CHAPTER III

RESULTS

3.1 Effect of *Klebsiella* R15 inoculation on root lectin and total proteins

The effect of *Klebsiella* R15 inoculation on root lectin were studied in 3 rice cultivars; RD7, NMS4 and KDML105, by comparative determination of lectin content and total protein between inoculated and non-inoculated seedling roots at every 7-day interval after inoculation until the rice plants were 28 days old. Figure 3.1 shows that inoculation of rice seedlings with *Klebsiella* R15 resulted in a significant increase of lectin content about 2-3 fold in the seedling roots (Appendix III), on day 28 in RD7 and KDML105 and day 21 for NMS4 (Figure 3.1 a, c, and e), but no significant increase in total proteins content except NMS4 on day 28 (Figure 3.1 b, d, and f). This increasing amount of lectin affected by *Klebsiella* R15 is apparently dependent on initial lectin content in the 3 rice cultivars and never exceed the initial lectin content on day 7.

As seedling development proceeds, in the presence and absence of associative *Klebsiella* R15, root lectin content declined as a function of time after germination, however the rate of decrease in root lectin content differed among 3 rice cultivars. In RD7, root lectin in both inoculated and non-inoculated rice seedlings steadily declined during the 4 weeks observed after germination. While in NMS4, root lectin content in both inoculated and non-inoculated rice seedlings decreased gradually comparing to RD7. In contrast to the first two

Figure 3.1 Effect of *Klebsiella* R15 on root lectin content and total proteins

Rice seedling (200 plants) were grown hydroponically in 1500 ml NF medium. On day 7 after germination, 50 ml of *Klebsiella* R15 suspended in 0.85% NaCl (about 10^9-10^{10} cells/ml) was added to the medium. Control plants received 50 ml 0.85% NaCl. Every 7 day interval after inoculation, 50 rice plants were determined for lectin content and protein content as described in Materials and Methods.

a and b show total root lectin (ng/50 plants) and total proteins in rice RD7

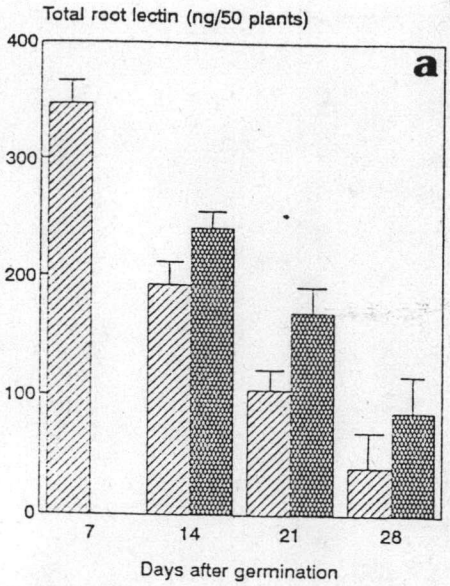
c and d show total root lectin (ng/50 plants) and total proteins in rice NMS4

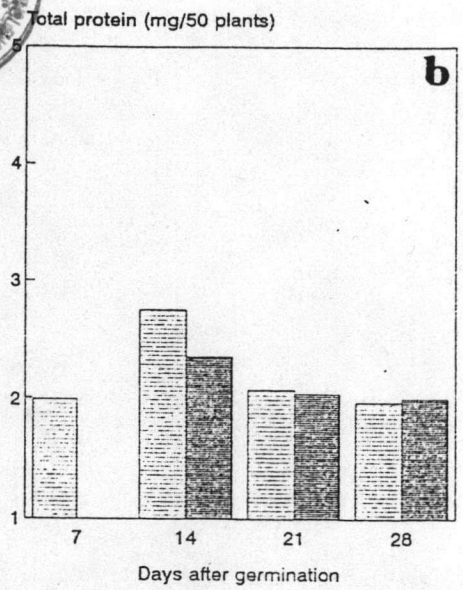
e and f show total root lectin (ng/50 plants) and total proteins in rice KDML105

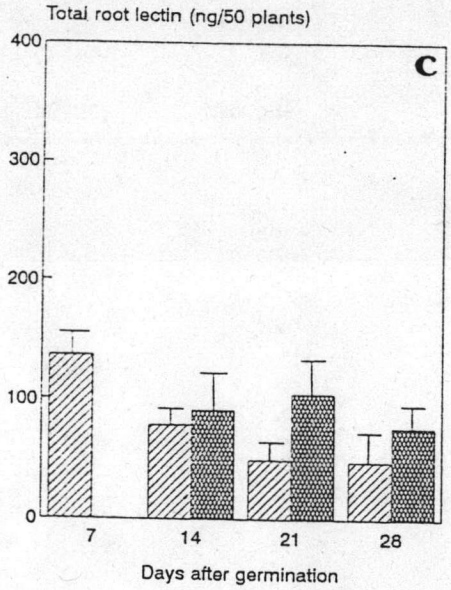
Histograms  and are conditions in the absence, and presence of *Klebsiella* R15 respectively

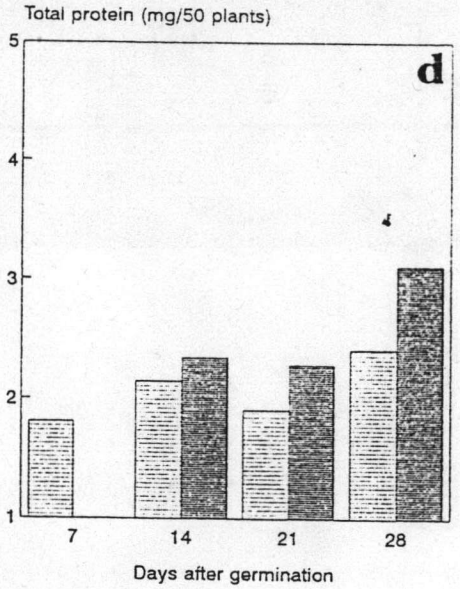
The total root lectin values presented are mean ± SE of 3 repeated experiments for each rice cultivar.

