CHAPTER 4

Discusion

4.1 Antisera preparation

To ensure that the antisera against Klebsiella, R15 and R17, and Azospirillum, R25 will not be contaminated with antibodies against constituents of yeast extract, tryptone and other protein antigens, cells were grown in defined NF medium. Cells suspension were heated to destroy the flagellar antigens (Davies,1951; Woff and Berker, 1968), so that only somatic antigen should be obtained.

In most serological studies with other associative N_2-fixing bacteria, for example Azotobacter spp., cultures were grown in defined medium (Jensen and Peterson, 1955; Norris, 1960; Holme and Zacharies, 1965; Zarnea et al.,1966; Tchan and de Ville, 1970). However, Dazzo and Milam (1975) immunized goats with Azospirillum lipoferum and Azotobacter paspali grown in a trypticase soy broth without the medium causing any apparent interference when the antisera were used in agglutination test and detection of antigens by

immunofluorescence.

Rabbits were used for antisera production against R15, R17 and R25 because they are easy to obtain, inexpensive, and giving high antibody titers production. However, the highest agglutination titer of 1:1,600, 1:2,400 and 1:3,200 were obtained in only one rabbit out of 2-4 rabbits immunized for R15, R17 and R25, respectively. This result corresponds to previous reports (Chase, 1967; Kishinevsky and Gorfel, 1980) showing that individual animal may differ in their response to identical injections of the same antigens.

4.2 Development of competitive IND-ELISA technique

The ELISA techniques are based on the adsorption of cellular antigens to the solid phase of the microtiter plate, and this adsorption is a major factor governing sensitivity and precision of the assay. Perce et al. ,1977 and Voller et al.,1979 reported that the non-specific adsorption of antigen to the plastic surface should be hydrophobic interactions between proteins and the polystyrene surface. Ahmad et al., 1981 and Kishinevsky and Maoz, 1983 successfully used plastic

microtiter plates (Dynatech M 129A) to passively adsorp the rhizobial antigens to the plate wells. Whereas Olsen and Rice, 1984 failed to attach steamed R. meliloti all to Immunlon2 (Dynatech) microtiter plates by passive adsorption. To achieve a film attachment of test antigens to the well surfaces, they found it was necessary to evaporate rhizobial cell suspensions in the plate wells at 85°C. Fuhrmann and Wollum II, 1985 also reported that plate type was a significant factor for analysis with ELISA methods. The passive adsorption of antigens to microtiter plate wells thus depends on both the nature of antigen and the quality of plastic material plate.

In this thesis, COM-IND-ELISA have been developed to overcome the efficiency of non-specific binding of antigens, for identification and especially for quantitative determination of R15, R17 and R25 strains. The suitable concentrations of antigen: R15, R17 and R25 ($6.75x10^7$, $3.38x10^7$ and $1.69x10^7$ cells/ml respectively) were coated to each well whereas the tested antigen was mixed with the antisera. Because, the adsorption of antigens to solid support is a passive process, antigen coating were performed for overnight

at 5°C. This selected incubation period was supported by Asanuma et al., (1985) who detected the nodule antigens of Rhizobium by IND-ELISA and showed that the highest absorbance occured at incubation period of 8 h, regardless of the temperature. Voller et al. 1973; Maat and De Bokx, 1978 also recommended that the incubation period in the coating step should be overnight at 4°C. The optimum titer of the first antibody (anti-R15, R17 and R25) were 1:25,600, 1:25,600 and 1:51,200, respectively, and the second antibody (conjugate) dilution of 1:1,500 provided the best results. The incubation time of each step is an interesting factor. In our experiment, the suitable incubation period for the first antibody was 1h at 30°C, the conjugate was 3h at 37°C and the substrate in enzymatic reaction was 50 min at room temperature. However, the different conditions on IND-ELISA were observed in various types of ELISA experiments. The appropriate conditions of IND-ELISA for N_2-fixing Pseudomonas strain H8 reported by Barraquio et al., 1986 were 10^9 cells/ml of the coating antigens, with suitable incubation time of 16-18 h at 4-6°C for antigen coating, 4h 37°C for antisera incubation, 3h 37°C for conjugate incubation. Fuhrmann and Wollum II, 1985 reported that the particular

incubations used for the analytical selectivity of R. japonicum were as follows: antigen coating, 1h at 37°C; anti-Rhizobium, 1h at 28°C; conjugate 1:1,500 dilution, overnight at 5°C; and substrate, 50 min at room temperature. Taxonomic analysis of Streptomyces by IND-ELISA was reportd by Kir and Rybicki, 1986 in which antigen coating was 2h at 37°C; antisera 1:100 dilution, 1h at 37°C; conjugate 1:1,000 dilution, 1h at 37°C; and substrate, 1h at room temperature.

