SCREENING OF *RHIZOBIUM* AS AN EFFECTIVE BIOINOCULUM IN ARHAR UNDER AGRO-CLIMATIC ZONE OF ODISHA

A Thesis submitted in the
Orissa University of Agriculture and Technology in partial fulfillment
of the Requirement for the degree of
Master of Science in Botany

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CERTIFICATE- I

This is to certify that the thesis entitled "**SCREENING OF *RHIZOBIUM* AS AN EFFECTIVE BIOINOCULUM IN ARHAR UNDER AGRO-CLIMATIC ZONE OF ODISHA** " submitted in partial fulfillment of the requirements for the award of the degree of **Master of Science** in Botany of the Department of Botany, College of Basic Science & Humanities, Orissa University of Agriculture and Technology, Bhubaneswar, is an authenticate record of bonafied research work carried out by **Ms. Khirabhi Tanaya Dash** (Adm no-14/BOT/ 15) under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma or published in any form. It is further certified that the evidence and help obtained by various sources during the course of investigation has been duly acknowledged.

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This is to certify that the thesis entitled, **"SCREENING OF *RHIZOBIUM* AS AN EFFECTIVE BIOINOCULUM IN ARHAR UNDER AGRO-CLIMATIC ZONE OF ODISHA"**submitted by the student bearing roll number **14/BOT/15** to the **Orissa University of Agriculture and Technology, Bhubaneswa*r***, in partial fulfilment of the requirements for the award of degree of Master of Science in Botany, has been approved by the students advisory committee after an oral examination of the same.

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<u>DECLARATION</u>

I hereby declare that the project work entitled **"SCREENING OF *RHIZOBIUM* AS AN EFFECTIVE BIOINOCULUM IN ARHAR UNDER AGRO-CLIMATIC ZONE OF ODISHA"** submitted by me for the partial fulfilment of the **Degree of Master of Science** in Botany to the **College of Basic Science & Humanities ,Orissa University of Agriculture And Technology, Bhubaneswar** is an original piece of research work, and no part of this thesis has been submitted for any degree or diploma to any other University/ Institution.

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INTRODUCTION

1. INTRODUCTION

In a country like India Pulses is one of the most important food crop which helps in supplementing the protein requirement of the majority of the population. With an increasing population and economy there is a great demand of different types nutritional reach food crops and pulses are one of them. Pulses are seeds of annual legumes that include plant such as different types of beans, black gram, green gram, arhar, lentils, cow peas, pigeon peas, horse gram etc. Pulses are versatile as they keep us healthy, they can be incorporated in daily menu right from soups and starters to salad and main dish even desert as the rich protein source and they also stabilize the soil for mineral accumulation in plants by fixing atmospheric nitrogen. Analyzing the recent worldwide protein necessity and availability there is a matter of huge concern to meet the challenge.

India is the most common largest producer as well as largest consumer of pulses in the world. Although, India has the distinction of being the world's single largest producer of pulses, the difference in production and population ratio is insignificant. The increase in population has pushed up demand of pulses, while the fall in availability has pushed up their prices. Although a large area of approximately 20-22 million hectares is under different pulse crops, their production is more or less stagnant for the last four decades, which ranges between 11 and 13 million tons (Ali and Kumar 2006). Madhya Pradesh is the leading state in producing pulses in India followed by Maharastra, Rajasthan, Andhra Pradesh. But there has been decline in annual production of pulses from 3488 million tons in 2003-04 to 2948 million tons in 2008-09 in Madhya Pradesh, and similarly in other states also. In 2003-04 pulses produced were 635 kg/ha in an area of 23.46 of million hectare that slightly decreased to 597 kg/ha grown in an area of 24.54 million hectares in 2008-09 as per report of Agriculture Ministry, Govt. of India (Goyal *et al*., 2010).

There are numbers of variety of pulse crops grown in India and accepted as a major player in pulses contributing around 25-28% globally**.** A liberal trade regime has kept imports in this region around 25 lakh tonnes per annum, *i.e.* 20-25% of domestic production (Goyal *et al.,* 2010).

Thus it is hugely recommended to analyze the recent demand of pulses per population and application of biofertilizers in general and *Rhizobium* in particular to enhance the potency of soil to grow efficient legume crops on it and increase productivity.

Arhar (pigeon pea/Tur, botanical name-*Cajanus cajan*,family-Fabaceae) is the second important pulse crop after Grams. Availability of pulses in general and Arhar in particular is decreasing day by day as a consequence of increasing population and almost static production level of pulses. Hence it is required to pay due attention for the development of production technology. Traditional Arhar varieties are of longer duration are unsuitable for intensive cultivation. In the recent pasts pulse scientists have tried to solve this problem by evolving short duration cultivars with high yield potential.

On statistical basis of India producing pigeon pea is 740.20tonne/2000hectars.Also in states like Maharastra crop yield is 39.27%,Karnataka is of 17.57%,Andhra Pradesh is of 16.97%,UttarPradesh is of 11.85%,Gujurat is of 10.65%,Madhyapradesh is of 3.5%.(Agropedia.iitk.ac.in). Then another study shows production of Tur per yearly analysis like India's export in 2014-15 is 1.22 tonne,2015-16 is 4.02 tonne,2016-17 is 9.22 tonne where as in case of import 2014-15 showing 4584 tonne,2015-16 has 5797.77 tonne,2016-17 has 5420.25 tonne.The domestic prices for Tur have breen higher than that of international prices except march to april due only of its heuge demand of consumption as importing from outside.(Commodity profiles for pulses).

Pigeon pea is mainly grown in rainfed upland areas and is one of the most important pulse crops of the state. The protein content of Pigeon pea is 22% to 24%. In addition it contains carbohydrates, minerals and vitamins. Pigeon pea, which is also a good source of essential amino acids, can be an excellent crop to promote food and nutritional security in Odisha. However, its productivity of Pigeon pea is low at 415 kg/ha in Odisha as compared to the national average of 700 kg/ha. It also has a very low seed replacement ratio of 2% to 3%. A large section of farmers in the rainfed upland ecosystems of Odisha have remained isolated from improved cultivars and management practices of pigeon pea for various reasons. There is ample scope for the expansion of high yielding short and medium duration pigeon pea varieties and hybrids in the rainfed areas for the development of sustainable livelihoods implemented (2014-15 annual accomplishment report on livelihood/ICRISAT).

As per the statistical features of both pulses and pigeon pea, the importing cost is much greater than exporting over years. It is only because of the excessive food need as source of proteins lying on the border line of poverty and demand. So as our purpose must the way towards to cultivate local varieties of legumes indegenous to natural biofertilizers in different states of India having suitable climates to produce significant result.

The Arhar/Pulse productivity skill in particularly Odisha needs consideration of its soil fertility, climatic barriers, seasonal input/output. Odisha is a state of coastal environment featuring acidic soil with the P^H ranging between 5.5-6.5 where the soil constituents needs an alkalinity for fertility in legumes. The high dried temperature can also inhibit legume yield. Thus it is recommended to sow the seeds in the low temperature climate and harvesting must be done in early winter seasons. Taking the leguminous crop to be studied like Arhar in the preference and criteria for cultivation, Odisha mainly meets 65% of productivity and yield in common though it is used worldwide especially in India as a heavy meal diet consuming Arhar as “toor dal” in everyday life. Thus Odisha need to produce Arhar in an appropriate manner.

Nitrogen Fixation is the ultimate mechanism which brings efficiency to the legumes in case of high protein content. Thus there are soil bacteria on their roots which facilitate nitrogen fixation by forming root nodules in their root hairs which have an excellent nutritive content called Leghaemoglobin. Mostly *Rhizobium* is the soil bacterium forming symbiotic association with the leguminous plants fixing atmospheric nitrogen. *Rhizobium* is host specific in nature. There are immense varieties of *Rhizobium* available and cultured in worldwide corresponding to different leguminous crops. *Rhizobium* can be inoculated with leguminous seeds as a biofertilizer suppliment for a better and healthier crop yield. Though cultivators in field applying different synthetic fertilizers like; N,P,K,Mo etc. to enhance the productivity, it is hazardous for the human body metabolism. Also soil treated with *Rhizobium* previously can maintain its effect for the upcoming seasonal yield as contrast to chemical biofertilizers. Hence attention required to be given to viable seed selection, showing time and temperature, required irrigation, respective Rhizoidal strain, nodulation period etc. including proper crop field management.

THE IMPORTANCE OF NATIVE *RHIIZOBIA*

Rhizobia are soil born bacteria present in varied climate. It is important to consider the numbers and types of native *Rhizobia* in the soil because they can affect the results of inoculating legume seeds with introduced *Rhizobium*. Native Rhizobial populations are diverse, containing effective and ineffective strains. These native *Rhizobia* can affect the results of inoculation in two ways. If there are already many native *Rhizobia* in the soil that can nodulate a legume crop and induce BNF, then inoculating seed may not produce any further benefits. On the other hand, native *Rhizobia* may compete with introduced one to form nodules on legume plants. In this case, the nodules on the legume are formed by the native *Rhizobia* and not by the *Rhizobium* introduced as inoculants. If the native *Rhizobia* are not as effective at fixing nitrogen as the introduced strains, then plants will not get the maximum benefit from biological nitrogen fixation. Therefore it is important to inoculate local suitable strains of *Rhizobium* both in soil and with seeds to avoid lesser crop yield.

