	-	-
FBR23-4	-	-	-	+	-	+	+	-	-	-
FBR 23-5	-	-	-	+	-	+	+	+	+	+
FBR 23-6	-	-	-	-	-	+	-	-	-	-
FBR 23-7	-	-	+	+	-	+	+	+	+	+
FBR 23-8	-	-	-	+	-	+	+	+	+	+
FBR 23-9	-	-	-	+	-	+	+	+	+	-
FBR 23-10	-	-	-	+	-	+	+	-	-	-
FBR 23-11	-	-	-	+	-	+	+	+	-	-
FBR 23-12	-	-	+	+	-	+	+	+	+	+
FBR 23-13	-	-	+	+	-	-	-	-	-	-
FBR 23-14	-	-	-	+	-	+	+	+	-	-
FBR 23-15	-	-	-	+	-	+	+	+	+	+
FBR 23-16	-	-	-	+	-	+	+	+	+	-
FBR23-17	-	-	-	+	-	+	+	+	+	-
FBR23-18	-	-	-	+	-	+	+	+	+	-
FBR23-19	-	-	-	-	-	+	-	-	-	-
FBR 23-20	-	-	-	+	-	+	+	+	-	-
FBR 23-21	-	-	-	+	-	+	+	+	+	+
FBR 23-22	-	-	-	+	-	+	+	+	+	-
FBR 23-23	-	-	-	+	-	+	+	+	-	-
FBR 23-24	-	-	+	+	-	+	-	-	-	-
FBR 23-25	-	-	-	+	-	+	+	+	+	-
FBR 23-26	-	-	+	+	-	+	+	+	+	+
FBR 23-27	-	-	-	+	-	+	-	-	-	-
FBR 23-28	-	-	-	+	-	+	+	+	+	-
FBR 23-29	-	-	-	+	-	+	+	+	+	+
FBR 23-30	-	-	-	+	-	+	+	+	-	-
Total growth	0	0	6	28	0	29	24	22	16	8
Percentage	0	0	20	95.5	0	96.7	79	73.3	53.3	26.7

Appendix 4.17: Heavy metals test and generation time of Isolates from strain FBR 23

isolates	Zn		Pb		Cu	Hg		Ni	Co	Cr
	25	50	100	500	100	5	10	60	100	100
FBR23-1	+	+	+	+	-	-	-	+	-	+
FBR23-2	+	+	+	-	+	-	-	+	+	+
FBR23-3	+	+	+	-	+	+	-	+	+	+
FBR23-4	+	-	+	+	+	+	-	-	+	+
FBR 23-5	+	+	+	+	+	+	-	-	+	+
FBR 23-6	+	+	+	-	+	-	-	+	-	+
FBR 23-7	+	+	+	+	+	+	-	+	+	+
FBR 23-8	+	+	+	-	+	-	-	+	+	+
FBR 23-9	+	-	+	-	+	-	-	+	+	+
FBR 23-10	+	+	+	+	+	-	-	+	+	+
FBR 23-11	+	-	+	+	+	+	-	+	+	+
FBR 23-12	+	+	+	+	+	+	-	-	+	+
FBR 23-13	+	+	+	-	+	-	-	+	+	+
FBR 23-14	+	-	+	+	+	+	-	+	+	-
FBR 23-15	+	+	+	+	+	-	-	+	+	+
FBR 23-16	+	+	+	-	+	-	-	+	+	+
FBR23-17	+	+	+	-	+	+	-	+	+	+
FBR23-18	+	-	+	-	+	-	-	-	-	+
FBR23-19	+	+	+	+	+	-	-	+	-	-
FBR 23-20	+	+	+	+	+	+	-	+	-	+
FBR 23-21	+	-	+	+	+	-	-	-	+	+
FBR 23-22	+	+	+	-	+	-	-	+	+	+
FBR 23-23	+	+	+	+	+	+	-	+	+	+
FBR 23-24	+	-	+	-	+	+	-	+	-	+
FBR 23-25	+	-	+	+	+	+	-	-	-	+
FBR 23-26	+	+	+	-	+	-	-	-	+	+
FBR 23-27	+	+	+	+	+	-	-	+	+	+
FBR 23-28	+	+	+	-	+	-	-	+	+	+
FBR 23-29	+	+	+	-	+	-	-	-	+	+
FBR 23-30	+	-	+	+	+	+	-	+	-	+
Total growth	30	22	30	16	29	13	0	23	22	28
Percentage	100	73.3	100	53.3	96.7	43.3	0	76.7	73.3	93.3

