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Rhizobial Status and Strain Identification of Some Pulse Fields at Ankober, Keyt, Mehal-Meda and Molale (Northern Shewa)

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Abstract

This study considers the rhizobial status of some localities in the Northern Shewa Zone, where faba bean yield has decreased. Composite soil samples were taken from representative areas of two yield depleted areas, Molale and Mehal-Meda and two yield sustained areas, Keyt and Ankober. Soil analysis was done on the four soil samples to know their nutrient content and status of rhizobial population. It was found that the number of rhizobia per gram of yield depleted area soils was very low where as it was very high in the yield sustained area soils. The phosphorus and potassium content of Molale and Mehal-Meda soils was also lower than that of Ankober and Keyt soils. However, the nitrogen content of all the four soil samples was high. Also four *Rhizobium leguminosarum* strains were isolated from these soil samples by planting the host plant faba bean. The isolates were identified as different strains by using PCR-based amplification of specific DNA fragments of nitrogen fixing promoter region of the *Rhizobium* genome. The relative symbiotic effectiveness of the four isolates and one exotic strain Tal 1397N was determined in sand culture. The results showed that all the four local isolates and the exotic strain haven't caused any significant improvement from the control in any of the growth parameters except nodule fresh weight. Pot experiment was conducted to know the effect of inoculation by the local isolates and the exotic strain (Tal 1397N) and nitrogen and phosphorus fertilizers (urea and DAP) on the growth of faba bean.

I. Introduction

Pulse crops in Ethiopia occupy 12 to 15% of the land under cultivation. They are grown for both domestic consumption and export (Badege, 1987). Faba bean (*Vicia faba*), cheak pea (*Cicer arietenium*), field pea (*Pisum sativum*) and lentil (*Lens culinaris*) are the major highland pulse crops in Ethiopia. Interms of hectarage they rank 1-4 respectively (Beniwal, 1987).

Ethiopia is dependent to a large extent on the export of agricultural products including pulses. In 1981 the share of pulses was 4.38% but this was decreased to 0.93% in 1991. Through these years the share of highland pulses in the export market has followed a declining trend, becoming nil in 1991. The main importers of Ethiopian high land pulse crops were Germany, Djibouti, France, Mauritius, Yemen, and Saudi Arabia (Hailu *et al.,* 1994).

Faba bean is most commonly found in the 'Weyna Dega' *i.e.*, between 1800 and 2400 meters elevation (Bond *et al.*, 1985). Its greatest concentration is in Shewa, Wollo, Tigray, Gojam and Gonder regions (Amare *et al.* 1987). Botanically, it has been divided into varieties minor, equina and major on the basis of seed size although there is no discontinuity in seed size between them (Witcombe, 1982). In Ethiopia, mainly medium and small-seeded types are known to occur though large size types were introduced in the country by Portuguese around 1900. Medium size

seeds are predominant in south Showa, Arsi, Bale, Hararge and Sidamo highlands where as small seeded ones are common in Northern Shoa. (Beniwal, 1987). Historically Ethiopia is considered as the secondary center of diversity and also one of the nine major agro-geographical production regions of faba bean (Bond *et al.* 1985).

Northern Shoa zone, which is found in the Amhara region was one of the major highland pulse producing areas of the country. However, in recent years pulse production in the area has been reduced, except at some places like Ankober and Keyt, even the farmers themselves could not get for their own personal consumption.

There are many factors, which affect growth and Yield of faba bean. Some of them are pests and diseases, weeds, frost damage, late maturity, drought, water logging, hot temperature, and pollination (Bond *et al.,* 1985). In Ethiopia, chocolate spot and rust are the two most important diseases on faba bean with occasional damage in some zones from root rot and powdery mildew. Among insects boll worm and aphids may become important depending on the season. Frost is another hazard almost every year especially on the higher altitudes and certainly every 3-4 year cycle. Higher flower and pod abortion rate is also recognized as important even though the cause for this has not as yet been learned (Imru, 1981). The other biological constraints that limit productivity of faba bean are weeds. A weed

population survey carried out in the 1986 crop season in the Yerer and Kereyu, Menagesha and Salale awraja of Shewa administrative region had shown that most faba bean farmers do not weed their faba bean fields as they preferred to weed their cereal crops. The very few who weeded did so only once and that too in the podding stage (Badege 1987).

To get good yield of faba bean, it is necessary that the plant can get adequate quantities of essential mineral nutrients like nitrogen, phosphorus, and potassium (Bond et. al., 1985). Faba bean like other legumes can fix nitrogen in an endosymbiotic relationship with root nodule bacteria, generally called rhizobia. This capability makes it able to grow on nitrogen depleted soils. Although faba bean fix nitrogen, its effectivity depends on the availability of enough number of rhizobia in the soil, their strain i.e., they are effective nitrogen fixers or not and other environmental factors.

All rhizobia which infect faba bean do not have the same effectiveness in fixing atmospheric nitrogen. Some strains may be more effective nitrogen fixers than others, some can be tolerant to different environmental stresses like high soil temperature, available nitrogen (Martensson *et al.,* 1987), acidity and salinity. Therefore, before application of rhizobial inoculants or any other soil correction measures, it is good to know the rhizobial status of the soil, the kind of strains found in it and the ones which are going to be introduced.

This study concentrated in the Northern Shoa zone, where faba bean yield has decreased. According to information gathered from the farmers, some places such as Molale and Mehal Meda were not able to grow faba bean for the past ten years. One of the plausible hypothesis is that the symbiont rhizobia might not be sufficient and/or effective in nitrogen fixation.

Therefore, a study was made to know the rhizobial status of the soils of representative pulse fields of these places and compare with the yield sustained areas. Soil samples were also taken, rhizobia were isolated and pot experiment was conducted in a green house by cross inoculating the isolates of yield depleted and sustained areas. Also strain identification test was done for the rhizobial isolates using DNA finger printing techniques.

II. Litrature Review

Biological Nitrogen Fixation

Nitrogen is one of the most important macro nutrients which limit plant growth and crop productivity. Proteinaceous matter of plant, animal or bacterial tissue, either structural or enzymatic, contains nitrogen. It is also a component of many secondary metabolities which have a variety of functions, from plant growth regulators to defense compounds (Mengal, 1992). Air is inexhaustible and supplies large quantity of di-nitrogen however, nitrogen should be combined with hydrogen and oxygen to be available for plant growth. Such combinations have been made possible in nature through biological nitrogen fixation and physical processes like ultraviolet radiaton, fires and lightening. The other way of nitrogen fixation is the manufacture of nitrogenous fertilizers. The industrial process through which nitrogenous fertilizers are produced is known as Haber-Bosch process. It requires a considerable quantity of energy, up to 800^0F temperature, a catalyst and high pressure (Chatt, 1980; Subarao, 1988).

Biological nitrogen fixation is the reduction of atmospheric dinitrogen into ammonia, by prokaryotic microorganisms. These organisms can be either free living (fixing nitrogen non-symbotically) or symbiotic (fixing nitrogen in association with host plant). The enzyme which catalyses the reduction of molecular nitrogen to ammonia

is called nitrogenase, it is apparently very similar in all species and has the requirement for large amounts of energy and an anaerobic environment. It consists two oxygen sensitive proteins, azoferredoxin and molybdoferrodoxin (Brown, 1982; Gottschalk, 1979).

Some of the prerequisites for biological nitrogen fixation are; large amount of energy (5-30 mol of ATP per mol N_2 reduced), exclusion of oxygen and optimum temperature. The most obvious problem arising from N_2-fixation is that of resolving the conflicting demands of a large energy requirement and the need to protect the enzyme, nitrogenase, from irreversible damage by oxygen. Therefore, prokaryotes have evolved methods of oxygen protection (Brown, 1982).

Soils get gradually depleted of available nitrogen if they are cropped continuously and if the nitrogen removed by plant parts is not restored. To solve the problem of soil infertility it is necessary to add nitrogen in the form of organic nitrogen, chemical fertilizer or biologically fixed nitrogen to soils. Today, the annual rate of industrially fixed dinitrogen amounts to $73x10^6$ tonn nitrogen, where as that of biological nitrogen fixation (BNF) is about $118x10^6$ tonn per year (Mengal,1992). This shows the amount of chemically fixed dinitrogen is about 60% of the biologically fixed one. Biological nitrogen fixation is environmentally friendly and cheap. The whole process takes place without causing environmental pollution and at a relatively low cost.

Nitrogen fixing microorganisms

The prokaryotic organisms which fix atmospheric nitrogen can be either free living or symbiotic which need host plant to supply the energy required for the process.