A half cup of served legumes is equivalent to 30gram of meat. World food crisis especially for proteins leads to threats like Malnutrition, Kwasierker, Xeropthalmia etc. Legume cultivation needs a stable guideline and work plan to minimize crop damage as it needs proper required irrigation every time as considering period for seed showing, germination, temperature, plant growth and root nodulation. In between these efforts, attention must be given to viable seed selection with the appropriate variety, respective *Rhizobial* strain. The present piece of work is designed to identify the most suitable *Rhizobim* strain for cultivation of Arhar and methods of application of *Rhizobium* either with seed coating or inoculation in soil. Finding of the work can be applicable in developing a protocol for application of *Rhizobium* strain in farmer's field across the state.

Thus the objective is to create betterment in the sense of a healthier, safer world with enough protein need that can combat nutrient deficiency. So legume plantation with proper working scheme need to be charged in all aspects for which required natural biofertilizer application is first and most important thing to be carried out for best productivity. Since the announcement of IYP2016, legume association of all the countries has geared up to promote the initiative especially by FAO in India. The demand of Arhar/Pulse in the end on last 10 years trends growth in global consumption, the demand of 2 years would increase to 75.9 million tons in 2020 and 81.9 million tons in 2030.

PLAN OF WORK

1.Selection of efficient strains of *Rhizobium*.

2.Mass culture and organism isolation.

3.Potray application/inoculation of organism for plants.

4.In final selection of one or two potent *Rhizobial* strains for pot inoculation method.

Chapter 2
REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Rhizobium and its benefits to mankind corresponding to the crop pigeon pea (Arhar) on its isolation of strains with nodulation, crop yield, bacterial population and nutrient status to soils is of significance owing to its nitrogen fixing ability. In the case of Odisha both the quality of soil and quantity of productivity differs due to acidic nature of soil. Some of the research publications available on the subject are reviewed here.

India is the most common and largest producer and consumer of pulses in the world. Although, India has the distinction of being the world's single largest producer of pulses, the difference in production and population ratio is significant. The increase in population has pushed up demand of pulses, while the fall in availability has pushed up their prices. Although, a large area of approximately 20-22 million hectares is under different pulse crops, their production is more or less stagnant for the last four decades, which ranges between 11 and 13 million tons (Ali and Kumar, 2006).

This fall in availability of pulses is attributed to many factors; pulses are mostly grown under rain-fed conditions where drought is a common feature. Other factors include their low harvest index, prolonged vegetative growth, low yield and their susceptibility to diseases. Madhya Pradesh is the leading state in producing pulses in India followed by Maharashtra, Rajasthan and Andhra Pradesh. There has been decline in annual production of pulses from 3488 million tons in 2003-04 to 2948 million tons in 2008-09 in Madhya Pradesh, and similarly in other states also. In 2003-04 pulses produced were 635 kg/ha in an area of 23.46 of million hectare that slightly decreased to 597 kg/ha grown in an area of 24.54 million hectare in 2008-09 as per report of Agriculture Ministry, Govt. of India (Goyal *et al*., 2010).

There are numbers of variety of pulse crops grown in India and accepted as a major player in pulses contributing around 25-28% globally. A liberal trade regime has kept imports in this region around 25 lakh tons per annum, *i.e.* 20-25% of domestic production (Goyal *et al*.,2010)

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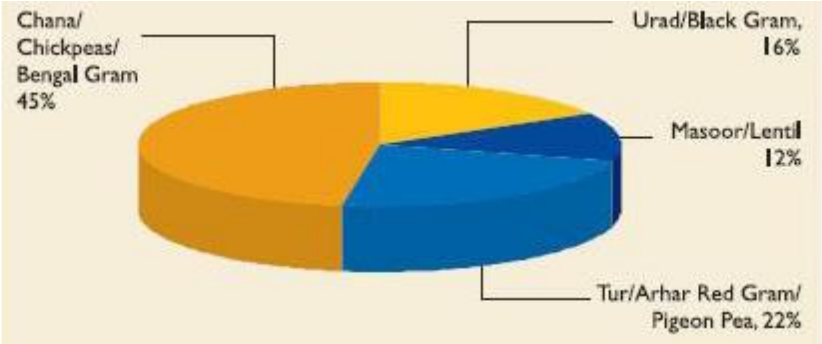

Share of various pulses in total production of India (*Source*: NB research).

The term biofertilizer is more appropriately commercial preparation of microorganisms termed as ‘the microbial inoculants' which have the capacity to fix nitrogen, solublise phosphorous and promote growth of plants. Inoculants were first made in USA and commercialized by private enterprise in 1930s (Smith, 1992). The beneficial micro-organisms, especially Plant Growth Promoting *Rhizobacteria* (PGPR), can be grown in the simple, cheap media and they are mixed with the appropriate carriers to produce biofertilizers. *Rhizobium*, also a PGPRs, fix nitrogen in association with legume crops like red-gram, blackgram, groundnut, cowpea, and soyabean which help in saving 20-40 kg chemical nitrogen. It is estimated that 50-300 kg nitrogen fixation from soil and helps in saving about 25-100 kg chemical nitrogen i.e. 55-220 kg urea per hectare. *Rhizobium* is a soil habitat Gram-negative bacterium, which can able to colonize the legume roots and fixes atmospheric nitrogen symbiotically. The symbiotic association between plant and the *Rhizobium* is initiated when bacteria in the soil attach to root hairs. This highly specific attachment process is mediated by plant proteins, the lectins that bind the bacteria to the surface of the root hairs, which is then penetrated by the microbes. The infected root cells divide and form a nitrogen fixing nodule which provides the anaerobic environment with oxyhaemoglobin necessary for nitrogen fixation (Stephens and Rack, 2000) . The morphology and physiology of *Rhizobium* vary from free-living condition to the asteroid of nodules. In addition to fixing the atmospheric nitrogen through nodulation, it shares many characteristics with other PGPRs including hormones production and solublization of organic and inorganic phosphate (Russell et. al., 1982). Through plant growth promoting substances, it helps in root expansion, improve uptake of plant nutrients, protects plants from root diseases and most important improves biomass production of fast growing at wasteland.

Rhizobia are a paraphyletic group that fall into two classes of the proteobacteria—the alpha- and beta-proteobacteria. Most of them belong to the order *Rhizobiales*, but several *Rhizobia* occur in distinct bacterial orders of the proteobacteria. Now *Rhizobia* are divided into *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Azorhizobium;* Gram-negative, nitrogen-fixing bacteria that form nodules on host plants. They also have symbiotic relationships with legume plants, which deprived of nitrogen without the essential nitrogen-fixing processes of these bacteria. The species of *Bradyrhizobium* falls under the family *Bradyrhizobiaceae*. However, root nodules of urad are formed by species of *Bradyrhizobium.* It is Gram-negative bacillus (rod shaped), motile with a single sub-polar or polar flagellum. They are a common soil dwelling microorganism that can form symbiotic relationships with leguminous plants where they fix nitrogen in exchange for carbohydrates from the plant. They are slow growing in contrast to *Rhizobium* species, which are considered fast growing *Rhizobia*. In a liquid medium, *Bradyrhizobium* species takes 3-5 days to create moderate turbidity, and 6-8 hours to double in population size. They tend to grow best with pentoses as a carbon source. The average G-C content of the genome is 64.1 mol % (Holt *et al*., 1994; Saharan *et al*., 2011).

Dubey *et al.* (2010) isolated 8 strains of endophytic root nodule *Rhizobia* from pigeon pea *(Cajanus cajan)* and identified as *Ensifer sinorhizobium* based on their physiological and biochemical characteristics; therefore, these strains were named as *Ensifer* spp. KCC1 to KCC4. KCC5 is placed in *Ensifer fredii* clade. Out of these, two isolates (KCC2 and KCC5) produced siderophore and showed strong antagonistic effect against *F. udum*.

There are number of abiotic factors such as temperature, pH, salt concentration, etc. that affects growth and survival of bacteria. Endophytic bacteria vary in their tolerance to these stresses. Temperature is a one of the limiting factors for legume- *Bradyrhizobium* spp. symbiosis and other endophytes (*Bacillus*, *Pseudomonas*, etc.). They are sensitive to temperature and other environmental factors such as pH, salt tolerance, etc. which can modify the plant growth or height and bacteria associated with plant survival.