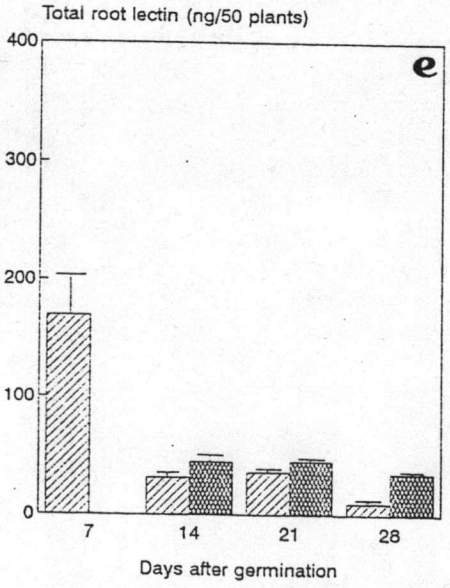
The total proteins, values presented are mean of 3 repeated experiments.

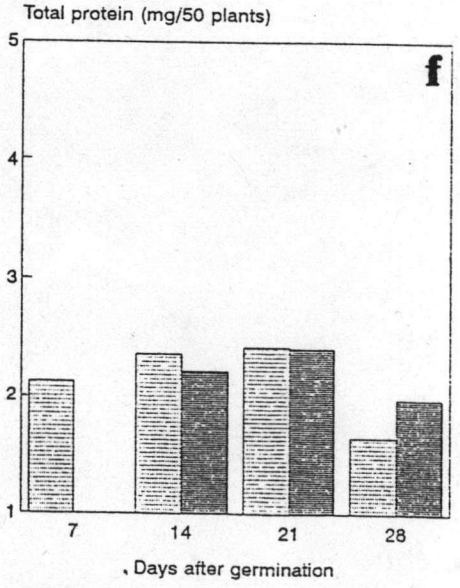

Total root lectin (ng/50 plants)
a
400
300
200
100
0
7
14
21
28
Days after germination


Total protein (mg/50 plants)
b
5
4
3
2
1
7
14
21
28
Days after germination


Total root lectin (ng/50 plants)
c
400
300
200
100
0
7
14
21
28
Days after germination


Total protein (mg/50 plants)
d
5
4
3
2
1
7
14
21
28
Days after germination


Total root lectin (ng/50 plants)
e
400
300
200
100
0
7
14
21
28
Days after germination


Total protein (mg/50 plants)
f
5
4
3
2
1
7
14
21
28
Days after germination

cultivars, root lectin content in KDML105 dropped sharply after 7 days of growth and maintained at rather constant amounts during the later 3 weeks.

Table 3.1 shows the average values of lectin concentration (mg% of total proteins) in the presence of *Klebsiella* R15 that RD7 shows consistently high response to bacterial inoculation throughout the experimental course. (the net increases are 0.0053, 0.0033 and 0.0023 mg% of total proteins on 7, 14 and 21 days after inoculation), where NMS4 has shown the moderate response i.e. the net highest detectable amount of lectin concentration is 0.0020% of total proteins (at 14 day after inoculation). In the least responsive cultivar; KDML105, the net increase in lectin concentration is only 0.0012% of total proteins at 3 weeks after inoculation.

3.2 Effect of *Klebsiella* R15 inoculation on shoot lectin and total proteins

Figure 3.2 and Table 3.2 show that there are no significant difference (Appendix IV) in the shoot lectin content between inoculated and non-inoculated rice seedlings in all 3 rice cultivars. In addition, the shoot lectin of both inoculated and non-inoculated rice seedlings decreased rapidly after 7 days of germination and remained rather constant at low amount during the last 3 weeks of seedling development. Similarly bacterial inoculation show no significant increase in total proteins excepts KDML105 on day 28.

These findings suggest that association between *Klebsiella* R15 and rice seedlings does not have significant effect on the shoot lectin content and total proteins under this experimental condition.

Table 3.1 Effect of *Klebsiella* R15 on root lectin concentration at different stage of growth

Days after germination	Condition*	Lectin concentration[a] (mg/100 mg total protein) ± (Difference)[b]		
		RD7	NMS4	KDML105
7	0	0.0170	0.0073	0.0080
14	-	0.0072	0.0035	0.0013
	+	0.0126	0.0039	0.0020
		+(0.0054)	+(0.0004)	+(0.0007)
21	-	0.0050	0.0026	0.0015
	+	0.0083	0.0046	0.0019
		+(0.0033)	+(0.0020)	+(0.0004)
28	-	0.0020	0.0021	0.0006
	+	0.0043	0.0025	0.0018
		+(0.0023)	+(0.0004)	+(0.0012)

* 0 = before inoculation, - = no-inoculation, + = inoculation

(a) values presented are the mean of 3 repeated experiments

(b) values in brackets are the difference value of lectin concentration between inoculated and non-inoculated rice seedlings

Figure 3.2 Effect of *Klebsiella* R15 on shoot lectin content and total proteins

Rice seedlings (200 plants) were grown hydroponically in 1500 ml NF medium. On day 7 after germination, 50 ml of *Klebsiella* R15 suspended in 0.85% NaCl (about 10^9-10^{10} cells/ml) was added to the medium. Control plants received 50 ml 0.85% NaCl. Every 7 day interval after inoculation, 50 rice plants were determined for lectin content and protein content as described in Materials and Methods.