Appendix 4.18: Intrinsic Antibiotics resistance of Isolates from strain FBR 23

isolates	Tetracycline					Erythromycin					Streptomycin				
	5	10	20	30	50	5	10	20	30	50	5	10	20	30	50
FBR23-1	+	+	-	-	-	+	+	+	-	-	+	+	+	+	+
FBR23-2	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
FBR23-3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR23-4	+	+	-	-	-	+	+	+	-	-	+	+	+	+	+
FBR 23-5	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
FBR 23-6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-8	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
FBR 23-9	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
FBR 23-10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-11	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
FBR 23-12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-14	+	+	-	-	-	+	+	+	-	-	+	+	+	+	+
FBR 23-15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-16	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
FBR23-17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR23-18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR23-19	+	+	-	-	-	+	+	+	-	-	+	+	+	+	+
FBR 23-20	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-
FBR 23-21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-22	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
FBR 23-23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-25	+	+	-	-	-	+	+	+	-	-	+	+	+	+	+
FBR 23-26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-27	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
FBR 23-28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-29	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-
FBR 23-30	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
Total growth	30	30	25	20	18	30	30	27	25	24	30	30	27	27	27
percentage	100	100	83.3	70	60	100	100	90	83.3	80	100	100	90	90	90

isolates	Penicillin					Chloroamphenicol					Neomycin				
	5	10	20	30	50	5	10	20	30	50	5	10	20	30	50
FBR23-1	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
FBR23-2	+	+	-	-	-	+	-	-	+	+	+	+	+	+	-
FBR23-3	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-
FBR23-4	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-
FBR 23-5	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
FBR 23-6	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-
FBR 23-7	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
FBR 23-8	+	+	+	-	-	+	+	+	+	+	+	+	+	-	-
FBR 23-9	+	+	-	-	-	+	-	-	-	-	+	+	-	-	-
FBR 23-10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-13	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-14	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-
FBR 23-15	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
FBR 23-16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR23-17	+	+	-	-	-	+	-	-	-	-	+	+	+	+	-
FBR23-18	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-
FBR23-19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-20	+	+	-	-	-	+	+	+	+	+	+	+	-	-	-
FBR 23-21	+	+	-	-	-	+	+	+	+	+	+	+	+	+	-
FBR 23-22	+	+	+	-	-	+	-	-	-	-	+	+	+	+	-
FBR 23-23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-24	+	+	-	-	-	+	+	+	+	+	+	+	+	-	-
FBR 23-25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-26	+	+	+	-	-	+	-	-	+	+	+	+	+	+	-
FBR 23-27	+	+	-	-	-	+	+	+	-	-	+	+	+	+	-
FBR 23-28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FBR 23-29	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
FBR 23-30	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Total growth	30	30	20	18	16	30	25	25	23	23	30	30	27	9	16
percentage	100	100	66.7	60	55	100	83.3	83.3	76.7	76.7	100	100	90	30	55