The developed COM-IND-ELISA methods, result in the sensitivity in the order of $1.95x10^6$ cells/ml of the soluble test antigens (Table 3.7 a-c). The accuracy observed as the per cent recovery of 90-120% were obtained for anti-R15 and R17, whereas the better values of 95-110% were obtained for R25. COM-IND-ELISA is hence a reliable method for quantitation of the cell number in ecological study. Moreover, the intra-assay precision in the range of 3-10%CV and inter-assay precision in the range of 3-15%CV were in the acceptable range recommended by Wall et al.,(1977).

The COM-IND-ELISA developed in this thesis required ca. $2x10^6$ bacterial cells/ml, for the detection

of highly significant positive reaction whereas as low as 10^4 to 10^5 cells/ml of rhizobia could be detected by double-antibody sandwich ELISA (Kishinevsky and Bar-joseph, 1978; Kishinevsky and Gurfel, 1980). Moreover, a modified ELISA could detect 10^3 to 10^4 rhizobia cells/ml in rhizobia both pure culture and in nodules (Morley and Jones, 1980; Kishinevsky and Maoz, 1983; Martensson et al., 1984). However, Asanuma et al, 1985 reported the same order of sensitivity to this work that rhizobia antigens could be detected at the concentration of 10^6 to 10^7 cells/ml. The lower sensitivity of which positive reaction by IND-ELISA was observed only at 10^7 cells/ml of Pseudomonas H8 was also reported by Barraquio et al., 1986.

4.3 Detection and identification of bacteria, R15, R17 and R25 by immunofluorescence and competitive indirect ELISA techniques

The antisera against R15, R17 and R25 were used as the first antibodies wheras the commercial FITC conjugated goat anti-rabbit immunoglobulin was used as the second antibody in both the IND-FA and theCOM-IND-ELISA techniques. The suitable titer of each

antiserum (anti-R15, anti-R17 and anti-R25) for IND-FA test was 1:100 whereas the titer for COM-IND-ELISA were 1:25,600, 1:25,600 and 1:51,200, respectively. The dilution of the second antibody was 1:10 for IND-FA but 1:1,500 for COM-IND-ELISA. Kielo et al., 1986 used a dilution of 1:10 of anti-type-1-FITC conjugate (Sigma) in adhesion assay of K. pneumoniae to plant roots by direct FA, whereas the FITC-labeled goat anti-rabbit immunoglobulins (Medac) was diluted 1:25 to detect diazotrophic rods in the root of grass by IND-FA (Barbara et al., 1986). The alkaline phosphatase conjugated to goat anti-rabbit IgG (Miles Laboratories or Sigma) was used at 1:500 or 1:800 dilutions for IND-ELISA of Pseudomonas H8 which were reported by Barraquio et al., 1986. Kirby and Rybicki, 1986 used goat anti-rabbit antibody conjugated to alkaline phosphatase (Miles Yeda) at a dilution 1:1,000 for taxonomy analysis of Streptomyces and related organisms by IND-ELISA.

Concerning the specificity, antisera R15 and R17 showed significant cross-reaction only with Klebsiella oxytoca NG13 (26-28%), but no cross-reaction with Klebsiella pneumoniae M5a1 which is the free-living

N_2-fixer of the same Genus. These serological studies indicate very close relationship among rhizobacteria which can be distinguished from the free-living species. Thus, R15 and R17 are Klebsiella-like strains, not the pneumoniae varient, based on their serological properties. This method cannot distinguish R15 from R17 because anti-R15 showed about 96-98% cross-reaction with R17 and vice versa. Antiserum against R25 showed no cross-reaction with either R15 or R17 but showed very strong (100%) cross-reaction with Azospirillum lipoferum strains FS and 34H. From its serological properties, R25 is an Azospirillum lipoferum strain. These serological properties comfirmed the identification of these bacteria by chemical means (mol% G+C content, DNA sequence homology and fatty acid composition) reported by Boonjawat et al., 1986. The finding concluded that R15 and R17 are very closely related strains by all means except for the fatty acid composition.

The results from this study suggest that the serological properties assayed by IND-FA and ELISA reactions with Klebsiella and Azospirillum are genusspecific. Species specificity of IND-FA reaction has also been reported by De-Po li et al.(1980) in

Azospirillum species belonging to different host-plant specificity groups, and by Ladha et al.(1982) in A. lipoferum and A.brasilense associated with wetland rice. On the contrary, strain specificity of ELISA has been reported by Berger et al., 1979, where three strains of Rhizobium in culture and from nodules of lentils were used as antigens. Strain-specific ELISA for identification of Rhizobium spp. in culture and from roots nodules has also been reported by Kishinevsky and Bar-Joseph, 1978; Morley and Jones (1980) ; Olsen et al (1982) and Fuhrmann and Wollum II (1985). However, two closely related strains of R.lupini and of R. trifolii could not be distinguished agglutination and ELISA test (Kishinevsky and Gurfel, 1980). They also found negative ELISA reaction in two strains of R.leguminosarum incubated with their homologous antisera.