Twelve strains of *Bradyrhizobium* spp. (pigeon pea and cow pea nodulating bacteria) were tested for temperature tolerance (20ºC/10 ºC, 30ºC/20ºC and 38 ºC/25ºC) and their temperature tolerating ability were observed on the basis of their growth. Only five strain of *Bradyrhizobium* were most temperature tolerant strains viz., USDA 3278, USDA 3362, USDA 3364, USDA 3458 and USDA3472. Growth of both crop were mainly dependent on the temperature variation not on the *Bradyrhizobium* strain, they are indepemdent *Bradyrhizobium* strain. It was recorded that at lowest temperature height of plants were the shortest and nitrogen fixation was inhibited (of pigeon pea). The optimum temperature was 30 ºC/20 ºC (Marsh Lurline E. *et al*., 2006). Temperature affects the legume *Bradyrhizobium* symbiosis either directly, by limiting the growth of the microsymbiont and/or indirectly, by regulating the growth of the acrosymbiont (Hashem *et al.*, 1998; Kuykendall *et al.*, 2000).

Kumar (2010) tested the temperature effect for number of *Rhizobium* strains among those only 7 strains could grow at maximum temperature (55ºC) and grew at 5ºC. The optimum temperature for all the isolates of *Rhizobium* spp. was 28ºC. The observed optimum temperature regime for endophytes (*Rhizobium, Bacillus* and *Pseudomona*s) was 28ºC. Minimum and maximum tolerated temperature regimes for isolates were 5ºC and 55ºC, respectively . Maximum temperature for survival of *B. japonicum* was reported from 33.7 ºC to 48.7 ºC (Kulkurni *et al.,* 2000).

In addition to temperature, pH also affects the survival and nodulation of the bacteria *Rhizobium*. Acidic pH is a significant reason for limited survival of bacteria and reduced nodulation. Low pH levels also affect the production and excretion of Nod metabolites (O' Hara and Glenn, 1994). The optimum pH for *Rhizobial* population is neutral to slightly acidic. Study reported that in acidic soil the *Rhizobial* population is often small and ineffective (Taurian *et al*., 1998). High pH may also have negative effects on survival of endophytic rhizobacteria (such as *Bradyrhizobium, Bacillus, Pseudomonas,* etc.).

Salt concentration is one of the abiotic factors whose fluctuation can negatively affect the growth of endophytic *Rhizobacteria*. Salinity is a hazardous to agriculture in arid and semiarid regions (Rao and Sharma 1995). Around 40% of the land surface of the world has potential salinity problems (Cordovilla *et al*., 1994). Optimum salt concentration for the isolates strains of *Rhizobium*, *Bacillus* and *Pseudomonas* was 2-3%, while the maximum and minimum salt concentration tolerated were 0.5% and 6% (Kumar, 2012).

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Nitrogen is an essential plant nutrient and its average content is up to 80% in atmosphere. It is deficient in soils; causative plants are unable to use this atmosphere nitrogen which leads to reduced agricultural yields. Biological nitrogen fixation system possibly is a good alternative. It makes available the nitrogen supply to the plants directly (as fixed nitrogen) without any loss. Treatment with nitrogen fixing microorganisms shows a considerable increase in growth.

Biological nitrogen fixation process by bacteria fix around 65% of the nitrogen currently utilized in agriculture, and will continue to be important in future sustainable crop production systems (Matiru and Dakora, 2004).

The biological conversion of N2 to ammonia performed by *Rhizobia* is highly energy consuming. N2 is reduced to NH3 under consumption of ATP and redox equivalents, and is associated with the formation of H2 as a byproduct (N2 + 8 H + 8 e- + 16 ATP → 2 NH3 + H2 + 16 ADP + 16 Pi). The enzyme that catalyzes the reaction is called nitrogenase and consists of the dinitrogenase reductase protein (Fe protein) and the dinitrogenase (MoFe protein) which actually catalyzes the reduction of N2. The formation of hydrogen gas is always accompanied by formation of ammonia and is a wasteful process. Therefore, some micro-organisms possess hydrogenases that recover this otherwise lost form of energy. In nature, nitrogen in soil is replenished and made available to plants through nitrogen fixation, a process which converts atmospheric dinitrogen to nitrogen compounds (Yu *et al.*, 2008).

The Leguminoseae that form a symbiosis with *Rhizobia* in root nodules are effective in fixing N2 in terrestrial systems. In addition to symbiotic N2 fixation, phyllospheric cyanobacteria and lichens, and *Rhizospheric* bacteria can also play an important role (Cleveland *et al.*, 1999; Vitousek *et al.*, 2002).

In response to symbiotic association with Rhizobia, flavonoid molecules are released as signals by the leguminous plant, which leads to induce the expression of *nod* genes (for nodulation) in *Rhizobia*, which in turn produce lipo-chitooligosaccharide (LCO) signals that generate mitotic cell division in roots leading to nodule formation (Dakora, 2003).

In legumes N2 fixation also benefits to linked non-legumes through transfer of fixed nitrogen to cereals growing with legume crops (Snapp *et al*., 1998) or to the following crops rotated with symbiotic legumes (Hayat *et al*., 2008). *Bradyrhizobium* fix more nitrogen that the plant can use. The excess nitrogen is left in the soil and available for other plants or later crops. Intercropping with a legume has the potential to decrease the need for applied fertilizer. Co-inoculation of PGPR and *Rhizobium* spp. have been shown to increase root and shoot dry weight, plant vigour, nodulation, and nitrogen fixation in various legumes. In the root surroundings presence of PGPR improve ability of *Rhizobia* to compete with indigenous populations for nodulation.

Sprent 2001 documents the many species in the legume family that have the ability to form intimate, intracellular associations with diverse nitrogen--fixing bacteria, known as *Rhizobia*. As Sprent, et al. (1987), a seminal review, explains, legumes begin life in a symbiont--free state and they acquire *Rhizobial* symbionts in the soil each generation. Typically, *Rhizobia* first infect legume seedlings soon after germination and form tumors (nodules) on the plant's roots. For a successful infection, a compatible pairing of legume and rhizobial genotypes is required. However, as the key review Denison (2000) describes, the degree of specificity varies widely for both the bacteria and the plant partners. Once a symbiotic nodule is formed, the bacteria fix nitrogen for the plant in exchange for plant derived photosynthates. Nodules eventually senesce, a process that Denison and Kiers (2004) argues is critical for *Rhizobia* to escape back into the soil; the nodule tissue softens and breaks down, after which a subset of the *Rhizobia* are released from the plant. An ecological meta--analysis shows how important legume--*Rhizobium* symbioses are at a global scale, because symbiotic *Rhizobia* convert atmospheric dinitrogen into compounds that are useable to legumes and ultimately to other plants (Cleveland, et al. 1999). In terms of agriculture, only a handful of legume species are cultivated on a large scale, but Ferguson, et al. (2010) pointed out that these crops contribute more than 25 percent of global production, including food staples, fodder for livestock, cover crops, and emerging biofuels.

There is a clear need to better understand the legume--*Rhizobium* symbiosis both in the wild and in agriculture. However, it is important that the legume--*Rhizobium* symbiosis is not unique. Other plants such as alders can also form nitrogen--fixing symbioses with bacteria; and diversity of bacteria that act as nitrogen--fixing bacterial endophytes and can provide growth benefits to many plants(Sturz, et al. 2000).

The presence of appropriate *Rhizobia* the soil is the first critical factor for nodule formation (Pepper, 1991; Pepper and Upchurch, 1991) with the relationship between certain bacteria and legumes being highly selective (Rending and Taylor, Taylor,1989). In some cases, only one *Rhizobium* spp. is effective on a particular species of legume (Salisbury and Ross,1992).

Rhizobial nod genes are important in the determination of host specificity, infection and nodulation and are involved in the exchange of signals between the plant and bacteria (Denarie *et al.*, 1992). Legumes release several flavonoids, some of which may be specific to a particular *Rhizobium*. Both *Bradyrhizobium* and *Rhizobium* spp. are attracted by flavonoids (Denarie *et al.*, 1992).

The attachment of the bacteria to the hairs is the start of the infection process (Rending and Taylor, 1989; Prescott *et al.*, 1996). The infection process involves a complex series of interactions, before the *Rhizobia* enter the root hairs of the host (Somasegaran and Hoben, 1994). *Rhizobium* spp. produces *nod* genes (nodulation genes) that promote the binding between bacteria and root hairs (Pepper and Upchurch, 1991; Prescott *et al.*, 1996). Attraction between the *Rhizobia* and root surface occurs due to van der Walls forces and leads to the linking of free carboxyl groups in the peptidoglycan of the *Rhizobial* cell to organic acidsfound on the root surface (Howieson, 1995). Specialized proteins, known as lectins, found on the surface of the roots are believed to act as recognition sites (Allen and Allen, 1981;). *Rhizobial* multiplication occurs in the *Rhizosphere* and on the root surface (Denarie *et al.*, 1992)

Tubular structures known as infection threads allow the invasion of the root hairs and the underlying cortical cells by the *Rhizobia*. The infection thread is important to the *Rhizobia* as it provides a means of avoiding the plant defence mechanisms (Pepper and Upchurch, 1991). Somasegaran and Hoben (1994) reported that the *Rhizobia* are released from the infection thread into the cytoplasm of the host cell, where bacterial cell multiplication takes place.