Free living nitrogen fixing bacteria are present in most soils and in plant rhizospheres. There are several different bacterial species that can fix nitrogen in laboratory culture and may do so in the rhizosphere. Bacterial species like *Azotobacter* and *Clostridium* fix nitrogen aerobically and anaerobically respectively (Brown, 1982). Several species of blue green algae can also fix nitrogen. The principal genrera are *Anabena, Nostoc* and *Cylindrospermum* (Meyer *et al.*, 1960). Photosynthetic bacteria such as rhodospirillum also fix nitrogen (Stevenson, 1986). Researchers show that the maximum amount of nitrogen added to soils by free living bacteria is not more than 6 kg N/ha (Stevenson, 1986). This shows that the amount of nitrogen fixed by non symbiotic N_2 fixers in soils under intensive cultivation would appear to be too low to have much practical impact.

In symbiotic nitrogen fixation two members are required for the association, a plant and a microorganism. The seat of the symbiosis can be intracellular with in nodules that appear on the plant roots and stem gland or extra cellular in leaf space (Bond, 1967). According to Silvester (1977) some examples of symbiosis of ecological significance are between:

i. Angiosperm genus *Gunnera* and Cyanobacterium *Nostoc.*

ii. Angiosperm genus *Alnus* (Alder) and actinomycete *Frankia.*

iii. Pteridophyta genus *Azolla* and blue green algae A*nabaena.*

iv. *Rhizobium* or *Bradyrhizobium* and leguminous association.

Nitrogen fixing Cyanobacterium, *Nostoc* functions as micro symbiont in various genera of the gymnosperm family *Cycadacea* and in the angiosperm genus *Gunnera* (family Haloragaceae). Association of blue green algae *Anabena* vs *Azolla*, a freshwater fern, is used extensively in parts of south east Asia as a green manure crop and substitute for N fertilizers in rice paddy culture. The alga inhabits a cavity on the ventral surface of azolla and lives symbiotically while fixing nitrogen (Stevenson, 1986).

Of all other types of biological nitrogen fixation, *Rhizobium* legume symbiosis and nitrogen fixation is the most important one, since it contributes to increase in yield of food legumes, forage and forestry products. The importance of this relationship is emphasized by the fact that, even with the tremendous expansion of facilities that produce fertilzier nitrogen, legumes are still the main source of fixed nitrogen for a large portion of the worlds soils over the past three decades (Mengel,1992).

The general aim of agricultural research is to increase food production per unit area of land or to improve the economic efficiency of production *i.e.,* maintain output at

a reduced cost. Research into N_2 fixation specifically aims to enhance rates and efficiency of fixation in order to increase production of protein per unit area, reduce dependence upon fertilizer nitrogen, and reverse the wide spread decline in the nitrogen status of many of our arable soils. Therefore, nowadays, many new inocula strains of Rhizobia are being produced and released in USA, Canada, China, India, Brazil, Italy, Finland and many other countries to improve pulse production (Pobell *et al.*, 1995).

Rhizobia

The name rhizobium is derived from two Greek words. "Rhiza" means root and "Bius" means life, together it means "which lives in the root" (Breed 1957). Rhizobia are a heterogeneous group of organisms with differences that are significant enough to form more than one genus. Therefore, they are diversified into three genera namely; Rhizobium, Bradyrhizobium (Jordan, 1982) and Azorhizobium (Dreyfus *et al.*,. 1988). Currently they are classified into many species.

There are several species of rhizobium associated with cool season food and forage legumes. They are:

Rhizobium leguminosarum biovar vicae, that nodulates *Lathyrus, Pisum, Vicia, Lens*

Rhizobium leguminosarum biovar trifolli, that nodulates trifollium.

Rhizobium tropica, noddulating *Phaseolus vulgaris* type II

Rhizobium etli, nodulating *Phaseolus vulgaris* type I

Rhizobium melliloti, nodulating *Medicago Trigonella*

Rhizobium lupini, nodulating *Lupinus Ornithopus*

Most of this group are fast growing forms and produce medium to large colonies (1-5mm), after three to five days at 25-28^{0}C. Colonies of slow growing rhizobia will be barely detectable at three to five days and even after ten days. They will commonly produce colonies not exceeding 1mm (Vincent, 1982).

Factors affecting the symbiosis

The symbiotic interaction between root nodule bacteria of the genus *Rhizobium* and leguminous plants leads to the establishment of nodules, specialized organs on the roots of the host plant in which the rhizobia fix atmospheric nitrogen. This symbiosis is affected by both biotic or abiotic environmental factors. Biotic factors can be competitiveness among species, compatibility of rhizobia with the plant, and rhizobial interaction with other soil microorganisms (Lindstrom, 1985; Gibson, 1977), inter and intraspecific interaction of the host plant (Wahua, 1984). Abiotic factors include amount of available macro and micro nutrients in the soil, pH, temperature, water stress, salinity, and irradiance (Gibson, 1977; Lindstrom, 1985; Mengel and Kaparath 1978)

Legumes like other higher plants require "essential elements" like C, H, O, N, P, K, Ca, Mg, S, Fe, B, Mn, Cu, Co, Zn, Mo, and Cl for growth. They get them either from the atmosphere or from their rooting medium. However the mineral nutrition of legumes is some what more complex than that of other plant species because of the special symbiotic relationship existing between the legume host and the associated rhizobia. Particular nutritional requirements are necessary for this extra physiological process as to operate efficiently (Smith, 1982). From an examination of direct comparative studies with a number of nutrients Munns (1977) has concluded that, when grown non symbioticaly there is no good evidence to indicate that the nutritional requirements of legumes differ consistently from any other large diverse groups of plants. Abundance of mineral nutrients affect survival and growth of the rhizobia in the soil, infection and nodulation of the host root, and functioning of nitrogen fixation reactions with in the nodule (Smith, 1982).

When trying to assess factors affecting legume *Rhizobium* symbiosis, it is important to consider the symbiosis as consisting of a number of different developmental phases, each of which can have specific nutrient requirements. The symbiosis can be divided into four major phases; host plant growth, rhizobial survival and growth, infection and nodule development, and nodule function (Loneragan, 1972).

a. Rhizosphere colonization and pre-infection stages

This phase is pH sensitive since the activity and survival of rhizobia in soils is affected indirectly by hydrogen ion concentration in the soil (Mengal *and Kaparath* 1978). Low pH causes calcium deficiency, unavailability of Mg, P and Mo and toxicity of Al or Mn (Alva *et al*,. 1987). Each of these factors can severely affect plant and rhizobial growth. Free living rhizobia have special requirement for calcium and magnesium (Lindstrom, 1985; Smith, 1982). Increasing calcium levels increases the number of rhizobia in the rhizosphere and stimulates nodulation even at low levels of inoculation (Lowther, 1970; Beck and Munns 1985).

Salinity also has an effect on the growth and survival of rhizobium in the soil. Tolerance to different environmental stresses differs in different strains of rhizobia. The competitiveness of a strain determines its fate in the rhizospehere. The difficulties in obtaining high recovery of inoculant strains due to competition of indigenous strains have been well documented (Howel *et al.*, 1987). Competitiveness means the ability to utilize organic carbon compounds in the soil, ability to withstand environmental stress and especially compatibility with the host plant (Lindstrom, 1985). Some times other micro organisms may also affect the survival and growth of rhizobia in the soil. For instance some fungi show antibacterial activity towards rhizobia and others are found to be antagonistic (Gibson, 1977).

b. Infection and Nodule Development

Infection and nodule development of the host plant is affected by availability of mineral nutrients in the soil and/or other factors like temperature, irradiance, pH and salinity. High or low light intensity also affects nodulation. Trang and Giddens (1980) showed that shading reduces number and dry weights of nodules in soya beans. The decrease in number of nodules per plant can be attributed to the lower production of roots and hence nodules (Gibson, 1977). High light intensity is also reported to be a cause of low level of nodule development (Gibson 1977).

Nodulation generally takes place at all soil temperatures tolerated by the free living plant, but nodule abundance is reduced at a cooler and warmer extreme (Alexander, 1960). High temperature can depress root hair formation, thus, reducing the sites for nodulation thereby affecting adherence of bacteria to hairs (Munns *et al.*, 1977). Low temperatures also seriously retard infection (Gibson, 1977) and early nodule development (Zhang *et al.,* 1995). Rhizobial infection and nodule initiation are also pH sensitive (Mengel and kaparath, 1978). Nodulation requires more calcium and higher pH than does nitrogen fixation and growth of plants with the already established nodules. For some legumes initiation of infection thread formation is the most sensitive step, that requires elevated pH. However, the range of pH in which nodulation is affected varies with the species and rhizobial strain (Munns, 1977).

Following infection, nodule formation involves the establishment of nodule meristem (nodule initiation), bacterial release into cortical cells, intracellular multiplication of the rhizobia and development of bacteroids (Vincent, 1980). After infection, rhizobia are completely enclosed by plant tissue and therefore, fully dependent on a supply of mineral nutrient through the host plant. In all infection processes and subsequent nodule development, the rhizobia are located physically within plant tissues but remain separated from the host cells (O'Hara *et al.*, 1988).

