CHAPTER 1

Introduction

1.1 Rice cultivation in Thailand

The approximated land area of Thailand is 60,399,700 hectares. About 8.6 million hectares, or 14.2%, are rice land, the main agricultural crop of Thailand (Sorasith and Yasuo, 1983). Rice serves as the main food stuff of Thai people and surplus is exported. The average yield of rice production in Thailand is about 1.2-2.0 ton per hectares, whereas Japan's or Korea's production yield per area is about three times higher than Thailand's. Investigation and research for the improvement of rice yield has been carried out in Thailand since 1940 (Sorasith and Yasuo, 1983). Nitrogen is usually the limiting nutrient in soils. Most field experiments conducted in rice growing countries indicated that nitrogen fertilization, either as organic or green manure or as a chemical fertilizer plays a dominant role in increasing rice yield (Patnaik and Rao,1979). In Thai paddy fields where only a small amount of fertilizer has been applied to the soil

(Swaminathan,1984), most of the nitrogen removed from the soil by crop consumption thus mainly comes from the soil nitrogen. In contrast, in a developed country, nitrogen source for crop use comes from applied nitrogen fertilizer. The avaiable form of nitrogen for rice plants are ammonia-nitrogen and nitrate-nitrogen. In general, ammonium phosphate (16-20-0) fertilizer, which was tested in the pilot demonstrations, and found to be profitable in the increasing of rice yield in Thailand is the recommended form of chemical fertilizer. The amount of 16-20-0 fertilizer imported into Thailand has therefore increased considerably since 1966 (Sorasith and Yasuo, 1983). Manufacture of nitrogen fertilizer requires high fossil energy inputs and results in increasing fertilizer price during energy crisis. Hence, most of the poor Thai farmers cannot afford to add any chemical fertilizer in their farms. As a consequence, the natural process so-called biological nitrogen fixation (BNF) is by far the most convincing process to substitute chemical fertilizer for the improvement of the yield of rice production in Thailand.

1.2 Biological nitrogen fixation (BNF)

The biological nitrogen fixation is an emzymatic reduction of atmospheric dinitrogen (N_2) to ammonia. The ability to fix dinitrogen by nitrogenase enzyme is fairly distributed among the procaryotic microrganisms so-called N_2-fixing microorganisms or diazotrophs. N_2-fixing microorganisms are classified according to their ecology into three groups: the obligatory symbiosis, associative symbiosis and asymbiosis or free - living nitrogen - fixers. The most agriculturally important group is the symbiotic association of nitrogen - fixing bacteria with roots of crop plants. Now more than one hundred species were reported as diazotrophs which are heterotrophs, phototrophs and cyanobacteria (Postgate, 1982). The three major groups of diazotrophs in rice paddy fields are free-living cyanobacteria, symbiotic Anabaena-Azolla, free-living and associative bacteria (Watanabe and Roger, 1984). Azolla has been used as organic nitrogen fertilizer in paddy field together with phosphorus application which was found to be necessary for Azolla cultivation (Sawatdee, et al., 1978 and Cholitkul, et al., 1980). Many studies have shown that wetland rice roots are inhabited by a large

number of nitrogen-fixing heterotrophic bacteria, which partly account for nitrogen-fixing activity in the paddy fields (Yoshida and Ancajas, 1971, 1973; Dommergues et al., 1973; Dommergues and Rinaudo, 1979). However, not much information is available on the composition and characterization of nitrogen-fixing bacterial population associated with the rice root. The presence in rice fields of Klebsiella (Dugrid, 1959; Yoo et al.,1986 and Gerlach et al.,1989), Beijerinckia (Dobereiner and Ruschel, 1961; Diem et al.,1978), Azotobacter (Purushothaman et al., 1976), Azospirillum (Kumari et al., 1976; Nayak and Rao, 1977; Silva and Dobereiner, 1978), and Enterobacter (Watanabe et al.,1977), have been reported. N_2-fixing activity by acetylene reduction assay of washed roots is much higher than in the adjacent soil (Yoshida and Ancajas, 1973). The nitrogen fixation by diazotroph attached to the root surface is termed "associative N_2-fixation" and believed to be a major source of nitrogen in nonfertilized rice fields. The rice plants associated with these N_2-fixing bacteria also excrete some carbohydrates and amino acids which enhance bacterial colonization (Rae and Castro, 1967). Among the three major groups of diazotropes in rice paddy field, the bacterial group is the most

interesting because they associate with rice roots. N_2-fixing bacteria associated with 3 regions of rice: i) outer rhizosphere, ii) rhizoplane, and iii) endorhizosphere or histosphere.