a and b show total root lectin (ng/50 plants) and total proteins in rice RD7

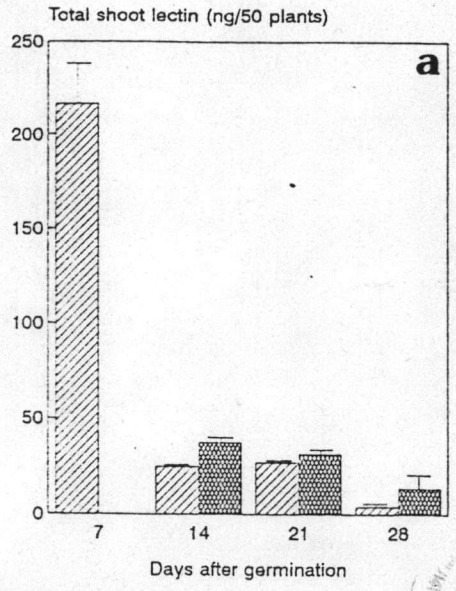
c and d show total root lectin (ng/50 plants) and total proteins in rice NMS4

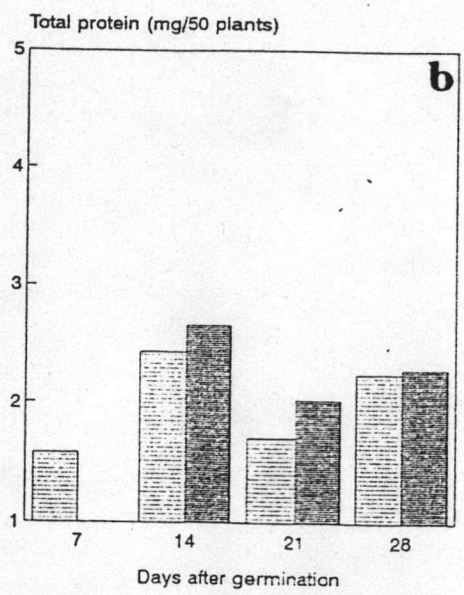
e and f show total root lectin (ng/50 plants) and total proteins in rice KDML105

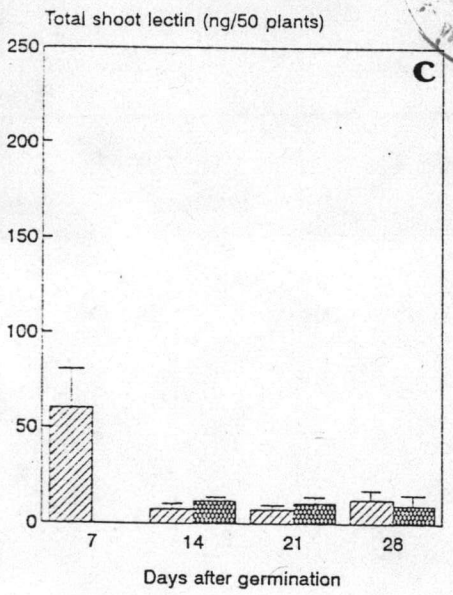
Histograms ▨ and ▩ are conditions in the absence, and presence of *Klebsiella* R15

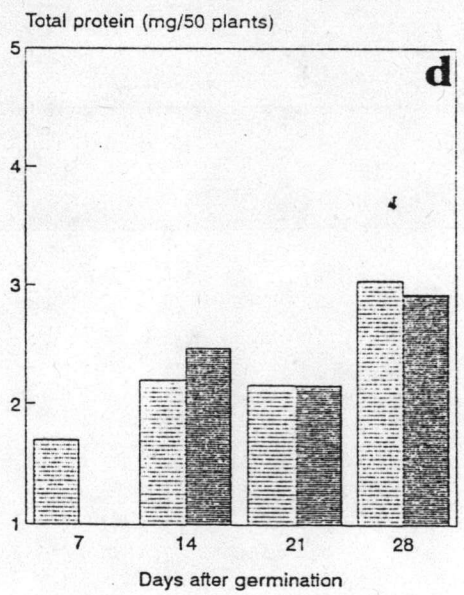
The total root lectin values presented are mean ± SE of 3 repeated experiments for each rice cultivar.

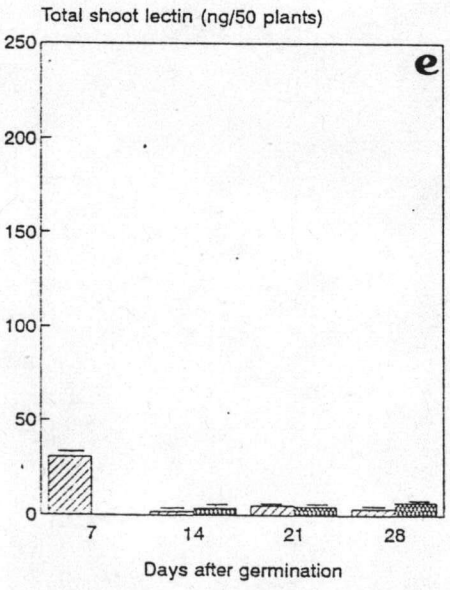
The total proteins values presented are mean of 3 repeated experiments.


Total shoot lectin (ng/50 plants)
a
250
200
150
100
50
0
7
14
21
28
Days after germination


Total protein (mg/50 plants)
b
5
4
3
2
1
7
14
21
28
Days after germination


Total shoot lectin (ng/50 plants)
c
250
200
150
100
50
0
7
14
21
28
Days after germination


Total protein (mg/50 plants)
d
5
4
3
2
1
7
14
21
28
Days after germination


Total shoot lectin (ng/50 plants)
e
250
200
150
100
50
0
7
14
21
28
Days after germination