Boron shows a direct involvement in nodule development. Appearance of both nitrogen deficiency symptoms and characteristic boron deficiency symptoms on boron deficient legumes suggests the requirements of this element for nodule development is similar to that for growth of the host plant (Munns, 1977). Under severe boron deficiency, nodulation is totally inhibited in peas and beans, whereas under less severe boron deficiency nodule development was affected. These observations may reflect the critical sensitivity of developing meristems to boron deficiency and indicate nodule growth and development as the most sensitive phase of the symbiosis to the above (O'Hara *et al.*, 1988). Plants require boron for nucleic acid metabolism, therefore, lack of it affects meristematic tissues. With boron deficency growing points of roots and shoots stop elongating, become discolored, disorganized and die. Nodules also fail to form properly, they remain small and lack vascular strands and bacteroids (Smith, 1982). Boron helps in maintaining the integrity of cell wall and membranes. Pea nodules developed under

low boron conditions were not functional and become prematurely senescent. Boron deprived nodules showed a generalized degeneration of cell walls and membranes including the peribacteroid membrane (PBM) which surrounds intracellular bacteroids (Bolanos *et al.,* 1996).

Iron deficiency decreases nodule number in a number of legumes (Trang, 1995). Results from studies on the response of peanut and soyabean symbiosis to correction of Fe deficiency indicate specific limitation of nodule development by iron stress (O'Hara *et al.*, 1988).

c. Nodule Function and Nitrogen Fixation

An efficiently functioning legume nodule is an intracellular association between legume root cells and rhizobium which fixes atmospheric nitrogen. The biochemistry of nitrogen fixation involves a specific requirement for several mineral elements. However, the nodules requirement for most of these nutrients is generally much less than that for optimum growth of the plant. Two important exceptions to this are Mo and Co. Both of these play specific roles in nitrogen fixation and are required in higher amounts by legumes grown symbiotically than by other plants (Smith, 1982). Molybdenum is a constituent of the nitrogen fixing enzyme, nitrogenase, therefore, a sufficient supply of molybdenum is essential for nitrogen fixation in legumes. The highest requirements and concentration of molybdonum have been shown in plants of the *leguminosae* family (Janssen and

Vitash, 1974). Bacteroids completely fail to fix nitrogen in the absence of molybdenum. Several researches show that when soyabean plants receive bound nitrogen, no cobalt is required whereas in the absence of bound nitrogen, plant growth is strictly dependent upon the size of cobalt supply (Shkolink, 1984). The principal effects of Co deficiency would appear to be through the reduced activity of the cobaltamin dependent ribonucleoside-triphosphate reductase, limiting DNA synthesis and bacteroid division (O'Hara *et al.,* 1988). Therefore, noduls of molybdenum and Cobalt deficient plants fail to fix nitroge. However, the resulting nitrogen starvation is correctable by supplying combined nitrogen (Munns, 1977). Calcium, Copper, Zinc, Iron and Sulfur are also known to affect nitrogen fixation of legumes directly or indirectly (Demeterio *et al.*, 1972; O'Hara *et al.*, 1988; Trang, 1995).

Addition of combined nitrogen to the root medium in large amounts reduce root hair infection (Allison and Ludwig, 1934), nodule mass, and N_2 fixing activity of nodulated roots (Eaglesham *et al.,* 1983). The inhibitory effect of inorganic nitrogen is plant mediated. Especially nitrate reduces root hair infection, available infection and lectin binding on the root (Dazzo *et al.,* 1985). It also suppresses nodule formation, or accelerates their senescence and inhibites nitrogenase activity. An investigation on the impact of mineral nitrogen applied at 0 and 100 kg/ha showed that the later rate inhibits nitrogen fixation in white clover (*Trifolium repens*) and completely cease at level of about 200kg/ha (Berthelsen *et al.,* 1994).

Effective nitrogen fixation by symbiotic bacteria is generally followed by factors which promote good vegetative growth of the host plant (Meyer *et al.*, 1960). The symbiotic relationship of host plant and *Rhizobium* requires a carbohydrate supply from the shoot for nitrogen fixation and subsequent amide and amino acid production for plant use. Therefore macronutrients like potassium, phosphorus and calcium may affect N_2 fixation directly by limiting the growth of the host plant or indirectly interfering with rhizobial nutrition (Collins *et al.*, 1986). Phosphate plays a key role in the energetics of metabolism. It's shortage reduces growth and plants become stunted. It was demonstrated that phosphorus deficiency reduces the energy status and functioning of soyabean nodules. This is accompanied by decreased specific nitrogenase activity by inhibiting at least one energy dependent reaction in the plant cell fraction of nodules (Sa and Israel, 1991). Phosphorus application stimulates nitrogen uptake, therefore increasing amount of applied phosphorus above the level required for maximum dry matter gain has shown an increase in nitrogen content of plant tops (Smith, 1982). Valunzuela *et al.*, (1995) showed that phosphorus application increases nodulation in the presence of high soil nitrogen content, which inhibited nodulation in the absence of phosphorus in pea plants.

Physical, chemical and environmental factors like temperature, soil pH, water stress and salinity also affect nitrogen fixation and nodule function by legumes. Hungria and Franco, (1993) showed that temperature is the most important factor affecting

biological activity. It affects nodule functioning and accelerate senescence in *phaseolus vulgaris*. Temperatures well above 30^0C which occurred in the soil under alfalfa has rapidly eliminated nitrogenase activity in established nodules (Munns *et al.*, 1977). Nitrogen fixation is highly sensitive to water stress in a number of legumes. It limits nitrogen accumulation, dry matter and yields (Albrecht *et al.*, 1984; Venkateswarlu *et al.*, 1989). Both soil and air humidity influence the growth of the plant and nitrogen fixation. Too high soil humidity limits the diffusion of oxygen to the nodules and causes a build up of Carbondioxide whereas air humidity affects transpiration and flow of fixed nitrogen from nodules to different parts of the plant (Lindstrom, 1985). Severe water stress causes very low respiratory rates, complete cessation of nitrogenase activity and pronounced structural changes in nodules, physical damage to the nodule and the deprivation of oxygen which is required for energy production by the bacteroids are the reasons for reduced nitrogen fixation (Albrecht *et al.*, 1984; Ismaili *et al.*, 1983).

Identification Methods

Both to evaluate competitiveness of rhizobial strains in the field and isolate them from soil or plant nodules, simple and rapid methods of identification are desirable. Some of the methods are listed below.

a. Cultural characteristics

Rhizobia can be described according to their growth on solid and in liquid media. The size, shape, color and texture of colonies and the ability to alter the pH of the medium are generally stable characteristics useful in defining strains of isolates. Usually colonies are discrete, round, varying from flat to domed and even conical on agar surfaces, they usually have a smooth margin. Colonies may be white, opaque or they may be milky to watery translucent. The opaque colony growth is usually firm with little gum, whereas the less dense colonies are often gummy and soft (Breed *et al.*, 1957).

Rhizobia do not absorb congored when plates are incubated in the dark. Colonies remain white opaque or occasionally pink, while contaminating organisms usually absorb the red dye. However, some strains of *Rhizobium melliloti* are an exception and absorb congo red strongly. Freshly prepared yeast extract mannitol agar media (YEMA) plates containing bromothymol blue have a pH of 6.8 and are green. Slow growing rhizobia show an alkaline reaction in this medium turning the dye to blue. Fast growing rhizobia show an acid reaction, turning the medium to yellow. Rhizobia grow poorly, if at all on peptone, glucose agar and cause little change in pH, when incubated at 25-30^{0}C. Heavy growth on this media indicates contamination (Somasegaran and Hoben, 1994).

b. Molecular markers

Traditional approaches to studying rhizobial competition employ indigenous molecules as markers. For example, induced or intrinsic antibiotic resistance is used to identify rhizobial strains (Josey *et al.,* 1979; Turco *et al.,* 1986). Two of the most commonly used resistance markers are resistance to Rifampicin and Streptomycin (Wilson, 1995).

The other traditional class of molecular markers used in rhizobial ecology are antigens expressed on the cell surface that can be recognized by specific antiserum (Graham, 1963). Serology has been used more than any other technique in identification, classification and characterization of rhizobia and is second only to gel-electrophoresis of proteins in discriminatory power (Eaglesham and Sinclair, 1993).

Marker genes are also used as a means of studying competitive ability of rhizobial strains. Nowadays gusA gene from the bacterium *Esherchia coli is being used (Wilson et al., 1992).* Other marker genes like introduced antibiiotic resistance genes like transposon Tn 5 (Pillal and Pepper, 1991), the lacZY (ß-galactosidase and lactose permease) gene (Katupitiya *et al.,* 1995) and different sets of luciferase genes are also used as marker genes in the studies of rhizobial ecology.

