CHAPTER IV

DISCUSSION

4.1 Effect of *Klebsiella* R15 inoculation on rice lectin

Inoculation of rice seedlings with *Klebsiella* R15 resulted in an increase in root lectin content but not shoot lectin. As quantitative determination by ELISA, comparing between inoculated and non-inoculated rice seedlings show that there is a 2-3 fold increase in root lectin content (Figure 3.1) when compared to the controls. While in shoot, there is no significant increase in shoot lectin content of inoculated seedlings (Figure 3.2). Moreover, characterization of molecular forms of root and shoot lectin has revealed that in the root, there is a significant increase of the 23-kDa and the 18-kDa polypeptides in those inoculated root extracts (Figure 3.4 a, b and c). In the shoot, there is no significant difference of the dominant 18-kDa polypeptide between inoculated and non-inoculated rice seedlings (Figure 3.4 d, e and f). From these results, it can be concluded that inoculation of rice seedlings with *Klebsiella* R15 has increased lectin content specifically in the seedling root and in the high molecular weight form of 23 kDa as well as the 18 kDa form.

4.2 Induction of root lectin by *Klebsiella* R15 inoculation

An increase in root lectin content in the inoculated rice seedlings during the 3 weeks after inoculation observed in all 3 rice cultivars (Figure 3.1) has raised the question, whether or not the increasing lectin content and concentration in the inoculated roots is

caused by an increase in *de novo* synthesis or accumulation of stabilized lectin. Characterization of molecular form of lectins in the root extracts of inoculated roots showed that there were significant increase in the 23-kDa form, which have been known as the precursor from of lectin polypeptide that the 18-kDa form or the lectin mature subunits, and the 10 kDa and 8 kDa forms were derived from (Stinissen et al., 1983).

This result is also supported by immunogold-protein A localization of lectin in the root tissues where lectins were found associated to the rough endoplasmic reticulum (Figure 3.12 a), which has been reported as the biosynthetic site, and in small vacuoles next to the Golgi bodies which are corresponding to packaging before transporting outside the cells (Stinissen et al., 1984a). The results obtained from these experiments, however, do not give the strong evidence for lectin biosynthesis, but it can support the Western blot analysis, and also explain the decreasing profile of root lectin by secretion as component of extracellular polysaccharides (Figure 3.7). The mechanism of action that *Klebsiella* R15 induced lectin biosynthesis in rice roots, at present, is not well understood, but one theory proposed by Okon and Kapulnik, 1986 is the hormonal effect of IAA-liked substances produced by associative N_2-fixing bacteria. Evidences in supporting this theory come from studies in *Azospirillum* which produces phytohormone-liked substances as indole-3-acetic acid (IAA), cytokinin, gibberellin and auxin (Tien et al., 1979, Jain and Patriquin, 1984). Among these phytohormone, indole-3-acetic acid shows the greatest stimulating effect on branching of wheat root hairs. In the same manner when rice seedlings were inoculated with *Klebsiella* R15, the bacterial inoculation caused more branching, denser and longer root hairs (Limpananont, 1987). This result suggests that *Klebsiella* R15 may

produce phytohormone-like substance as *Azospirillum*. At this point of view, it can be described that the hormone produced by *Klebsiella* R15 may effect root lectin content during the association, as it has been reported that biosynthesis of wheat and rice lectins in developing and germinating embryos are strongly promoted by abscisic acid (ABA), (Stinissen et al., 1984b; Raikhel et al., 1986). In wheat seedlings, Stinissen et al., (1985) demonstrated that root tips excised from seedlings grown in the presence of ABA exhibited a 3.5-fold increase in the relative rate of lectin synthesis. Rice Lectin gene has been cloned from the cDNA library of rice O. *sativa* cv. Nato as reported by Raikhel et al., (1989) that there is most likely only one single lectin gene in rice, but there are two transcripts (mRNA) of different lengths 1.1 kb and 0.9 kb. In situ hybridization showed the 1.1 kb rice lectin mRNA in root caps and specific cell layers of the radicle, coleorhiza, scutellum, and coleoptile. The protein encoded by the open reading frame of both mRNA encompasses 227 amino acids residues with the calculated M.W. 22,798, which corresponds with the 23 kDa band detected by Western blot analysis (Figure 3.4 a, b, c). In many rice cultivars, the 23 kDa preproprotein undergoes proteolytic cleavage of a COOH-terminal domain to yield the mature 18 kDa polypeptide, and smaller polypeptides of approximately 10 kDa and 8 kDa (Stinissen et al., 1983). In these 3 local rice cultivars the 18 kDa form is dominant in both root and leaf fractions, where the smaller 8-10 kDa bands are hardly observed, in both inoculated and non-inoculated conditions.