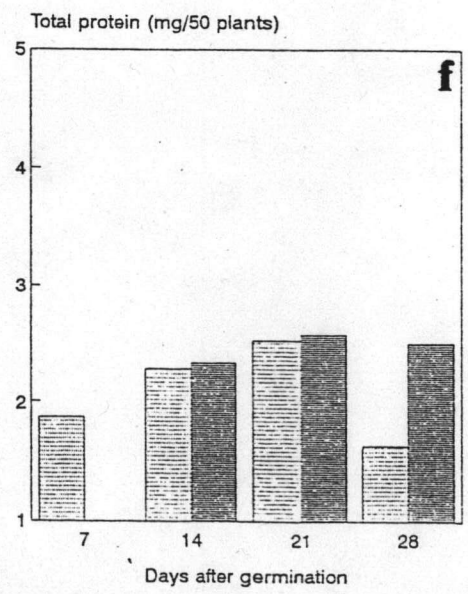

Total protein (mg/50 plants)
f
5
4
3
2
1
7
14
21
28
Days after germination

Table 3.2 Effect of *Klebsiella* R15 on shoot lectin concentration at different stages of growth

Days after germination	Condition*	Lectin concentration[a] (mg% total proteins) ± (Difference)[b]		
		RD7	NMS4	KDML105
7	0	0.0130	0.0035	0.0016
14	-	0.0010	0.0003	0.0001
	+	0.0014	0.0004	0.0001
		+(0.0004)	+(0.0001)	(null)
21	-	0.0015	0.0003	0.0002
	+	0.0016	0.0004	0.0001
		+(0.0001)	+(0.0001)	-(0.0001)
28	-	0.0002	0.0004	0.0002
	+	0.0006	0.0003	0.0003
		+(0.0004)	-(0.0001)	+(0.0001)

* 0 = before inoculation, - = no-inoculation, + = inoculation

(a) values presented are the mean of 3 repeated experiments

(b) values in brackets are the difference value of lectin concentration between inoculated and non-inoculated rice seedlings

3.3 Relationship between nitrogen-fixing activity and root lectin content

In this experiment the nitrogenase activity of (i) free-living rice seedlings, (ii) free-living *Klebsiella* R15, and (iii) associative rice-R15 were measured in parallel with root lectin content. Figure 3.3 shows the time-course of the nitrogenase activity after inoculation of *Klebsiella* R15 (10^9 cells/plant) into 3 rice cultivars a) RD7, b) NMS4 and c) KDML105. During the first week after inoculation, nitrogenase activity increased rapidly at the same rate ($r = 0.98$ Appendix V) and independent on the internal root lectin content. During the second week after inoculation, 14-21 days after germination the nitrogenase activity in the rhizosphere of three rice cultivars show trend of different patterns although not significantly different, because of the high variation beyond day 17. NMS4 seems to show slower increase with respect to the first week. The rhizospheric nitrogenase activity of RD7 and KDML105 seems to increase with the same rate as observed in the first week and reached the plateau at the end of second week although RD7 shows the higher maximum activity than KDML105. The rate of increasing nitrogenase activity during the second week after inoculation can be related to the initial lectin content in the root before and after inoculation. However the cultivar RD7 which contains the highest amount of root lectin, shows the highest nitrogenase activity and NMS4 which contains the lowest internal root lectin content shows the lowest nitrogen fixing activity. For KDML105 with the share decrease of internal root lectin during development faster than NMS4 either with inoculation or not could support for higher N_2-fixing activity during the second week by secreted lectin.

Figure 3.3 Relationship between nitrogenase activity and root lectin content

Nitrogenase activity was assayed by Acetylene Reduction Activity (ARA) concurrently with lectin determination in 3 different sets of 3x10 tubes; (i) free-living bacteria *Klebsiella* R15 (ii) free-living rice seedlings and (iii) associative condition between rice and *Klebsiella* R15. Values presented are mean ± SE of 10 tubes.

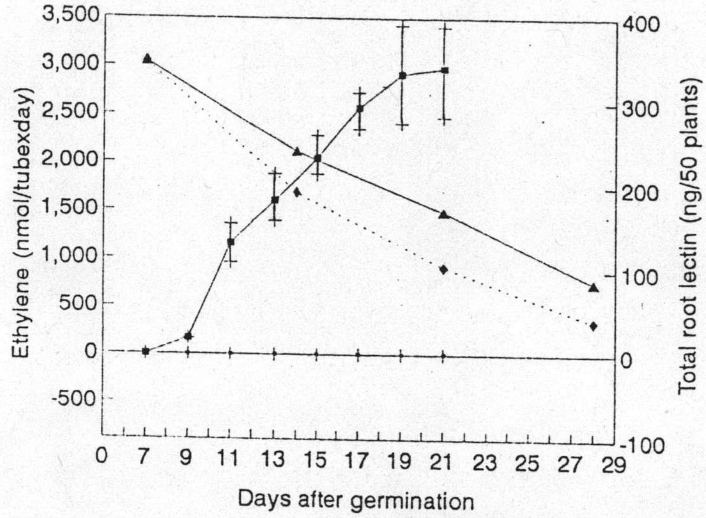
(a), (b) and (c) show relationship between associative nitrogenase activity and root lectin content in rice cv.:RD7, NMS4 and KDML105 respectively.