Barraquio et al., 1986 reported that 48 from 563 isolates of the rice roots tested, were not serologically different from Pseudomonas H8. Strains which were significantly different from P. H8 serological were both similar and different to P. H8 in cultural characteristics and acetylene reduction activity. One isolate which was cross-reactive with P.

H8 did not have N_2-fixing activity.

Increased specificity of IND-FA reactions were found when heated cell antigens were used. Heat treatment have reduced the agglutinability of the antigens as reported in Azospirillum by Ladha et al. (1982) and Rhizobium by Means and Johnson (1968). These phenomena are presumably due to the destruction of common flagellar antigen (Davis, 1951; Wolf and Berker, 1968).

The environment also affects serological properties as shown by shifting the culture medium from NF to RM, and addition of NaCl concentration into the culture media. When the rich medium grown-bacteria reacted with its homologous antiserum (produced from antigens grown in nitrogen-free medium), less sensitivity has been observed. This result suggests that the surface antigenic properties of these bacterial cells might change in fertile and non-fertile environments. When these bacteria were exposed to saline environments, such in NF and RM medium containing various concentrations of NaCl, they can survive by adapting their metabolic activity and cell wall

structure resulting in morphological change as observed by immunofluorescent technique (Fig. 3.7). Moreover the sensitivity of detection by IND-FA method decreased in high salt concentration especially in the NF medium (Table 3.4). Among these three strains, R17 is the best salt tolerant strain which corresponds with its origin of isolation from Tapra in Khonkaen Province where saline soil is a serious problem in Summer. This result suggests that environmental factors such as soil fertility and salinity can affect the sensitivity of detection and species identification by IND-FA method. Ladha et al. (1982) reported that stage of culture antigens also affected immunofluorescent reaction of Azospirillum. In contrat, Muyzer et al. (1987) reported that IND-FA reactions of equal intensity occurred with all the cultures of Thiobacillus ferrooxidans grown in five different energy substrates. Whether serological method can be applied suitably with a bacterial strain after inoculation in the field depends on the species.

IND-FA was used in adsorption and association study because this technique has the potential of detecting and identifying a specific bacterial cell antigen simultaneously. Moreover, this technique is

still the only effective technique available that provides the means of recognizing specific microorganism directly in their natural environment. However, the non-specific adsorption of labelled antisera by root tissue may interfere with the determination of bacterial cell antigens by FA technique, gelatin-Rhodamine gel was therefore used to suppress non-specific adsorption as described by Bohlool and Schmidt (1968).

Adhesion of R15, R17 and R25 on the root-pieces of 8 rice varieties showed that 1h of incubation period, and at least 10^7 - 10^8 cells/ml of bacteria were required. Similar results were obtained with longer incubation time (3h and 12h). This result showed that bacterial adsorption on the root surface was very rapid and can be used to screen for compatible pair of plant-bacterial interaction. The finding comfirmed the worked of Limpananont (1987) which reported the inoculation of R15 and R17 (10^8 cells) in 3 rice seedlings (7-day-old) grown in 5 ml sterile distilled water, and observed loose cluster of bacteria after 2 h of incubation.

The results in this study show that IND-FA has the potential of detecting and identifying a specific N_2-fixing bacteria simultaneously. Futhermore, this technique is effective to recognize the specific species directly in their natural environment.

Several advantages of the proposed ELISA procedure have already been emphasized. The ELISA can provide rapid and sensitive identification of associative N_2-fixing R15, R17 and R25. This technique enabled the detection of the three strains in suspensions containing $2x10^8$ cells/ml. In comparison to other immunological method, the sufficient concentration to ensure detectable bands in immumodiffusion technique was 10^9 - $5x10^{10}$ cells/ml (Dudman and Brockwell, 1968; Vincent 1970), whereas 10^9 - 10^{10} cells/ml are required in the agglutination tests. Furthermore, the cost of these developed COM-IND-ELISA can be significantly reduced by using small amounts of conjugate, which was used at the dilution of 1:1,500, whereas the conjugate dilution of 1:500 or 1:800 were used in Pseudomonas spp. as reported by Barraquio et al., 1986. The significant advantage is also noticed with IND-FA methods. The use of nonfractionated anti-R15, R17 and R25 antisera

eliminates the time-consuming purification procedure as reported in Rhizobium by Fuhrmann and Wollum II, 1985. Moreover, unlike direct ELISA which requires separate conjugates for each antiserum used (Voller et al., 1980), IND-ELISA procedures have the benefit from the availability of using high-quality, commercially prepared conjugated anti-immunoglobulins.