4.3 Correlation of internal root lectin accumulation and N_2-fixing activity

Although the molecular forms of root lectins are similar in 3 rice cultivars, the pattern of root lectin accumulation during

development are different. The N_2-fixing activity which occurred during the first week seems to depend on the more or less the same amount of bacterial cells inoculated into the rhizosphere, and did not depend on the internal root lectin concentration. Immuno-gold labelling of lectin shows that excreted lectin in the glycocalyx at epidermal cells of rice root and extracellular polysaccharides plays important role as associative factor between bacterial cell and root surface (Figure 3.8). The decrease in internal lectin by secretory mechanism via small vacuoles or directly as cluster of gold-labeled lectin towards the epidermal cells surface into the medium should facilitate better adhesion. Since it has been shown (Limpananont, 1987) that root exudate of 7-day-old rice seedling of RD7 exhibited the hemagglutination activity of lectin. So during the first week after inoculation (day 7-14), the nitrogenase activity in the rhizosphere of three rice cultivars increased sharply with the same rate. But in the later stage after inoculation, the rhizospheric nitrogenase activity might be due to the ability of each cultivar to synthesize lectin, to secrete lectin as adhesive factor for *Klebsiella* R15 or to facilitate invasion of bacteria into the root. As observed in Figure 3.3 RD7 and KDML105 show the higher rate of internal root lectin depletion than NMS4, consequently the nitrogenase activity in the rhizosphere of RD7 and KDML105 are higher than in the rhizosphere of NMS4 that contained higher amount of internal lectin than KDML105, but retained lectin in the root. However, the nitrogenase activity in rhizosphere of NMS4 has shown the tendency to increase in later time. Comparison between RD7 and KDML105, for the maximum N_2-fixing activity KDML105 showed lower nitrogenase activity than RD7 which might be due to less ability to synthesis lectin. Other possible explanation is the efficiency of KDML105 to excrete lectin into the medium which has been studied by ^{35}cysteine incorporate (personal

communication), so that nitrogenase activity has been facilitated by secretory lectins. The associative nitrogenase activity shows the significant increase (2,500-3,000 nmol/tube x day) over the free-living rice and the free-living *Klebsiella* R15 maintained under the same experimental conditions. The internal root lectin content which showed only 2-3 fold increase in these three cultivars did not showed direct quantitative correlation with the rhizospheric nitrogenase activity. However the demonstration by immunogold-labeling that rice lectins exported to the root surface bind to *Klebsiella* R15 and intercellular lectins bind to invasive bacteria in the exodermis layer, therefore lectin is the first plant protein which show direct interaction with N_2-fixing *Klebsiella* R15, and show increased concentration specifically in the root after bacterial inoculation. The nitrogen-fixing activity seem to required many more other factors besides lectins such as ATP derived from photosynthates. Since Sanchez. et al. 1991 reviewed in the control of nodulin genes in root-nodule development and metabolism that lectin cannot be considered nodulins because they are not specifically expressed in the nodule but just only required in the preinfection stage. Truchet et al, 1991, using a root hair deformation assay, showed that the common *nodABC* genes and host-range *nodH* and genes are involved in the production of extracellular signals. The major alfalfa-specific sinal is a sulphated and acylated glucosamine tetrasaccharide which elicits root hair deformation, cortical cell division and nodule formation on aseptically-grown alfalfa seedlings (Schultze, et al. 1991; Truchet et al. 1991). Since the bacterial signal from *Rhizobium meliloti* has been identified as derivatives of beta-1, 4-tetrasaccharide of D-glucosamine, legume lectins, which are characterized by specific binding sites for oligosaccharides and by conserved hydrophobic binding sites, may be good candidates for representing signal receptor molecules

(Kijne et al, 1990).