Nitrogenase activity — free-living bacteria *Klebsiella* R15

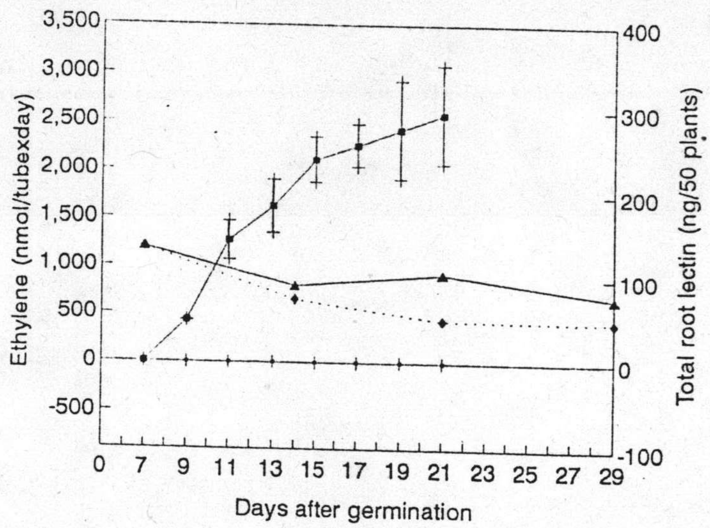
— free-living rice seedlings

— associative condition between rice and *Klebsiella* R15

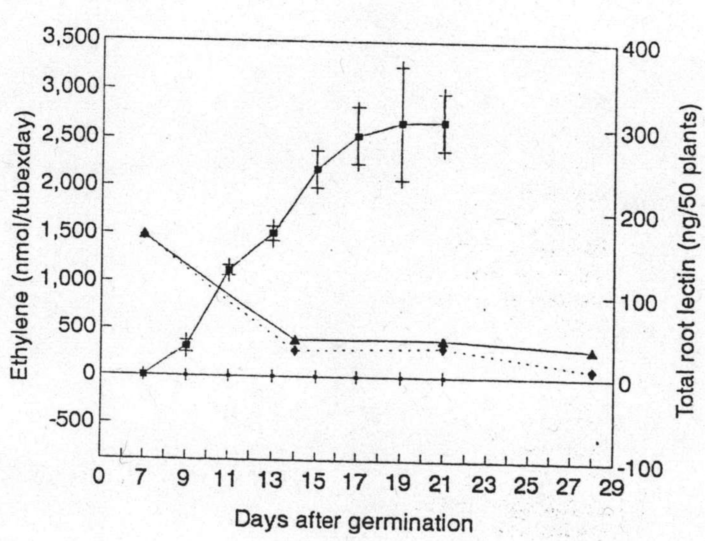
Total root lectin ◆ in the absence ▲ presence of *Klebsiella* R15



Ethylene (nmol/tubexday)
Total root lectin (ng/50 plants)
Days after germination



Ethylene (nmol/tubexday)
Total root lectin (ng/50 plants)
Days after germination



Ethylene (nmol/tubexday)
Total root lectin (ng/50 plants)
Days after germination

3.4 Effect of bacterial inoculation on total nitrogen content of rice plant

On day 21 or 2 weeks after inoculation, the dry weight and % N of rice plants (30) were measured after dividing into root and shoot fractions. Table 3.3 shows that there are no significant difference in dry weight, % nitrogen and total nitrogen in all 3 cultivars tested on day 21 after germination or 2 weeks after inoculation.

3.5 Molecular forms of root and shoot lectins characterized by Western blot analysis

In accordance with the quantitative determination by ELISA, molecular forms of rice lectin in root and shoot extracts were also investigated by Western blot analysis after separation on 12% SDS-polyacrylamide gel electrophoresis. The protein bands were specifically probed with antilectin and visualized by horseradish peroxidase immunostaining. The lectin polypeptides in root extracts show the similar patterns of molecular weight in all 3 rice cultivars (Figure 3.4 a, b, and c) that in associative condition, there are significant increase in the 23-kDa (lectin precursor polypeptide) and the 18-kDa polypeptide and also slightly stained in the 10-kDa and 8-kDa (Lane 4, 6 and 8). In free-living condition, the 23-kDa and 18-kDa polypeptides nearly disappeared since 14 days after germination, where the 10-kDa and 8-kDa bands can be observed at low intensity of staining and disappeared as a function of time.

Characterization of lectin polypeptides in shoot extracts of both free-living and associative conditions shows that only the 18-kDa polypeptide is dominant, and the intensity of staining is gradually decreased as a function of time after germination (Figure 3.4 d, e and f).

Table 3.3 Effect of *Klebsiella* R15 on total nitrogen content of rice plants two weeks after inoculation

Rice cv.	Dry weight (mg/30 plants)		% Nitrogen (mg/100mg dry weight		Total nitrogen (mg/30 plants)	
+ R15	shoots	roots	shoots	roots	shoots	roots
RD7 -	0.1414±.006	0.1084±.003	2.86±0.11	1.88±0.19	0.0040	0.0020
+	0.1265±.007	0.0987±.004	2.88±0.22	1.87±0.13	0.0036	0.0018
NMS4 -	0.1493±.009	0.1200±.007	2.31±0.15	1.60±0.21	0.0034	0.0019
+	0.1485±.011	0.1104±.007	2.39±0.19	1.84±0.18	0.0035	0.0020
KDML105-	0.1485±.009	0.0934±.006	2.12±0.09	1.60±0.23	0.0032	0.0015
+	0.1513±.010	0.0815±.005	2.17±0.21	1.19±0.19	0.0032	0.0013

Values presented are means of six replications

Figure 3.4 Western blot analysis with antilectin of crude extracts from rice root and shoot fractions

The crude extract was separately in 12% SDS-polyacrylamide gel and transferred to the nitrocellulose membrane and probed with antilectin (1:500). Antigen-antibody complexes were detected using goat-antirabbit IgG labeled with horseradish peroxidase (1:1500)

(A), (B), (C) are the Western blot analysis of 15 µl crude extracts, which contain 15 µg total proteins from root fraction of RD7, NMS4 and KDML105, respectively, at different stages of growth

(D), (E), (F) are the Western blot analysis of 15 µl crude extracts from shoot fraction of RD7, NMS4 and KDML105, respectively