In the rhizosphere of rice in Thailand, Boonjawat et al. (1982) estimated that the amount of total nitrogen fixed by associative bacteria is in the range of 20-80 kg N per hectare per crop. By using the acetylene reduction activity assay (ARA) of washed and surface sterilized roots, 259 isolates of N_2-fixing bacteria were collected from washed roots and rhizospheric soils of rice grown in acid soil and semi-arid soil of Thailand. All of them are Gram - negative rod. Only 22 strains showed ARA higher than 1 umol per OD420nm per day. Only three representative strains were selected for further study namely R15, R17 and R25. The chemotaxonomy of these three bacteria were reported by Boonjawat et al.,1986. On the basic of phenotytic characteristics and per cent G+C content, R15 and R17 are closely related to the <u>Klebsiella</u> spp. and R25 resembles to <u>Azospirillum</u> <u>lipoferum</u>. Boonjawat and Limpananont (1985), working with <u>Klebsiella</u>, R15, R17 and strain NG13 of <u>Klebsiella</u> <u>oxytoca</u> inoculated in

several varieties of rice seedling, reported the formation of enveloped micronodule structures in some of the local varieties of rice such as RD5, and RD7. A few bacterial clusters were also found in the epidermal cells of rice root tissue. Moreover, rice lectins which are epicuticular and secretory glycoproteins of rice were demonstrated to play an important adhesive function in these association. For these reasons, the interaction between rice roots and these N_2 - fixing bacteria has been described as "associative". The original information of these rhizospheric N_2 - fixing bacteria are shown in Table 1.1.

1.3 Strains Identification

The ability to detect and identify each specific strain of N_2 - fixing bacteria in both laboratory and field situation will be useful in ecological studies of the interrelationship between a particular bacterial strain and its plant host, with emphasis on compatability and competitive ability of that strain after inoculation into various paddy fields.

Table 1.1 Information on bacterial strains (Boonjawat et al., 1986)

Strain No.	Country	Rice cultivar	Isolation site
Indigeneous strains			
17R1a2 (R17)	Thailand (Tapra)	RD6	Rhizoplan (non-sterile)
15RR12 (R15)	Thailand (Rangsit)	RD7	Rhizoplan (non-sterile)
25Sa12 (R25)	Thailand (Tapra)	RD6	Rhizospheric soil
Exotic reference strains			
Klebsiella oxytoca (NG13)	Japan (NIG)	not known	Rice rhizosphere
Pseudomonas (H8)	Philippines (IRRI)	IR26	Histosphere
Pseudomonas (KLH76)	Philippines (IRRI)	Khao-lo	Histosphere
Azospirillum lipoferum (FS)	Philippines (IRRI)	-	Histosphere
Azospirillum lipoferum (34H)	Philippines (IRRI)	IR34	Histosphere

Strains of some Gram negative rod shape bacteria; such as Rhizobium spp., Klebsiella spp. and Azospirillum spp. have dramatic differences which are distinguishable from each other by direct examination under microscope. Fluorescent dyes (fluorochromes), such as fluorescein isothiocyanate (FITC) and acridine orange which react with proteins and nucleic acid, respectively, have been used to stain microbial cells and distinguish them from the environmental background with high sensitivity (Parkinson et al.,1971; and Ransey, 1978). But the nonselective staining procedure does not allow for differentiation of one species from other rod shape bacteria in complex microbial assemblages. Though microorganisms are nondescript in morphology, they do have unique chemical, physiological and genetic features which can be used to identify genera, species, and even strains of a particular microorganism.

1.4 Serological techniques

Serological techniques rely on differences in the chemical nature of particulate and soluble antigen of cells and provide a powerful tool for microbial

differentiation. The bacterial cell possesses a variety of specific antigenic determinants that may be used to identify specific strains. Of the two basic types, somatic and flagellar antigens, the former has proven very useful for routine identification of bacterial strains (Means and Johnson, 1964; Williams and Chase, 1977). Antigen - antibody reactions are highly specific in that the antibody reacts only with the antigen that elicited its formation. Antisera are the most specific reagents for identifying many strains of microorganisms.