4.4 Effect of bacterial inoculation on plant growth

Association between *Klebsiella* R15 and rice roots for 14 days did not show significant difference in dry weight, %N and total nitrogen. This part of results can not be concluded at present, that inoculation of rice seedling with *Klebsiella* R15 did not have advantage to rice seedlings. Since the rice plants were grown in nitrogen-free minimum medium with limited space and time. The population density of rice plants in the tray is much higher than the natural condition. In the field trials using cultivated rice, fixed nitrogen has not been immediately transported to the plant but it remained with the bacteria, whether in the rhizosphere or soil, for a period of time before release (Ito et al., 1980; Eskew et al., 1981). Comparative study of glutamine synthetase specific activity in free-living *Klebsiella* R15 and in *Klebsiella* R15 associated with rhizosphere of rice (Boonjawat et al., 1991) shows that when *Klebsiella* R15 are associated with rice seedlings, they can fix nitrogen more efficiently and assimilation of NH_3 to glutamine occurred in the bacterial cells, rather than transferring fixed-N to rice in the form of NH_3 as found in other symbiotic systems such as, *Azolla-Anabaena caroliniana* (Orr and Haselkorn, 1982), *Nostoc-Anthoceros* (Joseph and Meeks, 1987).

Moreover, study on the effect of *Azospirillum* inoculation on nitrogen incorporation in wheat, in field grown experiment, Döbereiner et al., (1983) reported that the significance increase in total N between treatment and control plants can be observed, but required at least 65 days after germination.

Therefore if this experiment has been performed in the field and extended until the maturity of rice plant such as 65 day, or longer the

significance in plant dry weight, %N and total N might be observed.

4.5 Role of lectin

By using immunogold-protein A technique to localize rice lectin in root tissues, the finding that in inoculated plants, lectins are located over the bacterial fibrillar glycocalyx, a few number were found stained over the bacterial cell. This finding indicates that the fibrilla network, might be lipopolysaccharides (LPS) and/or exopolysaccharides (EPS) that contain specific binding sites for lectins (Limpananont, 1987), they may also play an important role in attachment of bacterial cells to epidermal cells of root. In other associative system, such as *Azospirillum brasilense* and wheat the attachment of bacteria to root epidermal cells are mediated by the electron dense connection (Levanony et al., 1991). While in the case of invasion of bacterial cells into the intercellular spaces of root's epidermal cells, the fibrillar structure disappeared and merely lectins were found directly attached to the bacterial cells (Figure 3.8 b). From this observation, there are 2 possible suggestions: (i) lectins may be involved in translocation of bacterial cells into the exodermis of rice root, or (ii) lectins may serve to agglutinate and immobilize bacteria as a defense mechanism as proposed by Chrispeel and Raikhel, 1991. According to Chrispeel and Raikhel (1991), the most likely function for the vacuolar lectins with chitin binding domain is in plant defense for example delaying larval development of weevil (Murdock et al, 1990). In addition rice lectin may be involved in the plant protection against the invasion of plant pathogens, especially those with N-acetylglucosamine bearing on the extracellular polysaccharides or cell walls. The presence of lectins at the stoma and bulliform cells which are located on the infection sites of the leaf surface may block the

movement of some fungi. The role of lectins in plant defense have been proposed not only in rice but also in most Gramineae and other plants producing the chitin-binding lectins (Broglie et al., 1986; Stanford et al., 1989; Broekaert et al. 1990; Chrispeels and Raikhel, 1991). However the invasion of nitrogen fixing bacteria into rice root has been specifically localized at the exodermis and not systemic as those pathogens and should not be harmful for plants at this layer.

By comparing the pattern of lectin distribution between inoculated and non-inoculated root it has been found that the localization of lectin in these 2 conditions are similar, but the intensity in lectin accumulation is higher in inoculated root, this observation is correspond to the results that there is a 2-3 fold increase of lectin in the associative root. However the similar pattern of lectin distribution in the root, beside as vacuolar lectins, they are scattered in the middle lamella among the root cells at all layers from inside towards the epidermal cells, indicating that even in the absence of bacteria, lectins should have some physiological function as secretory proteins on the outer surface of root, as they are previously characterized extensively (Chrispeel and Raikhel, 1991). Specific localization of lectins in the xylem and phloem of root and leaf, and also in the intracellular spaces among these tissues indicates that lectins might be involved in the translocation of some nutrients or metabolites from root to leaf and vice versa. It is worthwhile to notice that the metabolites might be lipopolysaccharides glycolipids or glycoproteins that can be recognized by lectins according to their carbohydrate specific binding sites.