isolates	Ampicillin					Nalidixic acid				
	5	10	20	30	50	5	10	20	30	50
FBR23-1	+	+	+	+	+	+	+	+	-	-
FBR23-2	+	+	+	+	-	+	+	-	-	-
FBR23-3	+	+	+	-	-	+	+	-	-	-
FBR23-4	+	+	+	+	+	+	+	+	-	-
FBR 23-5	+	+	+	-	+	+	+	+	+	+
FBR 23-6	+	+	+	+	+	+	+	+	-	-
FBR 23-7	+	+	+	-	+	+	+	+	+	+
FBR 23-8	+	+	+	+	+	+	+	+	-	-
FBR 23-9	+	+	-	-	-	+	-	-	-	-
FBR 23-10	+	+	+	+	+	+	+	+	+	+
FBR 23-11	+	+	+	+	+	+	+	+	+	+
FBR 23-12	+	+	+	+	+	+	+	+	+	+
FBR 23-13	+	+	+	+	-	+	+	-	+	+
FBR 23-14	+	+	+	+	+	+	+	+	-	-
FBR 23-15	+	+	+	+	+	+	+	+	+	+
FBR 23-16	+	+	+	+	+	+	+	+	+	+
FBR23-17	+	+	+	-	-	+	+	-	-	-
FBR23-18	+	+	+	+	+	+	+	+	-	-
FBR23-19	+	+	+	+	+	+	+	+	+	+
FBR 23-20	+	+	-	+	-	+	-	-	-	-
FBR 23-21	+	+	+	+	-	+	+	-	-	-
FBR 23-22	+	+	+	-	+	+	+	+	-	-
FBR 23-23	+	+	+	+	+	+	+	+	+	+
FBR 23-24	+	+	+	+	-	+	+	-	-	-
FBR 23-25	+	+	+	+	+	+	+	+	+	+
FBR 23-26	+	+	+	+	+	+	+	+	-	-
FBR 23-27	+	+	+	-	-	+	+	-	-	-
FBR 23-28	+	+	+	+	+	+	+	+	+	+
FBR 23-29	+	+	-	+	+	+	-	+	+	+
FBR 23-30	+	+	+	+	+	+	+	+	+	+
Total growth	30	30	27	23	20	30	27	20	15	15
percentage	100	100	90	76.7	66.7	100	90	66.7	50	50

Appendix 5: Sampling sites of rhizobial isolates Ethiopia

Rhizobial Isolate	Region	Name of isolation site	Altitude of Isolation site (m.a.s.)	Latitude	Longitude	Colony color	Colony diameter (mm)	Growth rate	Growth on BTB
FBR11	Amara	Enemay	2470	10-39-49-N	38-10-36-E	Large Watery	5	Fast	Yellow
FBR15	Amara	Goncha Soso Enese	2450	37-51-00-N	11-02-00-E	Medium Watery	4	Fast	Yellow
FBR23	Amara	Enemay	2550	10-30-54-N	38-09-57-E	White	5	Fast	Yellow

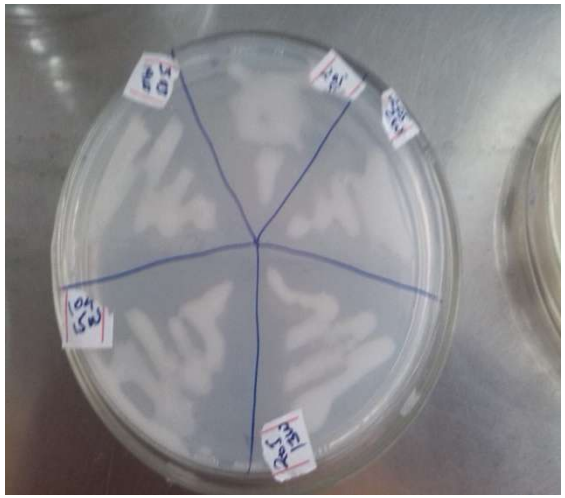
Appendix 6a: Rhizobial isolates Pre-history of biochemical and physiological test results