Characteristic patterns and sequences of nucleic acids

Nucleic acid based protocols are mostly less time consuming and effective in arranging strains into coherent groups. More recently, the development and application of these methods has led to significant advances in the area of rhizobial identification and phylogenetic studies. Different approaches of nucleic acid detection methods exist, based on either detection of specific DNA sequences by hybridization or an amplification of characteristic sequences followed where necessary by detection through hybridization or restriction fragment length polymorphism (RFLP) (Richardson *et al.*, 1995).

DNA-DNA hybridization protocols form the basis for the determination of genera and species of microbes (Wayne, 1987). Detection of nucleic acids using hybridization technique involves the use of characteristic DNA sequences (probes) in the DNA or RNA of the organism under study. DNA hybridization is a matching of homologous sequences of the probe with the ones in the test organism. A major disadvantage of this technique is its high cost and lack of precision. Since closely related strains or organisms have very similar genome, it may be difficult to differentiate strains of a single species with this approach (Wilson, 1995).

The most promising nucleic acid based approach for rhizobial ecology at present is the amplification of characteristic finger prints of DNA bands from bacterial genomes by polymerase chain reaction (PCR) technique. DNA amplification

provides a powerful tool for the study of genetic variation (Wilson, 1995). There are two broadly defined classes of PCR-based protocols for amplification of specific DNA fragments and analysis on gels (Schneider and Bruijn, 1996).

The first is amplification of one or a few specific DNA fragments like portions of the rRNA operon, the variable but characteristic intergenic regions between the 16s and 23s rRNA genes or variable tRNA gene regions, recognizable DNA segments that are diagnostic for a genus, species or strain (Schneider and Bruijn, 1996; Laguerre *et al.*, 1996). PCR amplification of specific DNA sequences, allows specific and sensitive detection of bacteria at the genus, species or strain level depending on the design of the oligo nucleotide primers (Pillai *et al.*, 1992; Richardson *et al.*, 1995).

The second aspect is the use of DNA sequences that serve as primers to amplify numerous distinct fragments, which are electrophoretically size fractionated, the resulting banding pattern yields species or strain specific finger prints. The multiple DNA fragments are amplified simultaneously. The primers can be either random or pairs derived from repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC) sequences and box elements (Laguerre *et al.*, 1996). The protocols are collectively known as rep-PCR (Schneider and Bruijn, 1996). The use of short DNA sequences that serve as primers to amplify numerous distinct fragments is known as randomly amplified polymorphic DNA (RAPD) analysis (Schneider and Bruijn, 1996). This analysis is based on the theory that if any

oligonucleotide sequence was chosen at random, its distribution within the genome of differing individuals would vary. Following amplification of DNA using a single, randomly chosen primer sequence, the amplification products derived from separate individuals would be different (Harrison, 1992).

III. Materials and Methods

The rhizobium isolation, plant and soil chemical analysis and the pot experiments were carried out at the National Soil Laboratory, Ministry of Agriculture. The strain identification test was done at the Department of Biology , Addis Ababa University and Armaur Hansen Research Institute (AHRI), Addis Ababa.

1. Soil sampling

Composite soil samples of 0-20 cm depth, each from representative areas of Mehal-Meda, Molale, Keyt and Ankober were collected for all analysis.

2. *Rhizobium* Isolation

The soil bulk composite samples of the four study areas were grounded and 3kg was taken from each. Plastic pots were surface sterilized by swabbing with 70% ethyl alcohol. The soil samples were then filled in the three pots from each.

Fababean seeds, from improved variety Bulga 70, were surface sterilized according to Vincent (1970) with 95% ethyl alcohol and 0.2% acidified mercuric chloride solutions for three minutes with each. After washing with several changes of sterilized distilled water, five seeds were planted on each pot of the four soil samples, and were later thinned down to three after germination. After 45 days of

growth, plants were uprooted, nodules were picked, surface sterilized and macerated. Then they were cultivated on yeast extract mannitol agar with congored.

Yeast extract monnitol broath (YEMB)

Mannitol	10g
Magnesium Sulphate	0.5g
Sodium Chloride	0.2g
Yeast extract	0.1g
Distlled water	1litre

Yeast mannitol agar (YEMA)

YEMB	1 liter
Agar	15g

plates contain 2.5g/litre congored

slant contains 3g/litre $CaCo_3$

The plates were kept for 4-10 days at 28^0C and single colonies were purified by re-striking on new YEMA congo red plates. Finally a single colony was taken by nichrome wire loop and streaked onto YEMA/$CaCO_3$ slants to be stored. After incubating them for four days they were kept at 4^0C, according to Vincent (1970).

3. Rhizobial status determination

Rhizobial population in the soils was estimated by plant infection count method (Vincent, 1970), planting in sterilizable poly carbonyl plastic pouches using modified Jensen's nitrogen free medium.

Improved variety Bulga 70, faba bean seeds were used for seedling making. Seeds were surfaced sterilized, as described before and they were allowed to germinate on petridish until both hyla and radicals were properly seen. Healthy seedlings were transferred into previously sterilized pouches filled with Jensen's nitrogen free medium and folded blotting paper immersed.

Modified Jensen's N-free medium

$CaHPO_4$	1.0g
K_2HPO_4	0.2g
$MgSO_4.7H_2O$	0.2g
NaCl	0.2g
$FeCl_3$	0.1g
Trace elements (stock solution)	1ml
Distilled water	1 liter

Stock solution

H_3BO_3	2.86g
$MnSO_4.4H_2O$	2.03g
$ZnSO_4.7H_2O$	0.22g
$CuSO4.5H_2O$	0.08g
$Na_2MoO_4.2H_2O$	0.14g
Distilled water	1 litre

The seedlings were made to rest on the top surface of the blotting paper while the radicals are immersed into the folds of the blotting paper protected from light.

Ten fold serial dilutions up to 10^{-8} of each soil were made in series of 9ml sterile distilled water by taking 10gm of soil and diluting it in to 90ml of sterile water so as to make the first (10^{-1}) dilution shaking for half an hour, and there after, taking 1ml aliquot from each dilution and transferring to the next 9ml sterile distilled water until 10^{-8} dilution was made from each dilution. 1ml, aliquot was used as inoculant for the pouches seedlings. After 45 days of growth, the plants were taken out of the pouches, nodules counted and rhizobial density estimated. The estimate of the count, pergram of soil was calculated by the following formula (Somasegaram and Hoben, 1994).

$$X = \frac{mxd}{vxg}$$

Where m = most probable number at dilution 1 of the series

d = dilution represented by tube 1

v = volume of aliquot taken

g = weight of soil sample

The most probable numbers were calculated from Fisher and Yates (1963) table, cited in Vincent (1970).

4. Strain Identification

For identification of rhizobial strains, polymeric chain reaction (PCR) in conjunction with a 20 base oligonucleotide primer (5'-3') AATTTT CAA GGG TCG TGC CA corresponding to a repetitive *Rhizobium* nif (nitrogen fixing) gene promoter region, was used to produce specific "finger print" profile for each strain when analyzed by gel electrophoresis.

Preparation of template DNA for PCR analysis

PCR amplifications were performed on purified total genomic DNA. DNA was isolated from cultures grown on TY (Trypton yeast) media.

Tryptone-yeast (TY) medium

Tryptone	5.0g
Yeast Extract	3.0g
$CaCl_2.H_2O$	0.87g
Distilled water	1000ml
Agar	12g

The rhizobial cultures were collected from plates, placed in Eppendorf tubes and washed by centrifuging at 13,000rpm in 500μl 1xTE buffer to avoid the extra cellular polysaccaharides. Then 200μl 1xTE buffer was added and the tubes were placed three times in a boiling water and ice for 5 and 3 minutes respectively, to lyse the cells.

1xTE (pH=7.5)

Tris - Base	1.21g.
$Na_2EDTA.H_2O$	0.37g.
distilled water	1000ml.

They were then centrifuged for three minutes at 13,000 rpm to precipitate the cell debris. The supernatant was collected and transferred to another tube and 300μl chloroform was added to each tube, vortexed and centrifuged at 13,000 rpm for five minutes. The supernatants upper layer was taken and DNA was precipitated by

adding 30μl, 0.5M NaCl solution and 300μl Isopropanol. The tubes were placed in ice for five minutes to allow the DNA precipitate, then they were centrifuged for 30 minutes at 13,000 rpm. The pellet was washed by 70% ethanol and allowed to dry at room temperature and was dissolved in 30μl sterilized water. Then the double stranded DNA concentration was measured by UV absorbance at OD_{260}nm, and was stored at -20 ^{0}C, until PCR was performed.