The movement of *Rhizobia* from the infection thread into the host cell results in the *Rhizobia* being surrounded by a membrane known as the peribacteroid membrane (Prescott *et al.*, 1996). This is the formation of nodules and leads to the proliferation of the enlarged rhizobia and cortical cells. The enlarged *Rhizobia* are often referred to as bacteroids in which the fixation of nitrogen by legumes occurs (Denarie *et al.*, 1992; Raven *et al.*, 1992; Salisbury and Ross, 1992; Somasegaran and Hoben, 1994

Leguminous plants are relevant economic and cultural important crops because their exceptional diversity, manifested in variety of vegetable forms that adapted to a wide range of ecological conditions, the high protein content of some grains, their use as pastures, increased world production and commodities. In this scenario, many farm investors, industries and researchers have focused attention in the development of biological and eco-friendly technologies for legume growth improvement and establishment. The ability of many legumes to form associations with bacteria that fix atmospheric nitrogen (the symbiotic association that improve growth) is thus a big matter of ecological and economic interest (Zahran, 2009).

Microorganisms are essential to the Earth's nitrogen cycle and to the Biological Nitrogen Fixation (BNF) process in leguminous plants, playing a very important role in terms of plant production in agriculture. Nitrogen fixing microorganisms could be used in live formulations (biofertilizer) that when applied to seed, root or soil colonize the *Rhizosphere*, or the interior of the plant, and promote growth by increasing the nitrogen supply to the host plant and building up soil health. The evaluation, in terms of economic and ecological costs, between chemical- and biological-nitrogen fertilizers support that BNF represents an economic, sustainable and environmentally friendly resource to guarantee the nitrogen requirement of an agro-ecosystem.

Chemical-fertilizer demand has historically been influenced by changing and often interrelated factors such as increasing populations and economic growth, agricultural production, prices, and government policies. In 2007, the production of chemical nitrogen fertilizers was 130 million tons which is likely to increase further in the coming years (FAO, 2011). Their production requires a great consumption of fossil fuels (1-2 % global fossil fuel) and is subjected of constant variations in prices (Vieira et al., 2010). Although their direct contribution to energy consumption seems minimal, it is unnecessary and unsustainable. On average, U.S. farmers apply 30-40 % more chemical nitrogen than is needed for optimal crop yield, thus wasting most of the applied chemical nitrogen. Given the rising cost of chemical nitrogen fertilizers, nitrogen fixation cover crops offer significant economic benefits. In 2006, the price of nitrogen fertilizers raised to 521 USD per ton in U.S. (Huang, 2007), estimating an over cost of 7 to 10 billion USD annually compared with FBN. For instance, the modest use of alfalfa in rotation with corn by U.S. farmers saved 200 to 300 million USD (Graham &Vance, 2003).In addition to the inconvenience of increasing prices, chemical nitrogen fertilization is associated with environmental problems because watershed contamination by nitrogen leaching, volatilization and denitrification. These problems could be avoided offering to farmers low-cost biofertilizer technologies. These are ecologically sound and their application could help to minimize the global warming as well as to reduce the fertilizer input in farming practices (Herridge et al., 2008a).

Chapter 3

MATERIALS AND METHOD

3. MATERIALS AND METHODS

This chapter deals with the description of the materials used and the methods or techniques adopted during the course of investigation.

3.1 Characterisation of *Rhizobium* Isolates collected from different organisations

Eleven Samples were collected from 3 different organisations

(1) Choudhary Charan Hariyana Agriculture University (CCHAU),Hissar- RPP10/16H1, RPP10/16H2,RPP10/16H3.

(2) Regional centre for organic farming Niladri Bihar ,BBSR - RBG10/16-B1,RAR-10/16-B2,RMB-10/16-B3,RGN-10/16-B4,RGN-10/16-B5

(3) Indian Institute of Pulses Research,Kanpur- RMB10/16K1,RLE10/16K2,RCP10/16K3

The collected samples were maintained in the laboratory and revived at regular interval. Fresh Culture of the isolates were taken for biochemical characterization.

3.2 Biochemical Characterisation

For confirmation following biochemical tests were carried out:

Catalase Activity

Different isolates which were 48 hours old were flooded with hydrogen peroxide and observed for liberation of effervescence of oxygen around the bacterial colonies according to Graham and Parker (1964).

Oxidase Activity

Few drops of p-aminodimethylaniline oxalate were added on the surface of isolates on YEM agar and observed for the production of color according to Kovaks (1956).

Acid from Glucose

Mannitol in the YEM agar was replaced by equal amount of glucose and bromothymol blue (25mg/l) was added to it, this modified media was inoculated with the strains and incubated. Change in color around the colonies was observed.

Methylene Blue and Gentian Violet Treatment

Methylene blue dye was added to the growth medium at a concentration of 0.1%, then inoculated with Rhizobium and incubated at 28°C ± 2°C for 2-7 days. Similar experiment was done with gentian violet at the concentration of 0.1% and observations were made according to Gao et al. (1994).

Starch Hydrolysis

Starch hydrolysis test was performed to determine the ability of micro organisms to use starch as a carbon source (De Oliverira, 2007). This medium was inoculated with Rhizobium and analyzed for starch utilization. Iodine test was used to determine the capability of micro organisms to use starch. Drops of iodine solution (0.1 N) were spread on 24 hours old cultures grown in Petriplates. Formation of blue color indicated non utilization of starch and viceversa.

Growth on Glucose Peptone Agar

Glucose Peptone Agar (GPA) plates were streaked with isolated strains and incubated. Presence of growth was observed after 48 hours according to Vincent (1970).

Urea Hydrolysis

YEM broth was amended with 2% (w/v) urea and 0.012% phenol red to check the urea hydrolysis. The broth was inoculated with log phase cultures and Journal of Agricultural Technology 2011, Vol. 7(6): 1705-1723 1709 incubated for 48 hours and observed for the production of color according to Lindstrom and Lehtomaki (1988).

Growth on Hofer's Alkaline Broth

Autoclaved Hofer's Alkaline Broth (HAB) was inoculated with log phase cultures and observed for growth after 48 hours (Hofer, 1935).

Gelatin Hydrolysis

Log phase cultures from YEM broth were swabbed on the surface of YEM agar plates containing 0.4% (w/v) gelatin to examine gelatinase activity. The plates were incubated at 28°C for 7 days (Sadowsky et al., 1983).

Citrate Utilization

Citrate utilization ability was determined, by replacing mannitol from YEM agar with equal amount of sodium citrate and bromothymol blue (25mg/l). The plates with modified media were inoculated and then incubated for 48 hours (Koser, 1923).

Growth in Presence of 8% kNO3

Strains were tested for the ability to grow in the presence of 8% KNO3 in YEM broth for a 7 days incubation period at 28°C (El Idrissi et al., 1996).

NaCl (2%) Tolerance

YEM agar media amended with 2% Nail (w/v) was inoculated and growth was observed after 48hours incubation.

Precipitation of Calcium Glycerophosphate

Precipitation of calcium glycerophosphate was carried out in YEM broth amended with calcium glycerophosphate. Formation of precipitate after the incubation indicates positive result (Hofer, 1941).

Antibiotic Resistance Test

The isolates were tested for antibiotic sensitivity by Kirby-Bauer disc diffusion method on YEM agar (Bauer et al., 1966). Cultures were inoculated Journal of Agricultural Technology 2011, Vol. 7(6): 1705-1723 1710 by swabbing with standard inoculums according to 0.5 Mc Farland tube over the entire agar surface. The agar surface was allowed to dry for 3-5 minutes before applying the antibiotic discs. Antibiotic discs were placed equidistantly on 90 mm Petri plate using sterile forcep. The plates were incubated aerobically at 28 ± 2°C for 48 hrs. Resistance to an antibiotic was detected by the inhibition zone formed around the discs. The antibiotics used were chloramphenicol (30µg), kanamycin (30 µg), tetracycline (15 µg), rifampicin (5 µg), nalidixic acid (30 µg), streptomycin (10 µg), neomycin (10 µg), vancomycin (30 µg), erythromycin (15 µg), cephalothin (30 µg), ampicillin (10 µg), gentamycin (10µg) and polyxifxin (300 units).

Utilization of Carbon and Nitrogen Sources

To determine the carbon and nitrogen utilization pattern, 80 µl of 10% w/v filter sterilized solutions of the carbohydrates (pentoses, hexoses disaccharides and trioses) and amino acids were added to 5 ml YEM broth in which yeast extract was reduced to 50 mg/l. The medium was then inoculated by the addition of exponentially (108 cells/ml) growing cultures of the isolates (Kumar et al., 1999). The inoculated broth were incubated at 28°C and kept at 150 rpm in an incubator shaker. Optical density was taken at 610 nm after 7 days incubation for measuring the growth.