In principle, the serological methods are devided into three steps as follow.

(i) Preparation of antigens for immunization

Growth of microorganisms are important in order to be able to use cells as antigens for immunization. It is necessary to understand antigenic structure of the microorganism and the requirements of the serological technique for which the antiserum is to be prepared. To obtain somatic antigens, subcultures are selected from smooth single colonies on agar, grown in broth, suspended in saline, then heated to destroy the flagellar antigens (Davies, 1951; Woff and Barker, 1968).

In principle, defined medium should be used whenever possible to grow the cells to be injected into animals, to ensure that the antisera thus obtained will contain only antibodies against bacterial antigens.

In most serological studies with Azotobacter species, cultures were grown in defined media (Jensen and Petersen, 1955; Norris, 1960; Holme and Zacharies, 1965; Zarnea et al., 1966; Tchan and de Ville ,1970). Although it is a good practice to use a defined medium, it does not mean that growth of immunizing antigens on complex media will necessarily lead to complications. Dazzo and Milam (1975) immunized goats with Azospirillum lipoferum and Azotobacter paspali grown on a trypticase soy broth without the medium causing any apparent interference when the antisera were used in a range of serological reactions. The strains that are to be identified serologically may then be grown on any convenient complex medium. Usually intact cells are injected, although for special purposes, disintegrated cells have been used (Dudman, 1964; Humphrey and Vincent, 1973). Zarnea et al. (1966) found that suspensions of Azotobacter cells disrupted by repeated freezing and thawing produced superior antisera.

Bacteroids from nodules of legume or bacterial cultures may be used as the test antigen. When working with nodule antigens, most investigators prefer to use fresh nodules. However, Somasegaran et al. (1983) demonstrated that dried nodules worked as well as freshly picked nodules as the antigen source. Nodules may be frozen as well as stored for future analysis without a loss of antigenic properties.

(ii) Preparation of antisera

To produce antiserum against any antigen, one must decide upon the animal species, injection schedule, and the manner of bleeding (Campbell et al., 1970).

Rabbits have been used almost exclusively as the animal species for production of antisera against diazotrophs, because high antibody titers can be achieved and maintained. In addition, they are easy to obtain, are docile, and are relatively inexpensive. However, other animals can be used, including mice, rats, sheep and goats. Immunization procedures fall into one of the two general classes: (1) primary immunization by repeated intravenous injection of the antigen without an adjuvant, at intervals of one or more days, followed

by a number of booster injections prior to bleeding , and (2) primary immunization with a single intramuscular injection of the antigen emulsified with an adjuvant, followed by one or more booster injections before bleeding. No systematic comparison has been made of the diazotroph antisera obtained by these procedures, but the first has been widely used in producing antisera for agglutination reactions and the second for antisera used in immunodiffusion (Bergersen, 1980; Schinghamer and Dudman, 1980).

(iii) Serological reactions

Reaction between antigens and antibodies may be detected by four techniques, agglutination, gel immunodiffusion, immunofluorescence (IF) and enzyme - linked - immunosorbent assay (ELISA).

1.4.1 Agglutination

One of the most widely used immunological procedures for strain identification is the agglutination reaction. The process in which the antigens are linked together by their corresponding antibodies is call "agglutination". The linked antigens

may be visible as clump, agglutinated or aggregate, either microscopically or macroscopically. The agglutination reaction depends on a firm structural relationship between an exposed antigen and the bacterium. Such reaction may be performed in different ways, for example with drops of antigens and antisera on glass slides for rapid results. However, for greater accuracy, agglutinations are generally performed in small test - tubes by mixing constant amounts of cell suspension with a dilution series of an antiserum; the highest dilution of antiserum which gives a detectable agglutination is taken as the end - point (the "titer") of the reaction. Thus, the comparison of the bacterial strains which are agglutinated by the same antiserum is made quantitatively by comparing their respective titers. Mean et al., (1964) were abled to identify antigens in single nodule by the agglutination test, but this method is insensitive when small nodules are examined. The agglutination test is sensitive for bacterial identification (Damirgi et al., 1967 and Vincent, 1970), but does not give such clear distinctions between reactions of antigenic identity and cross - reaction between closely related strains. Strains that react to the same titer with the same serum

are regarded as being closely related serologically, if not identical.