PCR Conditions

PCR amplifications were performed in a total volume of 20μl reaction mixture using Hybaid thermal cycler. At AHRI and Genius thermal cycler at the Genetic Research Lab. at AAU. Amplification reactions contained 2μl, 10xPCR Buffer (10mm Tris-Hcl, pH 8.3, 50mm KCl) 2μL, each of dATP, dCTP, dGTP and dTTP (200μM each), 1μl template (0.5μg/μl), 1μL sterile distilled water auto claved twice, 5mM $MgCl_2$, 2.5 units Amplitaq DNA polymerase and 1μL (2μM) primer. The temperature cycle for PCR was 1 cycle at 96 ^{0}C for 5 minutes followed by 45 cycles at 40^{0}C for 1 minute, 72^{0}C for 2 minutes, and 96^{0}C for 50 seconds, followed by a final cycle for 7 minutes at 72^{0}C.

Amplification products were separated on 2.0% agarose gel containing 1xTBE and Ethidium bromide. Picture of the gel was taken by polaroid camera and hierarchical agglo merative classification was performed for the rhizobial isolates using program package SYNTAX multivariate data analysis version 5.02 (Podani,

1988). Correlation coefficient was used as an index of similarity. A dendrogram was constructed using the average linkage option.

10XTBE Buffer

Tris - base	108g
$Na_2EDTA.2H_2O$	9.3g
Boric acid (H_3BO_3)	55g.
Distilled water	1 litre

Bacteriophage spp-1 DNA (Fisher Biotech) digested with ECORI and Ø 174X hae III fragments were used as DNA size markers.

5. Pot experiment

Variety Bulga 70 faba bean seeds were randomly selected and surface sterilized as described previously, and four seeds were planted in each pot. The four isolates and the exotic strain Tal 1397 N were prepared by growing them in YEM nutrient broth media for four days. The rhizobial number was adjusted to be 10^9 cells/ml and 4ml of the culture suspension was taken from each culture and incoluated on the pots in the following combination.

Ankober isolate (A1) on Molale soil

Molale isolate (M1) on Ankober soil

Keyt isolate (K1) on Mehal Meda soil

Mehal Meda isolate (M-M1) on Keyt soil

In addition to inoculating with rhizobial isolates the soil was also treated with fertilizer. Nitrogen fertilizer was applied as urea and phosphorus fertilizer was applied as DAP (diammonium phosphate). The seven treatments for each of the four soils are given below:

i. $N_{46}+P_{20} = T_1$

ii. local rhizobial isolate = T_2

iii. exotic strain (TAL 1397N) = T_3

iv. $N_{23}+P_{20} = T_4$

v. $N_{23}+P_{20}$+exotic strain (TAL 1397N) = T_5

vi. $N_{23}+P_{20}$+ local rhizoibal isolate = T_6

vii. Soil only (control) = T_7

N_{46}= 46kg nitrogen per hectare

Local rhizobial isolates were A1, M1, K1, M-M1 as presented eariler

N_{23}= 23kg nitrogen per hectare

P_{20}= 20kg phosphorus per hectare

To apply he above rate of fertilizer for the sols the following calculaion was done.

1kg DAP contains 230g N and P

1Kg Urea contains 470g N

The weight of one hectare top sol is 2,000,000Kg.

Therefre to apply nitrogen and phosphorus at 46Kgper hectare rate and 20Kg per hectare rate on 3Kg soil, 0.072g urea and 0.15g DAP was applied respectively. To apply $N_{23}P_{20}$ rate 0.04g urea and DAP was applied.

The pots were randomized, and placed in the green house according to 2x7 factorial experiment (Somasegaran and Hoben, 1994). When seedlings were visible above the surface of the soil, thinning was done leaving three plants per pot.

After 70 days of growth, pots were washed down to uproot the root of the plants from soil and measurements of plant shoot length, shoot dry weight and nodule fresh weight were made. Plant total nitrogen content was also analyzed using modified kjeldahl method, after the plant matter was dried an oven at 50^0C for 72 hrs and was later grounded.

The data obtained was analyzed using statistical soft ware M-stat (version c). Two factor randomized complete block design (RCBD) analysis and Dunkans' multiple range test were done.

To compare the relative nitrogen fixing capacity of the four isolates and the reference strain a separate experiment was done. For this purpose an imported acid washed sand was used. It was washed again with distilled water and 2kg of soil sample was filled into previously surface sterilized plastic pots. Faba bean seeds,

surface sterilized as described earlier, were allowed to germinate after which three seedlings were transferred in each pot. The experments were conducted in triplicate.

Culture suspensions of each of the five rhizobial strains were prepared by growing in YEMA broth for four days and rhizobial number per ml was determined and adjusted to 10^9 cell/ml (Somasagarn and Hoben, 1994). Then 1ml of culture suspension was taken and dispensed on to each seedling. Finally all pots were covered by acid washed gravel to avoid probable contamination from the air. Plants were irrigated by Jensen's nitrogen free media except the positive control which was supplied by KNO_3 in the media. There was also a negative control which was neither inoculated nor supplied with 0.05% KNO_3. After 70 days of growth, plants were uprooted and measurements for shoot length, shoot dry weight, nodule fresh weight and the planttotal nitrogen content were taken.

6. Plant and Soil analysis

Plant total nitrogen content was determined by modified kjeldahl method (Bremer, 1965). 0.2g of plant sample was weighed in 200 ml digestion tube, digested by sulphuric acid, distilled and percentage of nitrogen content calculated according to the following formula.

$$\text{Nitrogen \%} = \frac{(V_1-V_2)xNx0.014x100x\ mcf}{S}$$

Where: V_1 = ml of titrant used for the sample

V_2 = ml of titrant used for the blank

N = Normality of the acid

S = Weight of the plant material in gram

mcf = moisture correction factor

0.014= constant

For soil physical and chemical analysis, The soil samples were air-dried at room temperature by spreading on plastic trays. After drying, the samples were grounded in a grinding machine and sieved through a 2mm sieve.

a. **pH**

It was determined in 1:2.5 soil water suspension and after stirring, reading was made using corning 140 pH meter with combined electrode.

b. **Organic carbon**

It was determined by Walkley and Black method (Allison,1965).

c. **Soil total Nitrogen**

It was determined by modified kjeldahl method (Bremer,1965).

d. **Available phosphorus content**

It was analyzed by Olsen's procedure (Olsen and Dean, 1965).

e. **Potassium, Calcium and Magnesium**

They were analyzed by Walkley-Black wet oxidation method (Allison,1965).

IV. Result

1. Soil Analysis

Table 1: Soil Chemical Analyses

Parameters	Ankober	Keyt	Mehal Meda	Molale
PH	6.400	6.280	5.900	6.540
K meq/100gm soil[1]	1.088	1.271	0.51	0.958
Ca meq/100gm soil[2]	19.163	18.658	29.861	36.684
Mg meq/100gm soil[3]	4.087	5.425	9.695	10.305
T.N %[4]	0.224	0.194	0.202	0.125
O.C %[5]	2.086	1.683	1.348	0.934
O.M %[6]	3.596	2.901	2.324	1.610
C/N[7]	9.00	9.000	7.000	7.000
Av.P.ppm[8]	31.01	51.800	5.700	3.320
Av.K.ppm[9]	307.00	382.000	170.000	205.000

1. Total potassium content of the soil
2. Total calcium content of the soil
3. Total magnesium content of the soil
4. Total Nitrogen content of the soil in percent
5. Organic carbon content of the soil in percent
6. Organic matter content of the soil in percent
7. Ratio of carbon to nitrogen content
8. Available phosphorus
9. Available potassium

According to Desta and Angaw (1989) soils can be classified based on their Nitrogen content as :

Total N < 0.05% = very low

Total N 0.05 - 0.1% = low

Total N 0.1 - 0.15% = medium

Total N 0.15 - 0.20% = high

Total N > 0.20% = very high

The yield depleted areas of Molale and Mehal Meda have low available phosphorus but have medium and high nitrogen contents respectively, whereas Ankober and Keyt which are the yield sustained areas have very high level of available phosphorus and very high and high nitrogen contents respectively. According to Ngeborg (1986), soils are ranked according to the following phosphorus content in them.

Very high = 25ppm

High = 18 - 25ppm

Medium = 10 -18ppm

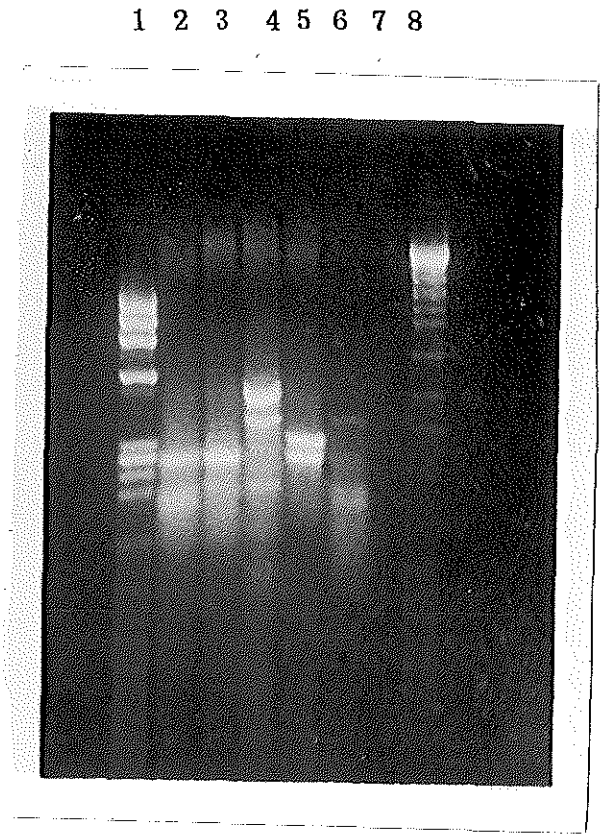
Low = 5 - 10ppm

Very low=5ppm

Also the organic matter and available potassium content of the yield sustained areas is higher than the low yield areas (Table: 1) .