Salt, pH and Temperature Tolerance

The ability of the isolated Rhizobial strain to grow in different concentration of salt was tested by streaking them on YEM medium containing 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5% and 5.0% (wt/v) NaCl. Differences in pH tolerance were tested in YEM agar by adjusting the pH to 3.5, 4.0, 5.0, 6.0, 6.5, 7.5, 8.0 and 9.0. All the plates were incubated at 28°C for 72 hours and YEM medium plates were used as controls. Difference in the range of growth temperature were investigated by incubation of bacterial cultures in YEM agar at 32°C, 34°C, 36°C, 38°C and 40°C. Control plates were incubated at 28°C. Strains were considered salt tolerant, resistant to acidity and temperature resistant when growth was similar to the growth in the control plates.

3.3 Experimental details

3.3.1 potray experiment

Preparation for nodulation experiment

Potray experiment was carried out to check the cross infection of different *Rhizobium* strains on Arhar. Soil was collected from O.U.A.T field and was sterilized to make free from microorganism at 121ºC and 15 lb pressure. For the purpose of nodulation, Only 3 kg of sterilized soils was taken in the potray.

Surface sterilization of seeds

Healthy seeds of Arhar collected from the College of Agriculture,,OUAT were taken for the experiment. Uniform sized seeds were rinsed with tap water. The seeds were then washed thoroughly with distilled sterilized water for at least five times. Seeds were surface sterilized with 2% sodium hypochloride(NaOCl) for 2 minutes then washed with sterilized distilled water for 1 minute.

Seed Sowing

About 24 hours before sowing, uniform irrigation was given. Seeds of Arhar were soaked with 11 different isolated *Rhizobium* strains and placed at a depth of 0.5-1cm in the po-tray. After 4-6 days of sowing, 2 healthy plants were maintained by thinning out the extra seedlings. The po-tray was then properly tagged and lebelled. Level of water maintained daily. After 15 days of seed sowing, fresh culture of the organism was prepared as in broth and reinoculated in potray to increase their viability for effective nodulation.

Physical parameter analysis

In the next step control sets were compared with the treatmented sets on the basis of different physical parameters i.e.shoot length, root length, leaf number, nodulation, biomass accumulation (Fresh and dry weight of shoots) of plants which were recorded after 45 days of seed sowing.

3.3.2 Pot Experiment

On the basis of nodulation in potray, the two most potent *Rhizobium* strains were selected in order to confirm the degree of effectiveness *Rhizobium* strain, a separate pot experiment was conducted (with same soil and same Arhar variety)

Preparation of culture pot

The soil was air dried and processed for ideal seed growth. Soil was sterilized to destory the microorganism at 121ºC and 15 lb pressure. This soil was filled in pots with1 kg soil per pot and watered equally.

Application type

Three different treatments were carried out for this pot experiment as follows

- **T1** as soil inoculation of the *Rhizobium*.
- **T2** as dual inoculation of the *Rhizobium*(Seed and Soil).
- **T3** as seed inoculation of the *Rhizobium*.

Seed inoculation

Healthy seeds of Arhar were taken for experiment. Uniform sized seeds were rinsed with tap water.The seeds were then washed thoroughly with double distilled sterilized water for at least five times.Seeds were surface sterilized with 2% sodium hypochloride(NaOcl) for 2 minutes then washed with sterilized distilled water for 1 minute.

Sowing

About 24 hours before sowing, all the pots were irrigated by unsterilized water. Five holes (1 to 2 cm deep) per pot were made with the help of sterilized glass rod maintaining equal distance from hole to hole. Sowing of 5 seeds was done on 12-04-17 by placing one seed in each hole with the help of sterilized forcep.

After germination a population of 2 plants per pot was maintained by thinning out the extra seedlings. Uniform irrigation to all pots was given.The pots was then properly tagged and labeled. After 15 days of seed sowing, fresh culture of the organism was prepared as in broth and reinoculated in po-tray to increase their viability for effective nodulation. As a preventive measure Chloropyruphus @ 2 ml in 100ml of distilled water was sprayed for the control of insects.

Observations recorded

The Arhar plants were uprooted after at 45 days sowing. Then the different physical parameters and physiological parameters were recorded.The chlorophyll content of leaf was estimated by spectrophotometry using acetone as the solvent for leaf extract .The percentage of nitrogen in soil was recorded by Kelplus method and the protein content of leaf was calculated by Lowry Method.

Chapter 4
RESULTS

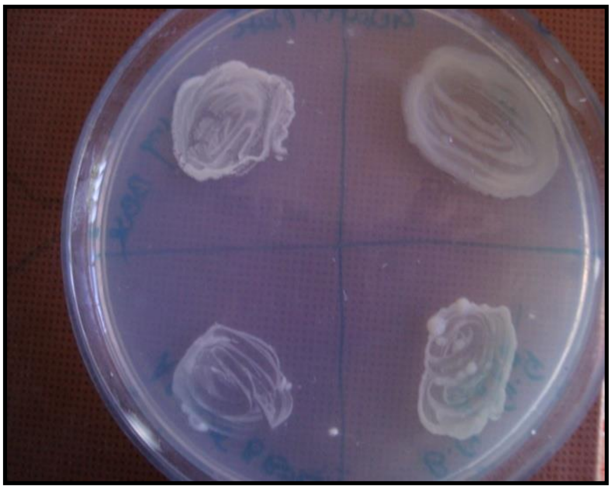
4. RESULT

The present experiment was designed especially (1) to observe nodulation behavior in order to screen effective *Rhizobium* isolate for better symbiosis.(2) experiment for evaluation of effective *Rhizobium* strain on growth performance of the crop.The results obtained from these studies are depicted below.

4.1 Biochemical and Enzymatic characterization for confirmation of *Rhizobium* strains.

A total of 11 *Rhizobium* sp. were collected and preserved in the Department of Microbiology, College of Basic Science & Humanities, OUAT were taken for their biochemical and enzymatic characterization. All the isolates were positive for oxidase and catalase test. None of the isolates were positive for citrate utilization test. All the *Rhizobium* sp. showed positive results for different enzymatic activities such as starch hydrolysis, urea hydrolysis etc. The isolates were also able to grow at 2% of NaCl concentration.

Figure-1:-Biochemical Test of collected samples



GELATINASE TEST

CASINASE TEST

4.2 Potray Experiment

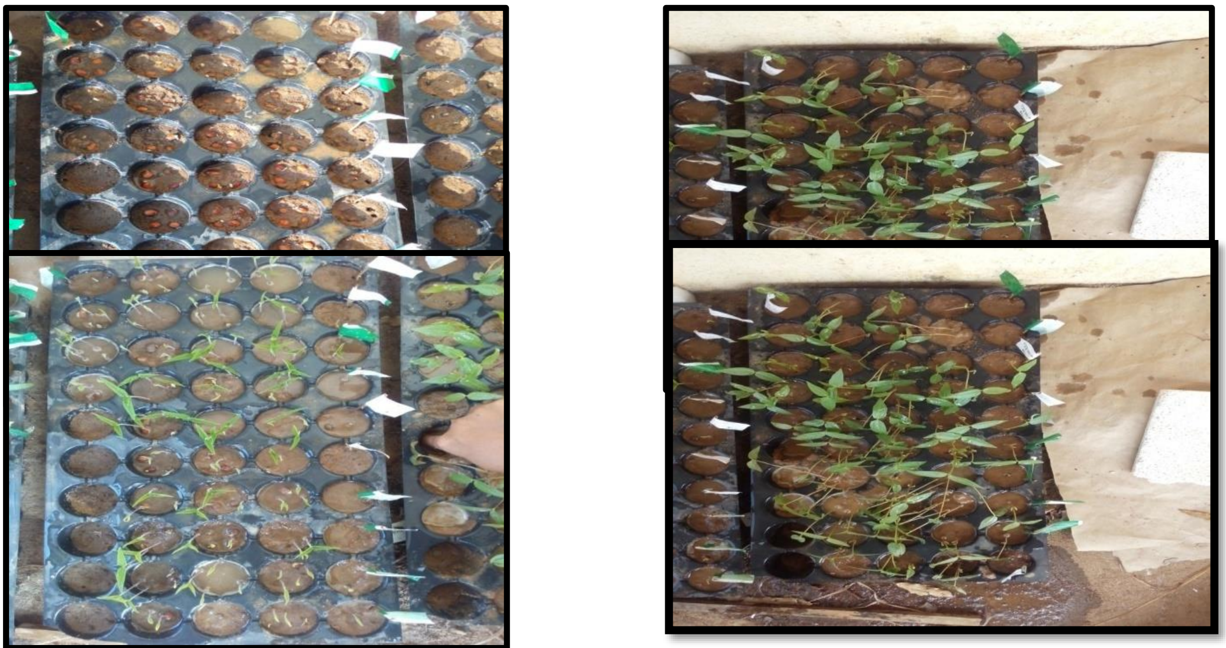
This experiment was conducted to screen the potent strains of nodule forming *Rhizobium* with Arhar from the 11 strains. Among all the isolates, only two *Rhizobium* sp. i.e. RMB and RCP showed maximum numbers of nodulation with respect to control. In case of strains like, RMB and RCP effective nodulations was found to be 31.66±11.66 & 27.66±1.45 respectively as compared to control where average nodule no. was 16.33±5.45. These two isolates were further tried with Arhar seeds to study their effect on growth and nodule formation under pot culture method.Potray experiment result was depicted in (Table-2) .