1.4.2 Gel immunodiffusion

Gel immunodiffusion is the precipitation reaction between certain soluble antigens and antiserum by diffusion on Noble agar, where antigens are brought into contact with the corresponding antibody. The precipitation resembles agglutination in many ways, but differs principally in the fact that the precipitating antigens are not whole bacterial cells (cellular) but are proteins or polysaccharides in solution.

1.4.3 Immunofluorescence (IF)

Immunofluorescence (IF) or fluorescent antibody (FA) technique provides means for the visualization of the antigen - antibody complex. Antibody molecules are covalently labelled with fluorescent substance so that their presence at any site to which they are attached can be readily detected by microscopy in ultraviolet light. This technique combines the specificity of immunological reactions with sensitivity and precision

of fluorescence microscopy. It alone among techniques, has the potential of detecting and identifying a specific microorganism simultaneously. Moreover, this technique is still the only effective technique available that provides the means of recognizing specific microorganisms directly in their natural environment.

The immunofluorescence technique is as precise as any serologically based test, the limitations being the levels of cross - reactivity of the strain to be used. It has been used successfully to identify the rhizobia in crushed nodules (Schmidt et al., 1968; Trinick, 1969) , in hand - cut section of nodules (Russell and Johes, 1975), and also for the quantitative determination of specific rhizobia in soils (Bohlool and Schmidt, 1970), Azotobacter in soil (Tehan and De Ville, 1970) and E. Coli in water (Pugsley and Evison, 1974). The immunofluoresence may be divided into two techniques. In the direct technique, the antibacterial antibodies are labelled, making it necessary to have fluorescent antibody for each of the strains to be studied. This requirement is eliminated by using the indirect technique, which uses a second antiserum prepared in an

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animal species different from that used to make the antibacterial antibodies; by labelling the antibodies in this second antiserum (e.g. made in sheep or goats) and using them to detect attachment of the antibacterial antibody. The second antiserum, required for the indirect technique, can conveniently be purchased with or without a fluorescent label. The two methods are illustrated diagramatically in Figure 1.1 and 1.2.

1.4.4 <u>Enzyme-Linked Immunosorbent Assay (ELISA)</u>

The introduction of the enzyme-linked immunosorbent assay (ELISA), initially described by Engvall and Perlmann (1971,1972) and Van Weeman and Schuurs (1971), offered a most promising alternative to the conventional serological methods mentioned previously. In principle, enzyme-linked immunosorbent assay (ELISA) is a term that refers to any procedure involving the use of a solid-phase carrier to facilitate the rapid separation of free antigen and antibody from an antigen-antibody complex and using an enzyme marker to detect that antigen-antibody reaction. The principles of ELISA are based on an important finding that antibodies can be conjugated to an enzyme in such a way