2. Strain Identification

The strain identification test using PCR-based amplification of specific DNA fragments of nitrogen fixing promoter region of the genome, showed that all the four isolates and also the exotic strain Tal 1397 N differ in their banding patterns.



Lane1:Ø174XhaeIII fragments

Lane 2: M1 isolate

Lane 3:A1 isolate

Lane 4: K1 isolate

Lane 5: TAL1397N

Lane 6:M.M1isolate

Lane 7:water control

Lane 8:bacteriophage spp-1 DNA

Fig. 1: Banding pattern of the five rhizobial strains

Table 2 Presence and absence of the five bands found by PCR amplification

band no	M1	A1	K1	Tal 1397N	M.M1
1	+	+	-	+	-
2	+	+	+	-	+
3	+	-	-	-	+
4	-	-	+	-	-
5	-	-	+	-	+
6	-	-	-	+	-

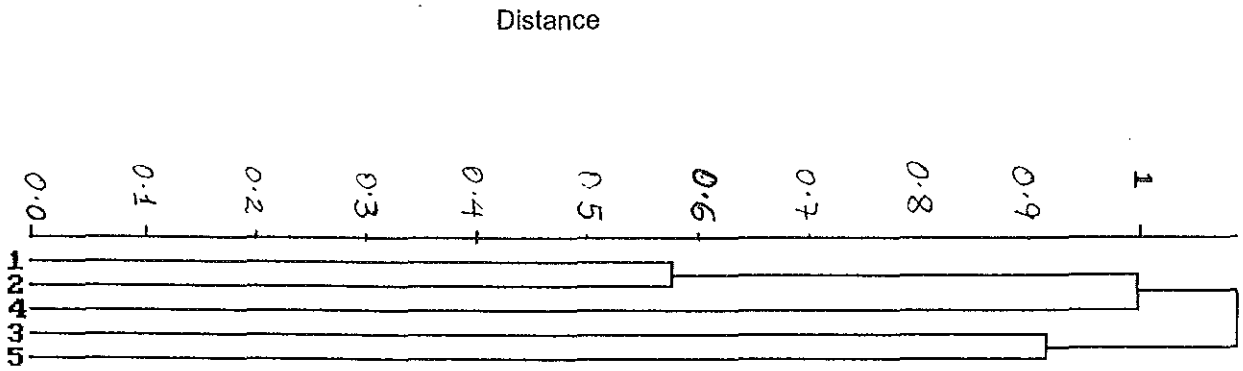


Fig. 2 Dendrogram of similarities between rhizobial isolates. Data was derived from PCR finger prints.1= M1isolate 2=A1 isolate 3= K1isolate 4=Tal1397N(reference strain) 5= M.M1isolate

Classification of the rhizobial isolates showed that rhizobial isolates M_1 and A_1 are close to each other, also rhizobial isolates K_1 and M.M1 were found to be close (fig. 2).

3. Rhizobial status

As it was observed from the density estimate, the number of rhizobia in the four soil samples was found to show the following.

Table 3 Rhizobial density estimate of the soil samples

Place	Number of rhizobia/gm of soil	
	minimum	maximum
Ankober	8.2×10^3	1.2×10^5
Keyt	8.2×10^4	1.2×10^6
Molale	2.6×10^1	8.8×10^2
Mehal Meda	1.5×10^1	2.2×10^2

It was observed that there is a general decreasing trend in the number of nodules per plant as the soil dilution factor increases, especially the Molale and Mehal Meda rhizobial density reached nil at 10^{-6} dilution. The number of nodules formed per plant by Keyt and Ankober soil dilutions was better than Mehal Meda and Molale.

4. Symbiotic effectiveness in Sand Culture

The relative Nitrogen fixation capacity of the five isolates *i.e* Tal 1397N, K1, M1, MM1 and A1 was tested by measuring plant total nitrogen content, total dry matter, shoot length and nodule fresh weight. The results indicate that inoculation of the plants with all the five isolates didn't show any significant improvement from the control in any of the parameters except nodule fresh weight (table 4).

Table 4. The effect of inoculation on total plant nitrogen content Shoot length and total dry matter.

Treatment	Shoot length /cm	Total dry matter /gm	Nodule fresh weight/gm	Plant total nitrogen content in percent
M.M1 isolate	34.9 ± 6.78	1.16±0.10	0.77±0.07 b	2.78±0.19
TAL1397N	28.5±1.81	1.54±0.41	1.07±0.12 a	3.24±0.47
K1`isolate	31.1± 2.81	1.08±0.29	0.4±0.16 b	2.43±0.32
M1 isolate	29.9±3.24	1.42±0.26	1.02±0.26a	2.95±0.35
A1 isolate	34.0±1.63	1.49±0.13	0.5±0.05 b	2.68±0.23
Control	31.7±4.49	1.17±0.10		2.20±0.31
KNO_3	39.1±9.71	1.63±0.16		2.56±0.04

Numbers in the same column followed by different letters are significantly different at 5% level (Duncan's multiple range test).

Numbers are means and S.D of three replicates (3 plants in each pot).

Letters in the columns (a,b,c) are rank of the means.

Nodule fresh weight was found to be significantly affected by inoculation of the different isolates as shown in the above table (Table 4).

5. Pot experiment

Table 5: Effect of treatment (fertilizer and inoculum) on growth of fababean plants grown on Ankober and Molale soils

Treatment	Shoot length in cm.		Shoot dry matter in gm.		Nodule fresh wt. in gm.		Total nitrogen contenet in %	
	Ankober	Molale	Ankober	Molale	Ankober	Molale	Ankober	Molale
T_1	74.40±0.8 a	73.1±2.04 a	3.85±0.19 a	3.46±0.39 a	0.56±0.04bc	0.2±0.008 cd	2.86±0.1 a	2.87±0.11b
T_2	71.00±1.7ab	60.0±1.92 b	3.62±0.22a	2.04±0.16 c	0.96±0.5 a	0.43±00.04b	2.78±0.22a	2.91±0.43b
T_3	68.00±4.32 ab	59.4±0.98 b	3.97±0.62a	2.17±0.26c	0.64±0.05b	0.40±0.05b	3.13±0.8 a	2.59±0.30b
T_4	69.3±6.43 ab	61.9±3.90 b	4.02±0.49 a	2.85±0.10ab	0.63±0.04b	0.27±0.04c	2.79±0.038a	2.99±0.14b
T_5	67.1±3.18 b	64.3±2.20 b	3.94±0.38 a	2.94±0.10ab	0.50±0.00c	0.64±0.03a	3.10±0.05a	3.53±0.06a
T_6	69.6±1.32 ab	62.3±0.89 b	3.95±0.16 a	2.59±0.20bc	0.63±0.04b	0.63±0.07a	3.29±0.24a	3.02±0.23b
T_7	67.2±3.18b	59.3±3.76 b	3.74±0.29 a	2.36±0.13bc	0.67±0.12b	0.10±0.00d	3.14±0.16a	2.86±0.10b

Numbers in the same column followed by different letters are significantly different at 5% level (Duncan's multiple range test).

Numbers are means and S.D of three replicates (3 plants in each pot)

Letters in the columns (a,b,c) are rank of the means.