TABLE-2 Effect of different *Rhizobium* strains on nodulation and growth performance of Arhar

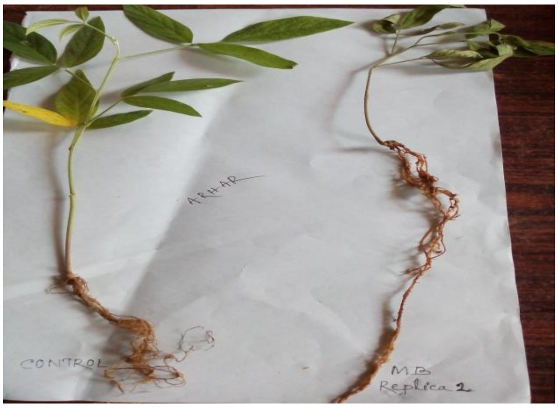
STRAIN	LEAF NUMBER	SHOOT LENGTH(in cm)	ROOT LENGTH (in cm)	FRESH WEIGHT (in gm)	DRY WEIGHT (in gm)	NODULE NUMBER
CONTROL	12±0.5	13.3±0.5	15.1±0.81	0.88±0.05	0.33±0.004	16.33±5.45
RGN 2	6.66±0.57	13.5±0.28	12.13±0.18	0.37±0.04	0.31±0.02	21.66±4.40
RGN 10/16	9.00±0.57	13.8±0.70	19.5±2.25	0.47±0.06	0.33±0.08	5.00±1.5
RPP 10/16 H1	12.33±0.57	16.2±1.43	10.96±1.03	0.41±0.02	0.27±0.02	14.66±3.52
RPP 10/16 H2	12.33±0.57	14.23±0.72	12.33±1.43	0.30±0.04	0.19±0.03	17.00±9.86
RRP 10/16 H3	9.00±0.57	13.16±0.44	18.40±2.88	0.28±0.02	0.19±0.006	16.00±6.42
RBG 10/16 B1	10.00±0.57	11.00±1.15	12.60±1.50	0.25±0.01	0.21±0.003	10.66±4.70
RAR	14.00±0.57	14.40±0.30	10.36±0.44	0.24±0.004	0.26±0.06	14.33±5.36
RMB	10.33±0.57	12.06±0.29	11.83±1.16	0.33±0.04	0.27±0.03	31.66±11.66
LE SOIL	12.00±0.57	14.76±0.39	10.56±0.52	0.45±0.01	0.18±0.003	8.00±2.08
CP SOIL	11.00±0.57	12.60±0.30	6.33±0.44	0.50±0.08	0.21±0.02	27.66±1.45
MB SOIL	10.33±0.57	10.90±1.06	16.76±1.74	0.37±0.01	0.16±0.01	17.00±7.23

(Values are mean of three samples and ± standard error. Figure in parentheses indicate percentage change over control. Result is significant at $p \leq 0.05$.)

Figure-2:-Potray Experiments for active crop growth

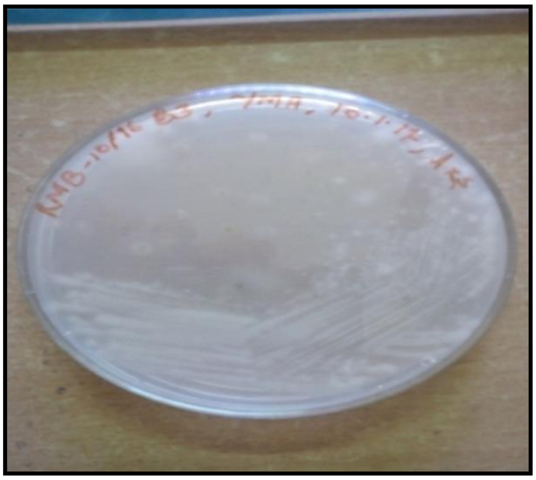


POTRAY EXPERIMENTS; VIEWS AT DIFFERENT GROWTH STAGES OF CROP

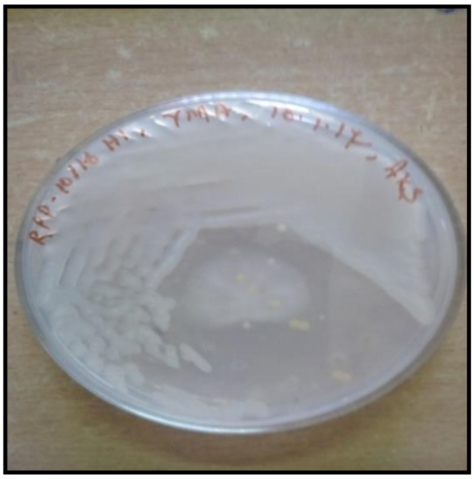


NODULATION PHASE

Figure-3:-Screening of suitable strains on the basis of nodulation



RCP



RMB

PURE CULTURE OF ORGANISMS

4.3 Pot Experiment

Two efficient Strains, selected on the basis of nodulation were applied with treatments like T1 Soil inoculation,T2 Dual treatments like(seed application and soil), T3 only seed treatment. All Physical growth Parameters of the crop plant along with biochemical parameters like Total chlorophyll, chlorophyll –a, chlorophyll –b, carotenoids and protein content of leaf and nitrogen content of soil were analysed to evaluate effect of the inoculated *Rhizobium* strain.The result obtained with respect to different parameters as follows.(Table-3)

(a)Shoot length T1 The RMB *Rhizobium* strain for treatment1 showed shoot length of 22.6±1.25 cm with increase in percentage of 33.25 over the control where the shoot length was 16.96±0.02cm. As two potent strains were taken, in the second strain RCP, shoot length was 20.83±0.28 cm with an increase of 22.81%.

TABLE-3:- Effect of selective *Rhizobium* strains on growth performance of Arhar in different treatment

TREATMENT	STRAIN	NO.OF LEAF	SHOOT LENGTH(in cm)	ROOT LENGTH(in cm)	FRESH WEIGHT(in gm)	DRY WEIGHT (in gm)
	CONTROL	7±0.57	16.96±0.02	5.13±0.34	0.169±0.0008	0.052±0.002
T1	RMB	12.33±0.88 (+76.14)	22.6±1.25 (+33.25)	7.66±0.28 (+49.31)	0.343±0.035 (+102.95)	0.074±0.003 (+42.30)
	RCP	11.66±0.88 (+66.57)	20.83±0.28 (+22.81)	6.58±0.40 (+28.26)	0.245±0.0008 (+44.97)	0.069±0.005 (+32.69)
T2	RMB	10.33±0.88 (+47.57)	19.90±0.20 (+17.33)	5.6±0.30 (+9.16)	0.234±0.061 (+38.46)	0.060±0.005 (+15.38)
	RCP	6.66±1.46 (-4.85)	18.93±1.03 (+11.61)	4.23±0.14 (-17.54)	0.194±0.004 (+14.7)	0.055±0.018 (+5.76)
T3	RMB	11±1.16 (+57.14)	18.96±0.03 (+11.79)	5.56±0.34 (+8.38)	0.240±0.009 (+42.01)	0.062±0.003 (+19.23)
	RCP	5.66±1.20 (-19.14)	15.0±0.11 (-11.55)	6.30±0.37 (+22.80)	0.193±0.005 (+14.20)	0.056±0.004 (+7.69)

(Values are mean of three samples and ± represents standard error. Figure in parentheses indicate percentage change over control. Result is significant at $p \leq 0.05$.)

Figure-4:-Pot Experiment

T1 RMB

T1 RCP

T2 RMB

T2 RCP

T3 RMB

T3 RCP

CONTROL

T2 In treatment 2, with RCP strain 19.90±0.20 cm shoot length was observed as compared to 16.96±0.02 cm in control with 17.33% increase. In the second strain RCP, with shoot length was 18.93±1.03 cm with an increase of 11.61% over the control.

T3 RMB strain for treatment3 the shoot length value of average was found to be 18.96±0.03 cm with increasing percentage of 11.79 over the control with average 16.96±0.02.Then with RCP, shoot length was found to be 15.0±0.11 cm. However, there was a decrease in the shoot length in this case with decreasing percentage of 11.55.

(b)Root length T1 RMB strain of treatment1 showing root length of average 7.66±0.28 with increasing percentage of 49.31 as compared to control having root length average 5.13±0.34.As we took two potent strains so the following strain is considered to be RCP with root length average of 6.58±0.40.with increasing percentage of 28.26.

T2 RMB strain of treatment1 having root length of average 5.6±0.30. with increasing percentage of 9.16 over control with root length of average 5.13±0.34.The second potent strain so was considered to be RCP with root length of average 4.23±0.14.with decreasing percentage of 17.54.