Lane 1 WGA 50 ng

Lane 2 Day 7, before inoculation

Lane 3 Day 14, free-living condition

Lane 4 Day 14, associative condition

Lane 5 Day 21, free-living condition

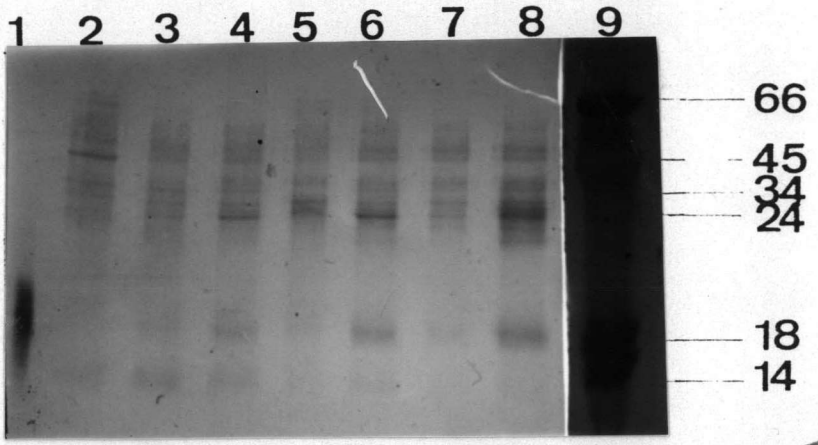
Lane 6 Day 21, associative condition

Lane 7 Day 28, free-living condition

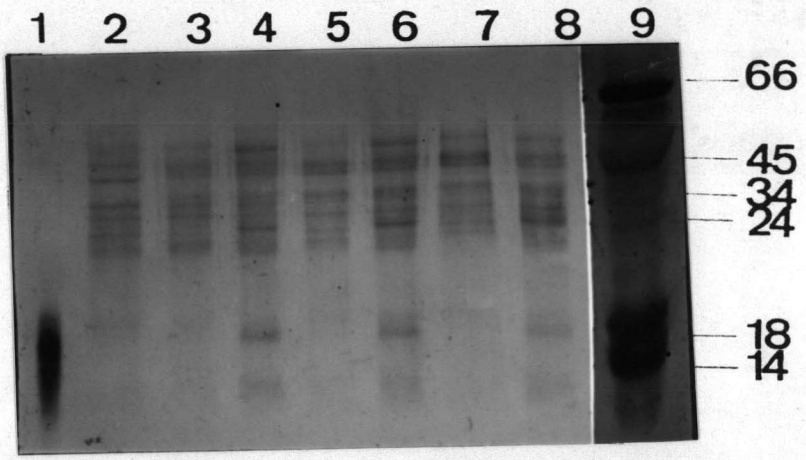
Lane 8 Day 28, associative condition

Lane 9 Protein markers; Albumin-Bovine Plasma 66-kDa, Ovalbumin 45-kDa, Pepsin 34-kDa, Trypsinogen, 24-kDa, ß- lactoglobulin 18-kDa and lysozyme 14-kDa

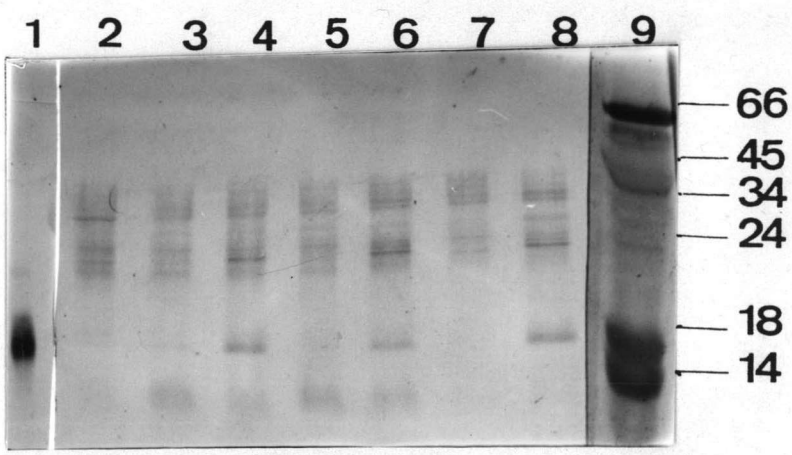
a)



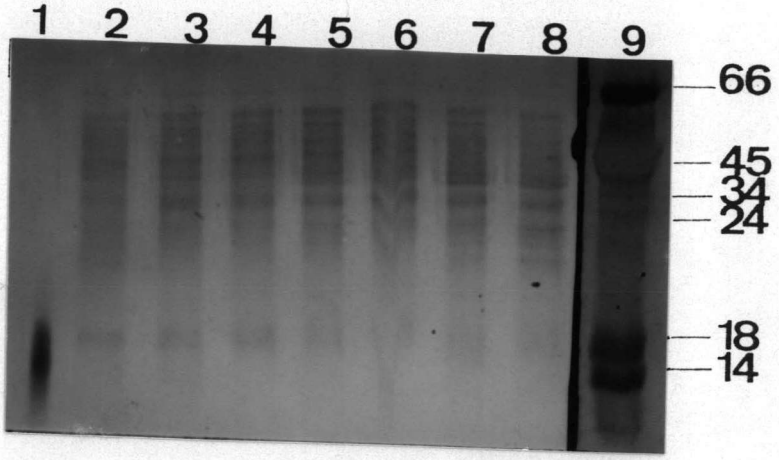
b)



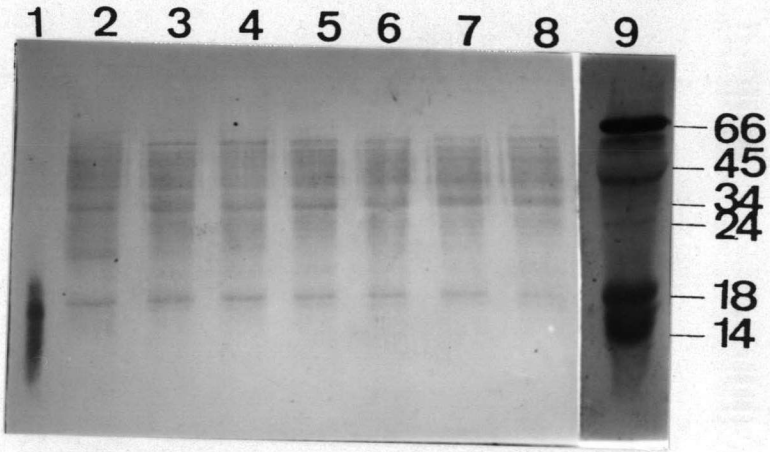
c)