Table 6:Effect of treatment (fertilizer and inoculum) on growth of fababean plants grown on Keyt and Mehal Meda soils

Treatment	Shoot length in cm.		Shoot dry matter in gm.		Nodule fresh wt. in gm.		Total nitrogen content in %	
	Keyt	Mehal-Meda	Keyt	Mehal-Meda	Keyt	Mehal-Meda	Keyt	Mehal-Meda
T_1	61.6±0.47a	52.5±2.9 a	4.14±0.6 a	3.3±0.48 ab	1.13±0.03a	0.20±0.04d	2.96±0.14	2.83±0.08
T_2	61.3±1.27a	43.2±2.7 b	3.96±0.05a	2.84±0.34b	0.71±0.02b	0.44±0.03 bc	2.97±0.29	2.94±0.11
T_3	61.9±1.59a	41.7±0.69b	3.9±0.23 a	2.56±0.09b	1.13±0.12a	0.39±0.05 c	3.05±0.22	2.75±0.21
T_4	60.4±3.90ab	52.1±1.55a	4.06±0.64a	3.84±0.19a	0.66±0.009b	0.17±0.08 d	2.58±0.95	3.15±0.20
T_5	58.9±1.48ab	53.8±1.63a	4.18±0.38a	3.92±0.30a	1.13±0.04a	0.6±0.04 a	2.78±0.19	2.95±0.17
T_6	62.2±0.73a	53.7±1.38a	4.16±0.36a	3.70±0.16	1.1±0.14 a	0.58±0.02 ab	2.99±0.06	2.71±0.16
T_7	56.9±2.04b	39.9±1.79b	3.94±0.31a	2.68±0.47b	0.7±0.08 b	0.16±0.04 d	3.0±0.39	3.07±0.18

Numbers in the same column followed by different letters are significantly different at 5% level (Duncan's multiple range test).

Numbers are means and S.D of three replicates (3 plants in each pot)

Letters in the columns (a,b,c) are rank of the means

The variability in shoot length, dry matter, nodule fresh weight and total nitrogen content due to the two factors i.e., soil type and the seven treatment levels was analyzed. The results revealed that total nitrogen content of plants grown on Keyt and Mehal Meda soils was not significantly affected by neither of the two factors. Whereas shoot length, dry matter and nodule fresh weight were affected at a highly significant level (P=0.01) by soil type (appendix 3). Comparison of the mean values of the above three parameters, on Keyt soils showed no significant difference (P=0.05) for shoot dry matter between treatments. Plant length was significantly improved for the six treatments at almost equal level compared with the control. Nodule fresh weight was highest for $N_{46}P_{20}$, inoculation by M.M1 isolate, Tal 1397N and $N_{23}P_{20}$. The other treatments were not significantly different from the control (table 5).

For Mehal Meda soil, treatments: N_{46} P_{20}, N_{23} P_{20} + K1 isolate, N_{23} P_{20} + Tal 1397N, N_{23} P_{20} have resulted in highest mean values of shoot length and dry matter. The other two treatments were not significantly different from the control. For the same soil nodule fresh weight was relatively highest for application of mineral fertilizer at N_{23} P_{20} rate with Tal 1397N and K1 isolate, followed by inoculation with K1 isolate and Tal 1397N only. The other treatments were not significantly different from the control, which has the smallest value (table:6).

For plants grown on Ankober and Molale soils plant height, dry matter and nodule fresh weight were affected at a highly significant level by soil type and

treatment (appendix 4). However, plant total nitrogen content was significantly affected only by treatment level (table 1). On Ankober soil all treatments have not shown any significant increase on shoot dry matter while shoot length was better than the control for: $N_{46}P_{20}$, M1 isolate, Tal 1397N, $N_{23}P_{20}$ and $N_{23}P_{20}$ + M1isolate. Mean value of $N_{23}P_{20}$ + Tal 1397N treatment was similar with the control. Nodule fresh weight was higher for plants inoculated with M1isolate only, the other treatments were found to be better than the control in nodule fresh weight mean value. On Molale soil shoot length was better than the control only for mineral fertilizer application at a rate of $N_{46}P_{20}$. Shoot dry matter was highest for the above treatment followed by $N_{23}P_{20}$ and $N_{23}P_{20}$ + Tal 1397N. The other treatments were not significantly different from the control. Nodule fresh weight was highest for $N_{23}P_{20}$+Tal1397N and $N_{23}P_{20}$+A1, followed by inoculation with Tal 1397N and A1 isolates only. $N_{23}P_{20}$ and $N_{46}P_{20}$ treatment means were low but better than the control.

V. Discussion

1. Rhizobial Status

Rhizobial number in the soil differs depending on availability of nutrients, organic matter and the presence or absence of the host plant. The work of Loutfi *et al,.* (1980) has showed that leaving the soil fallow for extended periods caused a considerable decrease in the population of *Rhizobium leguminosarum* in Egyptian soils. According to Date (1982), soils can be classified depending on their Rhizobial population status as:

<100 g $^{-1}$ soil = low number of rhizobia in the soil

100 - 10000 g $^{-1}$ = medium number of rhizobia in the soil

>10000 = very high number of rhizobia in the soil

Based on the above ranking the four soil samples of Ankober and Keyt, have very high number of rhizobia per gram of soil where as Mehal Meda and Molale have low rhizobial population. Differences in rhizobial population size vary usually with site factors. Usually numbers reflect the presence or absence of host plant. Larger numbers of rhizobia are often estimated in the soil surrounding the roots of a homologous host than in the soil of other legumes or non-legume plants (Date, 1982). In the case of this experiment pulse crops were not grown on the farmers

fields, for almost eight years. This must be one of the factors, which has contributed for the decrease of rhizobial number in these soils. Field experiments conducted in the Sudan also showed that there was no response for inoculation on traditional faba bean growing areas, whereas on areas where faba bean is not grown, the native *R. leguminosarum* population was low and a positive response was observed for inoculation with effective strains (Saxena and Stewart, 1983). The other factor, which might have contributed for the low rhizobial population number in the yield depleted areas, is the low availability of soil nutrients like organic matter, phosphorus, and potassium (Table 1). This difference might be due to dung manure application exercises at Ankober and Keyt, that is not common at Molale and Mehal Meda. Application of manure was found to increase faba bean yield in the Sudan (Yousif, 1987), which may be associated with the enrichment of the soil with nutrients and increase in rhizobium population.

2. Strain Identification

DNA amplification provides a powerful tool for the study of genetic variation. This study can be achieved through the use of randomly amplified polymorphic DNA (RAPD) fragment patterns or specifically amplified DNA fragment patterns. Richardson *et al.* (1995) was able to get different banding patterns for four different strains of *Rhizobium leguminosarum* bv. Vice. They used a 20-base oligo-nucleotide single primer (5'-3') AAT TTT CAA GGG TCG TCG CA, corresponding to a conserved nif (nitrogen fixing) gene promoter region. Newburry and Ford-Lloyd

(1997) have also mentioned that single primers in polymeric chain reaction can provide multi-locus markers. The same primer with Richardson *et. al.,* (1995) was used to see variation among the four rhizobial isolates and the exotic strain Tal 1397N used in this experiment. The banding pattern obtained was different for all the five rhizobial isolates (Fig. 1). Classification of the rhizobial isolates depending on the banding patterns produced showed that they are all different strains (Fig. 2).

3. Pot experiment

The main soil nutrients which affect plant growth are N, P and K. Field experiments carried out on various field crops in Ethiopia suggest that the main limiting nutrients in most parts of the country are N and P (Desta and Angaw, 1989). In this experiment the soil test results showed that all four soil samples have high soil total nitrogen content (Table 1). The possible reason why any significant difference was not observed in shoot dry matter between treatment for Ankober and Keyt soils could be the high availability of both nitrogen and phosphorus in both soils.

However Mehal Meda and Molale soils have showed a significant response to phosphorus and nitrogen fertilization at N_{46} P_{20}, N_{23} P_{20} rates alone or together with rhizobial isolates or exotic strain Tal 1397N. For Mehal Meda soil, shoot length and dry matter were higher for these treatments compared with the inoculated ones and the control (Table 6).

This result demonstrated that phosphorus caused an increment in shoot length and dry matter, since the soil is deficient of it. Many other experiments also showed that phosphorus application on phosphorus deficient soils improve shoot dry matter, plant nitrogen content and even nodulation. Duguma and Okali (1987) in their pot trial conducted two experiments on acidic soil, one by inoculation only and the other by inoulation and phosphorus application with calcium carbonate, inoculation only did not increase growth, while application of lime, phosphorus and inocula did. In India on a field trial of phosphorus and sulfur deficient vertisol it was shown that addition of Phosphorus and Sulfur increases grain (Dwivedi and Nayak, 1995) and straw yield and also nutrient composition of N, P, K, Ca, Mg and S (Nayak and Dwivedi, 1990; Majumdar *et al.*, 1994).

Inoculation with rhizobial strains A_1, K_1 or exotic strain Tal 1397N without phosphorus on Molale and Mehal Meda soils was not significantly different from the control. This may be due to the high available soil nitrogen and the ineffectiveness of the rhizobia (Table 6). There was also no significant difference between the four phosphorus treatments although the level of nitrogen fertilizer rate differs (Table 6). This strengthens the above result that nitrogen is not the cause for improvement of the above listed parameters.