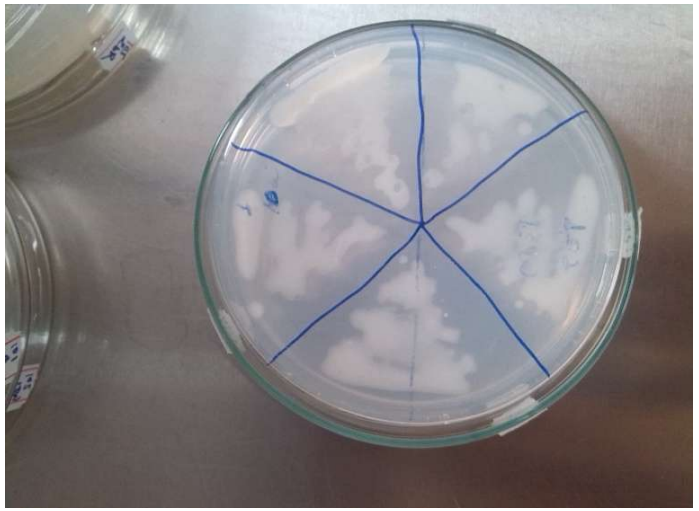
Rhizobial isolate	C&N-sources utilization(%)		Antibiotics resistance (µg/ml)								Salt tolerance in %
	C	N	Penic	Amp	Strept	Eryth	Chloro	Neo	Tetra	Nalid	
FBR11	80	74	5-50	5-50	5-50	5-50	5-50	5-50	5-50	5-50	0.5-6%
FBR15	90	90	5-50	5-50	5-50	5-50	5-50	5-50	5-50	5-50	0.5-6%
FBR23	82	72	5-50	5-50	5-50	5-50	5-50	5-50	5-50	5-50	0.5-6%

Appendix 6b: Rhizobial isolates Pre-history of biochemical and physiological test results

Rhizobial isolate	Heavy Metal tolerance (µg/ml)							pH tolerance	Temp. tolerance
	Cu	Ni	Hg	Cr	Co	Pb	Zn		
FBR 11	100	60	5	100	100	100	25-50	5-9	15-35
FBR 15	100	60	5	100	100	100	25-50	4.5-9	15-35
FBR 23	100	60	5	100	100	100	25-50	5-9	15-40

Appendix 7: Laboratory, green house and field pictures

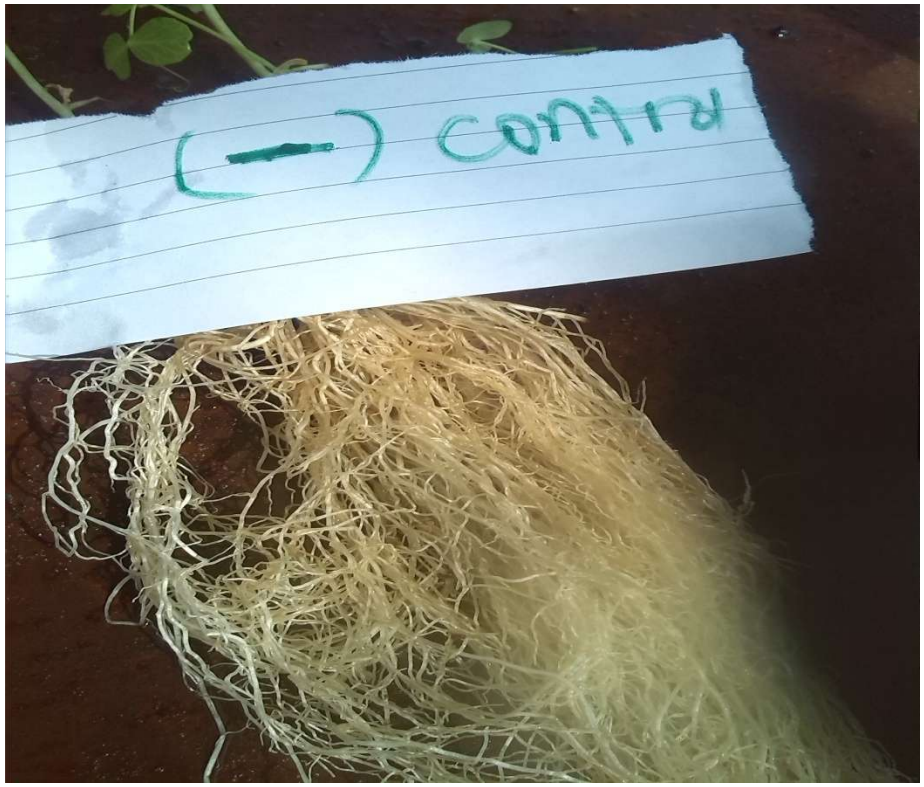




1. Pure culture

2. Field pea under field condition

Field pea under green house condition



3. Inoculated

Non inoculeted