4.4 Application of quantitative COM-IND-ELISA to select the compatible pair of plant-bacteria interaction

The colonization potential, nitrogen-fixing potential and plant vigor index are the 3 criteria which have been used in this thesis to classify the compatible pair of plant and bacteria into 2 catagories; (1) compatible pair with high colonization potential (10^8 - 10^9 cells/g dry wt. of root) together with associative N_2-fixation (0.28 - 111 umol C_2H_2/ g dry wt of root) and net increase in plant vigor index (633-2,558 cm.%), and (2) compatible pair with relatively lower colonization potential (10^7 - 10^8 cells/ g dry wt of root) without net gain in associative N_2-fixation, but still gain plant vigor index above control. The third type or possibly the worst

interaction was the pairing between SPT-R17, where high rate of colonization occurs with low rate of N_2-fixation and negative plant vigor index. Kloepper et al. (1989) and Lambert and Joes (1989) reviewed that root colonization by inoculated with free-living bacteria such as Azotobacter chrococcum, various Bacillus spp. and nitrogen fixing strains of Azospirillum may also influence the symbiosis between microorganism and plants, and thereby stimulated plant growth promotion (PGP) significantly. This primary mechanism is not nitrogen-fixation and may be similar to the second type of plant-bacterial interaction observed between RD6-R25 , RD6-R17 or RD25-R17. In the first group of plant-bacteria interaction, where net associative N_2-fixation has been observed, the increase in plant vigor index was tentatively higher than the second type, especially the pairing between RD7-R17. In this category both PGP substance and associative N_2-fixation should be advantage to plant-bacterial partners.

4.5 Relationship between colonization potential and root lectin

A hypothesis that specificity in plant-bacterial

interaction is mediated by lectins is well known. The lectin binding hypothesis of bacterial R15 and R17 to rice cv.RD7 were demonstrated by Limpananont (1987). Recently, Chaopongpang (1989) reported that rice cv. RD7 contained high lectin content in roots (58 ng/100plant), RD23 and RD25 contained moderate amount of lectin content in roots (17-34 ng/100plants) whereas the lectin content of KDML was the lowest amount (5 ng/100plants). This study demonstrates high colonization of R15, R17 and R25 in rice cv. RD7, but low colonization in KDML. These evidences suggest that the lectin content in roots should relate to colonization potential by which it may act as the adhesion function of plant-bacterial interaction.

4.6 Summary of the results

From this thesis, the results could be summarized as follows:

4.6.1 Production of antisera against 3 strains of nitrogen-fixing bacteria R15, R17 and R25 from female rabbits were achieved the high agglutination titer of 1:1,600, 1:2,400 and 1:3,200, respectively.

4.6.2 For IND-FA, anti-R15, R17 and R25 were used as the first antibodies at the dilution of 1:100 for all antisera and the commercial FITC conjugated goat antirabbit immunoglobulin at the dilution of 1:10 was used as the second antibody, giving the sensitivity in terms of the coating antigens in the order of 10^6 cells/smear.

4.6.3 Development of COM-IND-ELISA using anti-R15, R17 and R25 as the first antibody at the dilution of 1:25,600, 1:25,600 and 1:51,200, respectively, and commercial alkaline phosphatase conjugated goat anttirabbit immunoglobulin as the second antibody, giving the sensitivity of detecting homologous antigens in the order of 1.95×10^6 cells/ml, with the accuracy demonstrated by the per cent recovery of 90-120. The precision of intra assay and inter assay were in the range of 3-10%CV and 3-15%CV, respectively.

4.6.4 The specificity of IND-FA and COM-IND-ELISA tested by heated cells suspension of homologous and non-homologous strains at the concentration of 2.5×10^7 cells/ml showed corresponding results for IND-FA and COM-IND-ELISA. It sugguests that

R15 and R17 are Klebsiella-like strains, showing cross-reaction with K.oxytoca NG13 (26-28%), while R25 is an Azospirillum lipoferum, showing 100% cross-reaction with A.FS and 34H. Strong serological homology between strains R15 and R17 has been observed although they differ in salt tolerance and colonization potential.

4.6.5 By the 3 criteria, the colonization potential, nitrogen-fixing potential and increase in plant vigor index, the compatible pair of plant and bacteria was classified into 2 catagories; (1) compatible pair with high colonization potential, nitrogen-fixing potential and the increase in plant vigor index. RD7-R15 is the best pair for this catagory. (2) compatible pair with relatively lower colonization potential, without net gain in associative N_2-fixation, but still gain plant vigor index above control. The best compatible pair plant-bacteria of this type is RD6-R25. The third type or possibly the worst interaction is the pairing between SPT-R17, where high rate of colonization occurs with low rate of N_2-fixation and negative plant vigor index.