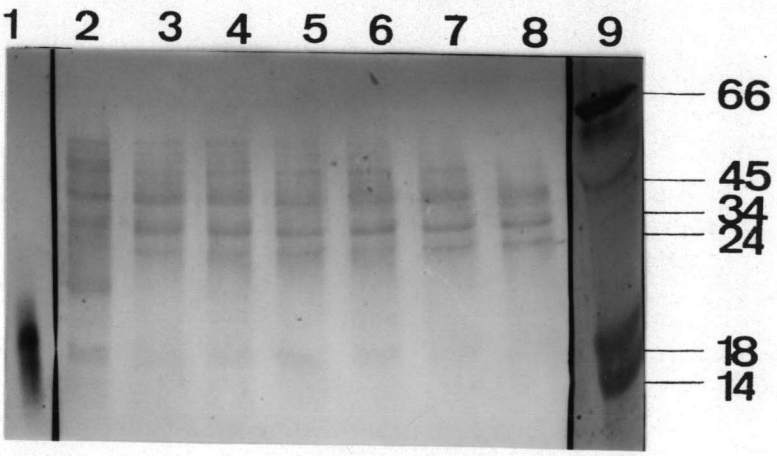
d)


1 2 3 4 5 6 7 8 9
66
45
34
24
18
14

e)


1 2 3 4 5 6 7 8 9
66
45
34
24
18
14

f)


1 2 3 4 5 6 7 8 9
66
45
34
24
18
14

These findings support the results obtained from the quantitative determination of lectin by ELISA that inoculation of rice seedlings with *Klebsiella* R15 leads to biosynthetic induction of root lectin, as evident by the 23 kDa de novo precursor form, while the molecular form of leaf lectins are similar in the presence and absence of *Klebsiella* R15 inoculation

3.6 Immunogold localization of lectin in rice root and leaf tissues

By using, immunogold-protein A labeling technique, specific lectin localization at the cellular and subcellular level was investigated. The specificity of immunogold staining is confirmed by Figure 3.5 (a and b), where no gold particles were observed in the tissues which were previously treated with non-immune serum or without the antilectin. Only in the presence of antilectin that the immunogold-labeled lectin can be observed in the root and shoot tissues of all 3 rice cultivars at specific locations demonstrated by the representative views of different cell types (Figure 3.6)

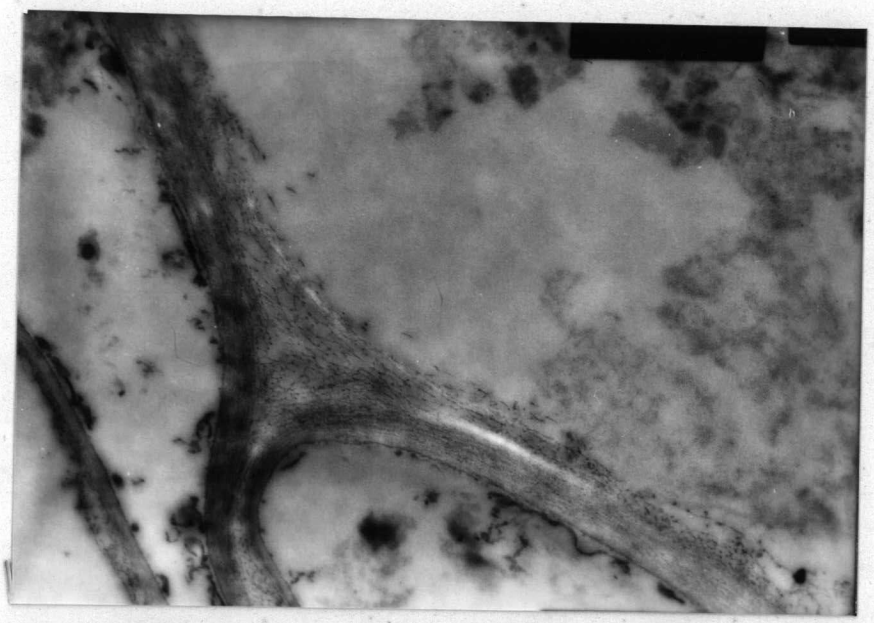
3.6.1 Localization of rice lectin in root tissues

3.6.1.1 In the presence of bacterial inoculation

At the cellular level, electronmicrographs of the root tip (Figure 3.7) show specific localization of epicuticular lectin attached to the extracellular polysaccharides (mucin) or the glycocalyx, the network-like structure adjacent to the surface of epidermal cells (Figure 3.7 a). Mostly gold granules were observed on secretory mucin or extracellular polysaccharides on root tips (Figure 3.7 b).

Colonized *Klebsiella* R15 observed on the surface of epidermal cell shows specific localization of lectin on the bacterial