Figure 1.1 Direct immunofluorescence

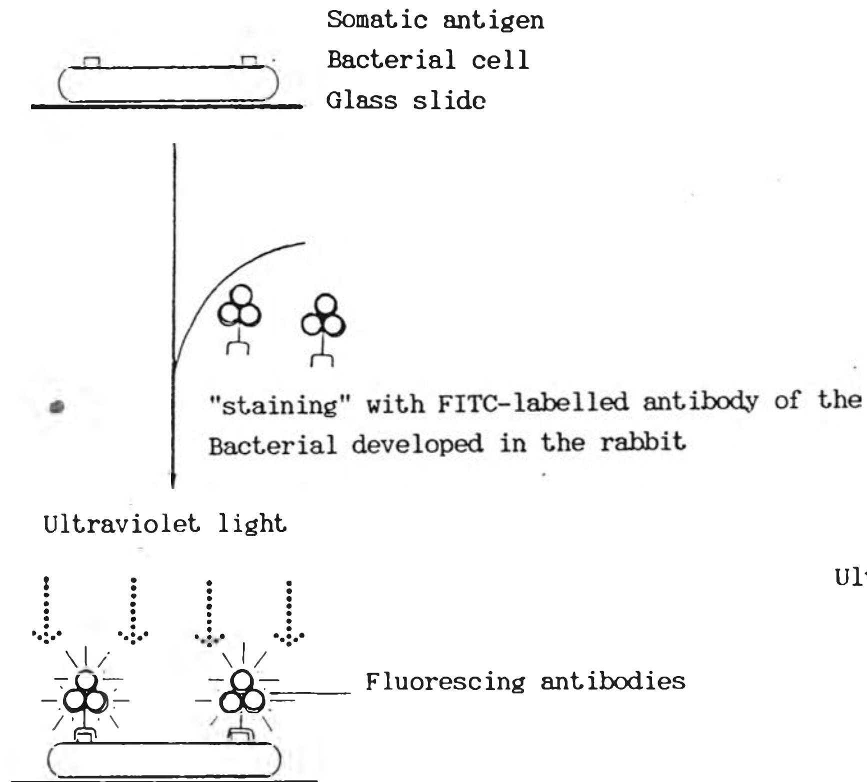
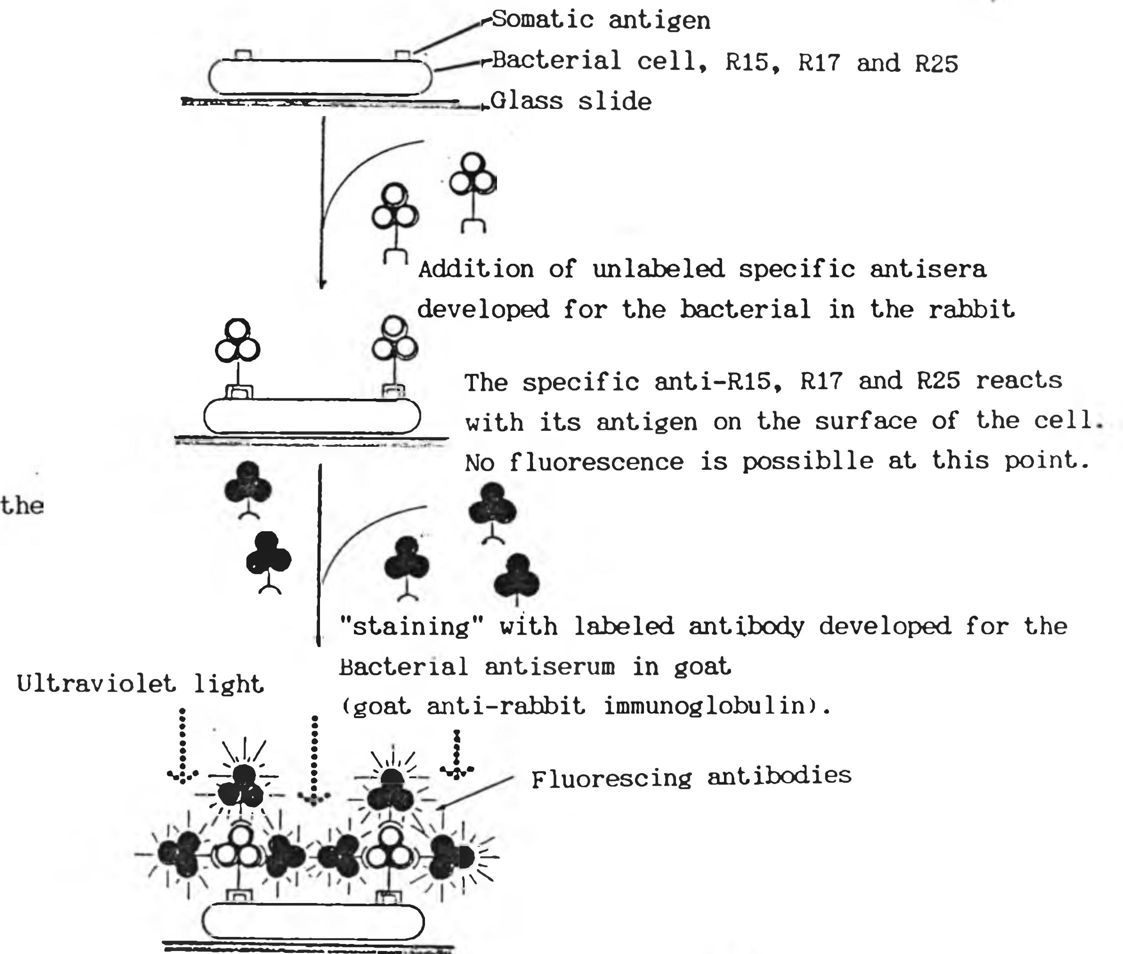