T3 In RMB strain of treatment1 root length of average was found to be 5.56±0.34. with increasing percentage of 8.38 as compared to control having root length average 5.13±0.34.As we took two potent strains so the following strain was considered to be RCP with root length average of 6.30cm±0.37.with increasing percentage of 22.80.

(c)No.of leaf T1 RMB strain of treatment1 showing leaf no. of average12.33±0.88 with increasing percentage of 76.14 as compared to control having leaf no. average 7±0.57.As we took two potent strains so the following strain was considered to be RCP with leaf no. average of 11.66±0.88.with increasing percentage of 66.57.

T2 RMB strain of treatment1 giving leaf no .of average10.33±0.88 with increasing percentage of 47.57 over the control which having leaf no.of average 7±0.57.As we took two potent strains so the following strain was considered to be RCP with leaf no. average of 6.66±1.46.with decreasing percentage of 4.85.

T3 RMB strain of treatment1 was found to be leaf no. of average 11±1.16 with increasing percentage of 57.14 as compared to control having leaf no. average 7±0.57.As we took two potent strains so the following strain was considered to be RCP with leaf no. average of 5.66±1.20.with decreasing percentage of 19.14.

(d)Fresh weight T1 RMB strain of treatment1 showing fresh weight of average 0.343±0.035 with increasing percentage of 102.95 as compared to control having fresh weight of average 0.169±0.0008.As we took two potent strains so the following strain is considered to be RCP with fresh weight average of 0.245±0.0008.with increasing percentage of 44.97.

T2 RMB strain of treatment1 showing fresh weight of average 0.234±0.061 with increasing percentage of 38.46 as compared to control having fresh weight of average 0.169±0.0008.As we took two potent strains so the following strain is considered to be RCP with fresh weight average of 0.194±0.004.with increasing percentage of 14.7.

T3 RMB strain of treatment1 showing fresh weight of average 0.240±0.009 with increasing percentage of 42.01 as compared to control having fresh weight of average 0.169±0.0008.As we took two potent strains so the following strain is considered to be RCP with fresh weight average of 0.193±0.005.with increasing percentage of 14.20.

(e)Dry weight T1 RMB strain of treatment1 showing dry weight of average 0.074±0.003 with increasing percentage of 42.30 as compared to control having dry weight of average 0.052±0.002.As we took two potent strains so the following strain is considered to be RCP with dry weight average of 0.069±0.005 .with increasing percentage of 32.69.

T2 RMB strain of treatment1 showing dry weight of average 0.060±0.005 with increasing percentage of 15.38 as compared to control having dry weight of average 0.052±0.002.As we took two potent strains so the following strain is considered to be RCP with dry weight average of 0.055±0.018.with increasing percentage of 5.76.

T3 RMB strain of treatment1 showing dry weight of average 0.062±0.003 with increasing percentage of 19.23 as compared to control having dry weight of average 0.052±0.002.As we took two potent strains so the following strain is considered to be RCP with dry weight average of 0.056±0.004with increasing percentage of 7.69.

TABLE-4:-Changes in Biochemical characteristics of Arhar under various conditions of *Rhizobium* application.

TREATMENT		Chla mg/ml	Chlb mg/ml	TOTAL chl mg/ml	CAROTENOID mg/ml	PROTEIN mg/ml
CONTROL		9.79±0.01	3.68±0.04	13.47±0.05	4.49±0.005	0.19±0.001
T1	RMB	10.51±0.02 (+7.35)	4.44±0.06 (+20.65)	14.90±0.03 (+10.61)	5.43±0.008 (+20.93)	0.36±0.001 (+ 89.47)
	RCP	8.21±0.03 (-16.13)	3.17±0.04 (-13.85)	11.38±0.03 (-15.51)	4.08±0.01 (-10.24)	0.03±0.001 (-84.21)
T2	RMB	7.27±0.03 (-25.74)	2.71±0.03 (-26.35)	9.99±0.02 (-25.83)	3.37±0.008 (-24.94)	0.20±0.001 (+5.26)
	RCP	20.21±0.25 (+106.43)	11.71±0.03 (+218.20)	31.91±0.21 (+136.89)	4.51±0.16 (+0.44)	0.26±0.001 (+36.84)
T3	RMB	8.39±0.01 (-14.30)	2.41±0.03 (-34.51)	10.80±0.04 (-19.82)	2.89±0.007 (-35.63)	0.21±0.001 (+10.52)
	RCP	4.42±0.01 (-54.85)	1.77±0.03 (-51.90)	6.19±0.04 (-54.04)	2.04±0.006 (-54.56)	0.21±0.001 (+10.52)

(Values are mean of three samples and ± standard error. Figure in parentheses indicate percentage change over control. Result is significant at $p \leq 0.05$)

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Chlorophyll and Protein Estimation of leaf

Chlorophyll content of leaf were estimated using spectrophotometry and the optimum density of pigments like chla.(663nm),chlb.(645nm),carotenoid(470nm)were obtained from the spectro reading and protein by Lawry method(at 660nm).Subsequently total chlorophyll value were estimated as adding both the chl.a and chl.b value in the given table as follows. The percentage of efficiency was compared with control which had chl a.value of 9.79±0.01mg/ml,chl.b value 3.68±0.04mg/ml,total chlorophyll 13.47±0.05mg/ml,carotenoid 4.49±0.005mg/ml and protein value 0.19±0.001mg/ml.T1 RMB for chl.a was with increasing percentage of 7.35,chl.b with increasing percentage of 20.65, total chlorophyll of increasing percentage of 10.61,carotenoid with increasing percentage of 20.93 and protein with increasing percentage of 89.47. T1 RCP for chl.a with decreasing percentage of 16.13,chl.b with decreasing percentage of 13.85,total chlorophyll with decreasing of 15.51,carotenoid with decreasing percentage of 10.24 and protein with decreasing percentage of 84.21.Similarly for T2RMB for chl.a was with decreasing percentage of 25.74,chl.b with decreasing percentage of 26.35, total chlorophyll of decreasing percentage of 25.83,carotenoid with decreasing percentage of 24.94 and protein with increasing percentage of 5.26. T2 RCP for chl.a with increasing percentage of 106.43,chl.b with increasing percentage of 218.20,total chlorophyll with increasing of 136.89,carotenoid with increasing percentage of 0.44and protein with increasing percentage of 36.84.Following T2, T3RMB for chl.a was with decreasing percentage of 14.30,chl.b with decreasing percentage of 34.51, total chlorophyll of decreasing percentage of 19.84,carotenoid with decreasing percentage of 35.63 and protein with increasing percentage of 10.52.T3RCP for chl.a with decreasing percentage of 54.85,chl.b with decreasing percentage of 51.90,total chlorophyll with decreasing of 54.04,carotenoid with decreasing percentage of 154.56 and protein with increasing percentage of 10.52.(Table-4)

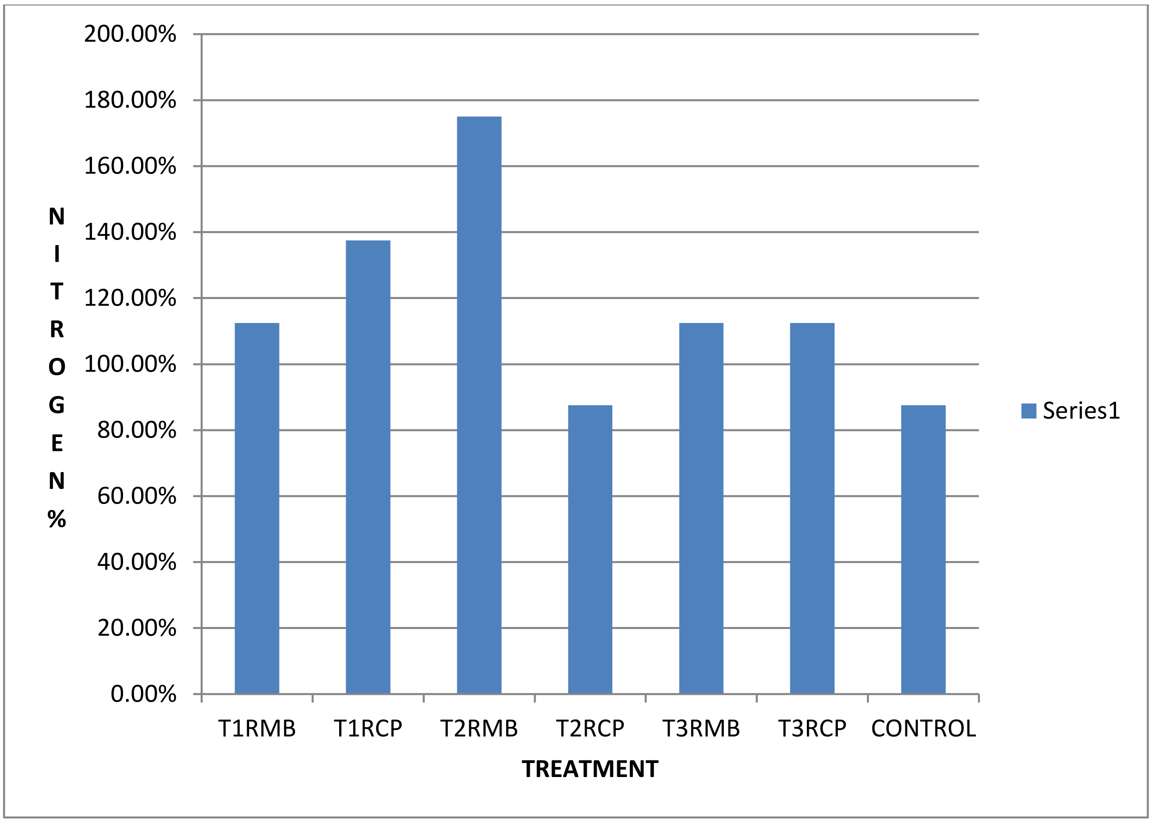
Nitrogen Estimation(%N_2) by Kelplus Method

Soil Nitrogen was estimated as taking difference between final and initial burette reading and the percentage was calculated by multiplying the difference with 125.