For Molale soils the best treatment in increasing both shoot length and dry matter was application of nitrogen and phosphorus at a rate of $N_{46}P_{20}$. Here it is clear that

only phosphorus is not responsible for the increment since, treatment of the soil at N_{23} P_{20} level was significantly different from the first, still receiving the same level of phosphorus (Table 5). The rest of phosphorus receiving treatments either together with nitrogen or inoculum were significantly better in shoot dry matter increase than inoculum only treated ones and the control. The inoculum only treated plants were not different from the control. This showed both nitrogen and phosporus contributed to the increase in shoot dry matter. This response for application of nitrogen can be due to the medium nitrogen content of the soil. In fertilizer trials conducted in different parts of Ethiopia, it was found out that the response of pulses to NP fertilizers is location specific and pulses generally appeared to show relatively higher response to P than to N (Desta, 1986).

Shoot length and dry matter was also highly affected by soil type (Appendix 4). The higher values were obtained for plants grown on the yield sustained area soils. This can be due to the availability of nitrogen, phosphorus and also potassium in higher amount than the yield depleted areas.

For Keyt and Mehal Meda soil grown plants, soil type or treatment did not affect total nitrogen content significantly (Appendix 3,Table 6). This is because both soils have high nitrogen content. As the efficiency test indicates (Table 4), all the five isolates did not significantly affect shoot dry matter, length and total nitrogen content . This agrees with the fact that inoculation of any of the soils did not give better

result than the control. Responses to inoculation may not be observed if the inoculant strain is not effective, non-competitive (Howel *et al.*, 1987) or the soil is rich in nitrogen (Vincent,1982). On field experiments conducted at ten locations in Upper Egypt, responses to inoculation in terms of seed yield was observed only at three out of the ten locations (Saxena and Stewart, 1983).

No significant difference in plant total nitrogen content was observed between Ankober and Molale soils. There was also no significant difference betweeen treatments for plants grown on Ankober soil, but the ones on Molale soil have shown higher plant nitrogen content for plants treated with nitrogen and phosphorus fertilizer at a rate of N_{23} P_{20} together with exotic strain Tal 1397N (Table 5). All other treatments were not significantly different from the control. From all other strains used for inoculation, Tal 1397N is a better nitrogen fixer (Table 4), although the difference is not significant. It has shown significantly higher nitrogen fixation ability, with application of mineral fertilizer at $N_{23}P_{20}$ rate only on Molale soil from the four soil types. This showed application of starter dose nitrogen and phosphorus has increased the nitrogen fixation ability of this strain. In a similar experiment conducted in India at an experimental farm, with low nitrogen and high P and K contents, most growth parameters were significantly improved by inoculation. A significant effect of starter nitrogen (20kg/ha) and up to 50 kg/ha phosphorus application was also observed (Kumar *et al.*, 1993).

Nodule fresh weight was very highly affected by soil type (Appendix 3). It was higher for Keyt and Ankober soils compared to Mehal Meda and Molale. This can be attributed to the good plant growth in the former two areas. There is also significant difference between treatments. For the yield depleted areas (Molale and Mehal Meda) nodule fresh weight was higher for the inoculated ones in the order N_{23} P_{20} + Tal 1397N and N_{23} P_{20} +K1or A1 isolates, K1 or A1 and Tal isolates only (Table 5 and 6).

Uninoculated treatments were not significantly different from the control except T4 in Molale. This difference indicated that inoculation improved nodule fresh weight since the indigenous rhizobial population is very low in the soil.

Starter nitrogen application with phosphorus ($N_{23}P_{20}$) has increased nodule fresh weight above the inoculum only treated ones. In a similar experiment Abdulsalaam and Tahir (1991) have also found phosphorus fertilization increasing nitrogen fixation, giving a better and efficient nodulation and assimilation of nitrogen by plant tissues and seeds. Sahlemedhin and Desta (1986) have also observed the same effect on Phosphorus.

VI. Conclusion and Recommendation

In this study it was found out that there is a very low number of rhizobial population in the yield depleted areas. No increase in any of the growth parameters was demonstrated by inoculating the studied fababeans with indigenous rhizobial isolates, exotic strain or by applying nitrogen fertilizer to the soils collected from Northern Shoa localities. However Phosphorus fertilization has shown a significant increase in shoot length, dry matter and nodule fresh weight. This implies that application of phosphorus together with inoculation of proper and effective rhizobial isolate, it will help to increase faba bean growth and yield in these areas. Inorder to screen effective rhizobial strains and determine fertilizer rate for these areas, field trial is recommended. The information gathered from the farmers at Molale and Mehal-Meda showed that farmers do not use mineral fertilizers, nor dung manure and ash to their soils which is common experience in Keyt and Ankober. This must have helped in the accumulation of high amount of nitrogen, organic matter and phosphorus in these soils. Based on this experience application of dung manure and ash can be taken as an alternative to mineral phosphorus application. The other good alternative to mineral phosphorus application is inoculation of soil by phosphate solublizing bacteria (PSB) and vesicular-arbuscular mycorrhizal (VAM) fungi. Tekaligne and Hque (1986) found that VAM inoculated leucerine (*Medicago sativa*) flowered earlier and produced more nodule and dry matter than control plants. An experiment conducted in Egypt showed inoculation of fababean

and lentil with VAM fungi to increase plant dry weight and nitrogn content almost equally as it was found by fertilization of super phosphate (El-Din and Moawad, 1988). Many researchers have found synergestic interaction between VAM fungi and PSB (Barea *et al.,* 1975; Piccini and Azcon 1987; Sreenivasa and Krishinara, 1992) in increasing dry weight, nutrition uptake and growth of plants. A synergestic effect was also observed between PSB and rhizobium (Asfaw, 1988). In parallel with rhizobial strain screening and soil fertilization, it is also necessary to asses the presence of any other yield constraints like, faba bean diseases and pests.

From this study it can be concluded that the reason for lower values of growth parameters like shoot length and dry matter, nodule fresh weight on soils of the yield depleted area soils is not neither due to deficiency of nitrogen or the low rhizobial number in the soil. Rather the low availability of phosphorus and the response observed upon its application to these soils indicate it might be this factor which has caused the retarded growth of the plants.

VII. References

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VIII. Appendices

Appendix 1. Number of positive pouches obtained by inoculation with soil dilution

Dilution	Molale	Mehal-Meda	Keyt	Ankober
10^{-1}	3	2	4	4
10^{-2}	3	1	4	4
10^{-3}	2	1	4	4
10^{-4}	2	1	4	3
10^{-5}	1	1	3	3
10^{-6}	0	0	2	2
10^{-7}	0	0	2	1
10^{-8}	0	0	2	0
Total	11	6	25	21.00

Appendix 2. Average number of nodules per plant formed by each locality soil dilution

Dilution	Molale	Mehal-Meda	Keyt	Ankober
10^{-1}	25	63	67	94
10^{-2}	45	2	44	103
10^{-3}	42	4	26	60
10^{-4}	16	12	16	57
10^{-5}	4	5	3	32
10^{-6}	0	0	5	15
10^{-7}	0	0	9	20
10^{-8}	0	0	7	0

Appendix 3. Showing the effect of the two factors soil type (Keyt and Mehal-Meda) and treatment on variability of shoot length, dry matter, nodule fresh weight and plant nitrogen content

		Shoot length		Shoot drymatter		Nodule fresh weight		Plant total N content	
Source of variation	d.f.	mean square	F value	Mean square	F value	Mean sqaure	F value	Mean square	F value
Replication	2	13.9	2.74 ns	0.55	2.99ns	0.009	1.33ns	0.04	0.56 ns
Soil type (A)	1	1593.5	314.24**	6.47	35.31**	3.452	508.8**	0.01	0.18**
Treatment (B)	6	74.1	14.61**	0.68	3.73**	0.073	10.73**	0.03	0.47 ns
AXB	6	52.6	10.37**	0.34	1.83ns	0.194	28.56**	0.07	1.09 ns
Error	26	5.1		0.18		0.007		0.07	
		CV 4.15%		CV=11.70%		CV=12.59%		CV=8.67%	

ns = not significant

* = significant at 5% level ** = significant at 1% level

16 15	8	1.7 1.0	1.7 1.0	1.8 1.0	$>7x10^8$
14 13	7	$5.8x10^2$ 3.1	$5.8x10^2$ 3.1	$5.8x10^2$ 3.1	6.9 3.4
12 11	6	1.7 1.0	1.7 1.0	1.7 1.0	1.8 1.0
10 9	5	$5.8x10^1$ 3.1	$5.8x10^1$ 3.1	$5.8x10^1$ 3.1	$5.8x10^1$ 3.1
8 7	4	1.7 1.0	1.7 1.0	1.7 1.0	1.7 1.0
6 5	3	5.8x1 3.1	5.8x1 3.1	5.8x1 3.1	5.8x1 3.1
4 3	2	1.7 1.0	1.7 1.0	1.7 1.0	1.7 1.0
2 1	1	0.6 <0.6	0.6 <0.6	0.6 <0.6	0.6 <0.6
0	0				

Approx. range 10^9 10^7 10^5 10^3

Factor 95% fiducial limit (x,÷): n=2 6.6 n=4 3.8