Figure 1.2 Indirect immunofluorescence



The pre-labeled goat anti-rabbit globulin fluoresces upon irradiation with ultraviolet light after reacting with the unlabeled specific antibody of the bacteria.

that the immunological and enzymatic activity of each moiety is retained. Enzymes are extremely active and one molecule can initiate chain reactions involving many molecules of colorless substrate, resulting in the formation of highly colored products. In this way, enzymes can easily be detected at very low concentrations and therefore facilitate the recognition of binding extremely small amounts of antibodies and antigens which may not be detectable by agglutination and immunodiffusion tests. The general principles of ELISA are similar to those of radioimmunoassay techniques except that instead of using a radiolabel-linked antigen or antibody to quantitate antigen-antibody reactions, an enzyme is used.

ELISA differs from other serological techniques in that it involves the consecutive immobilization of antigens and antibodies on an insoluble carrier surface followed by the removal of non relevant substances or interfering factors. The ELISA technique combines simplicity of operation with specificity, reproducibility, and rapidity of performance. Moreover, the enzyme-linked reagents are highly stable and have a long shelf life if stored correctly. Thus, the ELISA

technique was used extensively for the identification of viral and bacterial plant pathogens (Thresh et al., 1977; Clark and Adams, 1977; Bar - Joseph and Solomon, 1980; Koenig, 1978; Saunders and French, 1983) as well as in clinical bacteriology (Nassau et al., 1976) and immunology, where it was reported to have sensitivity comparable with radioimmunoassay (Maolini and Masseyeff, 1975). Kishinevsky and Bar - Joseph (1978) were the first to utilize ELISA for the identification of Rhizobium strains. The ELISA technique was modified to conveniently and rapidly handle large numbers of sample both in the laboratory and in field studies, without any loss in sensitivity. Successive workers have modified the technique in attempt to make it even more sensitive (Berger et al., 1976; Morley and Jones, 1980; Fuhrmann and Wollum, 1985). More recently, ELISA has been used in studies examining serological diversity of Rhizobium strains (Ahmad et al., 1981), their competitive ability (Nambiar et al., 1984; Rice et al., 1984; Martensson and Gustafsson, 1985) and for the quality control of inoculants (Olsen et al., 1983; Nambiar and Anjaiah, 1985).

There are many types of the ELISA techniques for strain identification. Indirect - ELISA, which overcomes the problem of different antibody conjugated preparation for each culture. It allows the detection by using a single antiglobulin enzyme conjugate. In this technique, the test antigen is nonspecifically bound to microtiter plate wells by passive or non-specific adsorption (Ahmod et al.,1981; Kishinsky and Mooz,1983), followed by a specific binding of antiserum to the antigen layer. The immobilized antigen-antibody complex is then used to "capture" the enzyme-labelled anti-immunoglobulin molecule. The amount of substrate degraded is proportional to the amount of enzyme present, which, in turn, is indirectly relative to the quantity of antigen on the solid surface. The passive adsorption of antigen to microtiter plate wells depends on both the nature of antigen and on the quality of each plastic material plate. One of the ways to overcome this problem is to use the Competitive-indirect-ELISA method (COM-IND-ELISA), which each well is coated with the tested antigen at the same amount and add the mixture between various concentration of test antigen and its homologous antibody. In this thesis, a COM-IND-ELISA has been developed for routine quantitative analysis of

Klebsiella R15, R17 and *Azospirillum* R25 in the rhizosphere of rice.

1.5 The aims of this thesis

One approach to increase the rice yeild is via enhancing biological nitrogen fixation. From the rhizosphere of Thai rice, R15, R17 and R25 have been selected according to their high N_2-fixing potential. Inoculation trials of these bacteria in the rice field need a sensitive, specific and rapid method for detection and identification of these bacterial strains, therefore, the objectives of this thesis are:

1.5.1 To produce antisera specific for each bacterial strain and use these antisera to detect and identify the N_2-fixing bacteria, R15, R17, and R25 in free - living and associative conditions by fluorescent antibody method.

1.5.2 To develop the COM-IND-ELISA method for qualitative and quantitative analysis of these bacterial strains.

1.5.3 To study the colonization ability of these bacteria in associative system after inoculation in rice seedlings grown in the laboratory level.