% of N_2= D×125 where;D=FBR-IBR

The reading estimated as like T1 RMB was 112%,T1 RCP was 137%,T2 RMB was 175.0%,T2 RCP was 87.5%,T3 RMB was 112.5%,T3 RCP was 112.5% where as control gave 87%.The result was analysed below in the given graph.

Figure 5:- Percentages of Nitrogen contains in soil.


200.00%
180.00%
160.00%
140.00%
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
NITROGEN %
T1RMB
T1RCP
T2RMB
T2RCP
T3RMB
T3RCP
CONTROL
TREATMENT
Series1

CHAPTER-5

DISCUSSION

5. DISCUSSION

The results obtained from the screening of different *Rhizobium* strains for effective nodulation on Arhar showed significant increase in various parameters studied. The present investigation is important in selection of effective *Rhizobium* strain for Arhar to increase productivity of the pulse. From the biochemical test it was confirmed that all the 11 collected strains were gram negative type bacterium i.e. *Rhizobium*. Convenient to this finding, Amarger et al. (1994); Gupta et al. (2000a); Gupta et al. (2000b); Chowdhury and Gupta, (2003); Shamseldin and Werner (2004) and Gupta et al. (2005) conducted isolation-screening experiments and selected effective location specific *Rhizobium* isolates of different legumes on the basis of Gram's reaction, biochemical variability and BNF (Biological nitrogen fixation) parameters as per our findings.

Among the leguminous seed crops, Arhar is one of the most important leguminous crop as it is the richest protein source consuming on daily diet basis in world wide. (Tiwari, 1999). Therefore, the study is compulsory in order to select effective natural biofertilizers like, *Rhizobium* strains for enhancing sustainable Arhar production.

5.1 Potray Experiment

The potray experiment, designed to screen for effective nodulation in 11 strains of *Rhizobium* showed significant result. Data presented in Table 2 and in Figures reveal that plant height,root length,leaf number,biomass and considering mostly the nodulation phase of Arhar in one month increased significantly due to inoculation of *Rhizobium* strains under optimum environmental condition.For e.g- difference in the height was non-significant in between 15 to 30 days, because inoculation of Rhizobium strains bring effective measures after 15 days of seed sowing,Gupta et al. (2005).

According to the study on Arhar the impotance was given mostly to the nodulation capacity of legume plants by the host specific behavior of Rhizobium which forms symbiosis with the legumes to fix atmospheric nitrogen.

5.1(a) Nodulation

Nodulation data after 45 days, presented in Table 2, indicate that among all the nodulated plants highest number of nodules was associated with plants achieved from seed inoculation with Rhizobium strains like RMB and in RCP. Gupta (1996) observed that number of nodules increases by inoculation with effective *Rhizobial* strains. This observation is also supported by the findings of further researchs like,Katre et al. (1997); Gupta et al. (2000a) and Gupta et al. (2000b); Egamberdiyeva et al., (2004); Gupta et al. (2005); Appunu and Dhar (2006), Singh and Kumar (2007); Sudaric et al. (2008); Sikora et al. (2008); also screened *Rhizobium* strains for different legumes basing on the nodulation study to increase productivity.

5.2 Pot Experiment

Pot experimental result of the two selective *Rhizobium* strains are depicted in (Tables -3) . From this experiment, potentiality of selected *Rhizobial* strains from the potray experiment on the basis of nodulation were carried out as taking three types of treatments like T1 (soil inoculation),T2 (both soil and seed treatment),T3(only seed treatment). Screening through such process with local isolates resulted in recommendation of most suitable strain of *Rhizobium* (Siddaramaiah and Bagyaraj, 1981; Kumar and Srivastava, 1994) . Gupta (1995) and Katre et al. (1997) also expressed the similar views and mentioned that microbial isolates even within same species, same place; vary greatly in their effects on plant.

5.2(a) Plant Height

Data presented in (Table 3), reveals that plant height after 15 days of seed sowing increased significantly over control due to inoculation of *Rhizobium* isolates. Control plant height 16.96 cm as shoot height and 5.13cm as root height was recorded as lowest, while 22.6cm and20.83cm was considered as highest shoot length respective to strains RMB and RCP with T1. Similarly incase of root length both the same strains of T1 type were measured as 7.66cm,6.58cm respectively.Sikora et al. (2008).The result showed that soil inoculation is the suitable treatment to make plant growth by the application of *Rhizobium* concominant to the findings of Shamseldin and Werner (2004).

5.2(b) Plant Biomass

Results of fresh weight and dry weight of plant (Table-3), increased significantly on inoculation of two selected *Rhizobium* isolates in the 3 different treatments. The fresh shoot weight and dry weight i.e. 0.343gm and 0.074gm respectively was recorded as highest, of the strain RMB with Treatment 1 and 0.245gm and 0.069gm was considered the second highest fresh and dry weight of the strain RCP. Chowdhury and Gupta, (2003) reported similar increase in plant biomass following inoculation of suitable *Rhizobial* strain.

5.3 Biochemical estimation

Also the Total chlorophyll, Chlorophyll-a, chlorophyll-b & carotenoid contents and protein content (Table-4) increased with inoculation of the *Rhizobium* strains under different experimental conditions. Significantly high increase in these parameters indicate effectiveness of the strain for Arhar and can be most suitable for application in crop field. It was observed that in case of seed treatment i.e.T2 strain of RCP showed better effect than other treatments. Similar positive effect was reported by Egamberdiyeva et al. (2004).

Subsequently increase in soil N_2 content of all 3 treatments indicate competitiveness of the augmented microbe in the soil and it is efficient in undertaking N_2 fixation and release to the soil. So as the result in figure.1 indicated the highest peak in case of strain RMB by treatment 2.Thus mainly from the above physical parameters it was concluded that the treatment1 found to be significant by the effect soil inoculation was carried out by the *Rhizobium* strain RMB and RCP. So as It was evident from the experiment that only inoculation in soil with amendment of *Rhizobium* is more effective in undertaking N_2 fixation, which can decorate the Indian economy as producing more quantity of legumes with helping the farmers of the country in general and state in particular to enhance the protein power in food supply in terms to quality check.

CHAPTER-6
SUMMARY AND CONCLUSION

6. SUMMERY AND CONCLUSION

The study on **“Screening of *Rhizobium* as a bioinoculum in Arhar under Agro-climatic zone of Odisha”**was undertaken with eleven different *Rhizobium strains* collected from various authroised organizations..The established fact is that growing of pulse crops in addition to both soil and seed inoculation as differently with Rhizobium,a natural organic fertilizer which provide effective nodulation by symbiotic nitrogen fixatation due to its host specific nature with legumes.As the work done to screen Rhizobium and characterized them with the help of different biochemical tests.Then to observe the nodulation quality of strains were taken in the potray ,selected potent strains as RMB and RCP again processed for pot experiment with three different treatments like T1 as soil inoculation,T2 as soil with seed inoculation and T3 for seed inoculation only.In the next step one of treatment was considered as the most efficient one on the basis of different physical parameters as mentioned in result table along with different physiological parameters taken in to observations.The result shown like T1 as the most qualitative one to proceed with future experiment for better crop production.

CONCLUSION

The following conclusion could be done from the experiment conducted on the study**“Screening of *Rhizobium* as a bioinoculum in Arhar under Agro-climatic zone of Odisha”**

- The work plan was proceeded on the basis of *Rhizobium* inoculation as it resulted significantly with respect to different physical and physiological parameters as well as in nodulation.
- Hence it is required to pay due attention for the development of legume production technology .Thus the objective is to create a healthier and safer world with enough protein amendment that can defend nutrient deficiency .
- So the future aspect must to focus on better and secured production of native *Rhizobium Sp.* that can facilitate more legume production in the country as sustaining stable economical status up to the mark.

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