a



b

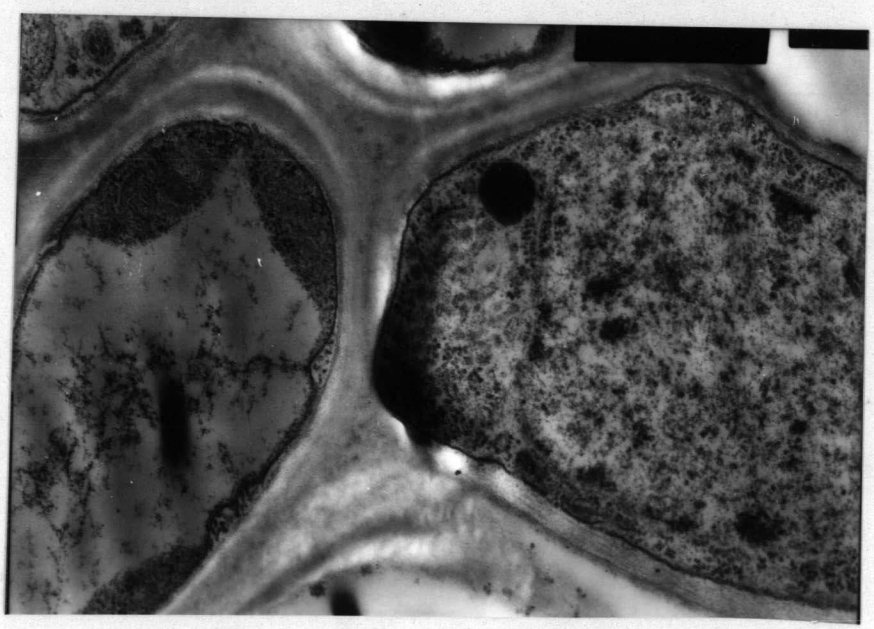
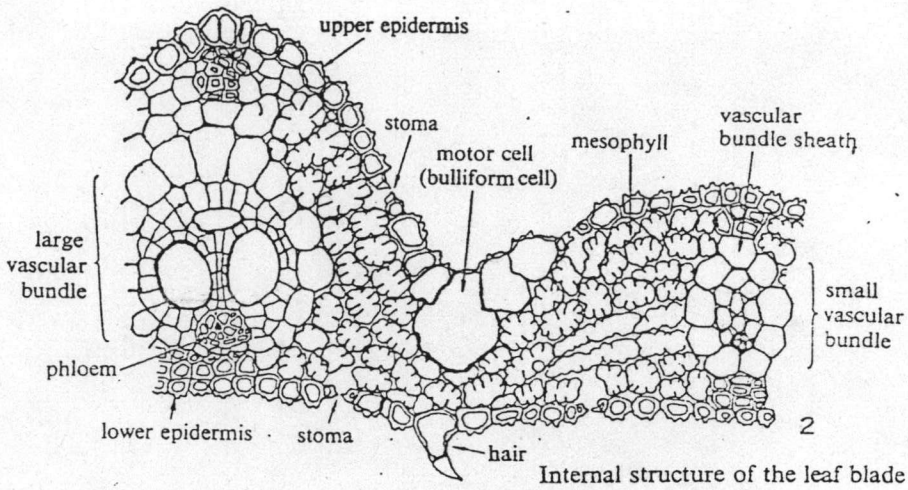
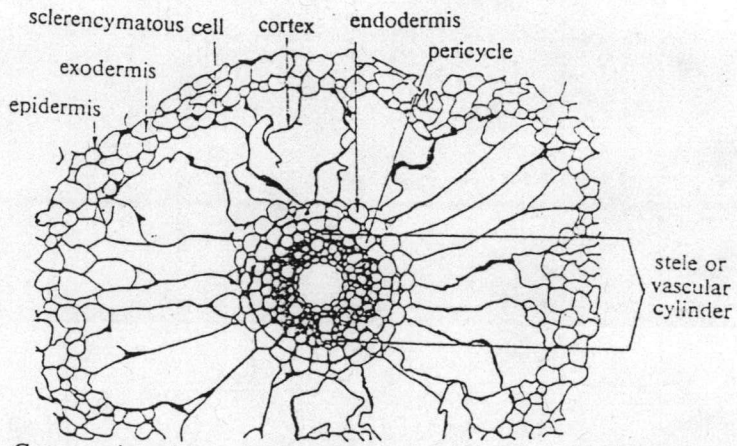


Figure 3.5 Ultrathin sections of control root and shoot tissues treated with non-immune serum

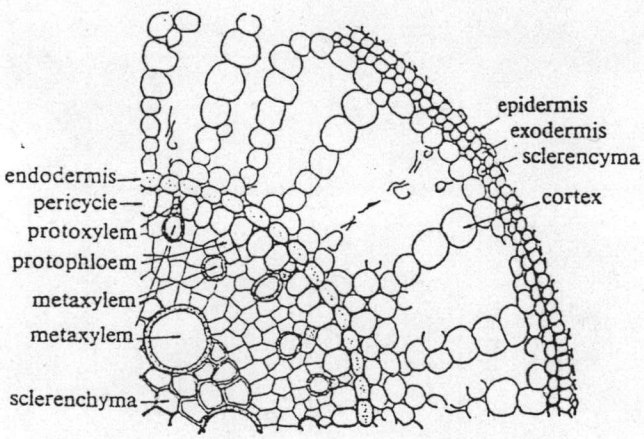
Antilectin was substituted with 0.01 M Tris-HCl buffer, followed by the protein A-gold complex. In general, no gold particles, are present in the root epidermal cells (a) x 28,000 and cell wall of xylem (b) x 28,000



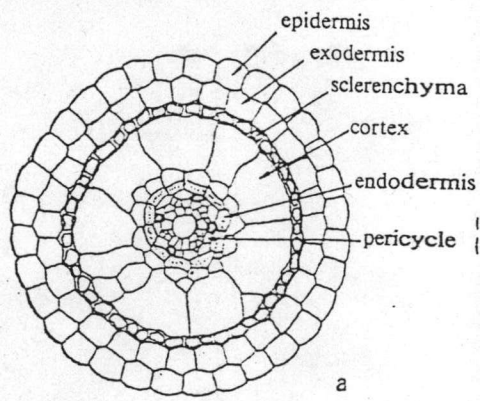
Internal structure of the leaf blade (× 300).



Cross-section of a seminal root (mature part) of the rice plant (× 160).



Internal structure of a crown root (1/4 of a cross-section through the maturation zone is shown) of the rice plant (× 155).



Internal structure of a branch root of rice (× 140).

Figure 3.6 Diagram of cross section of rice root (A) and leaf (B)

(Taken from Hoshikawa, 1989)

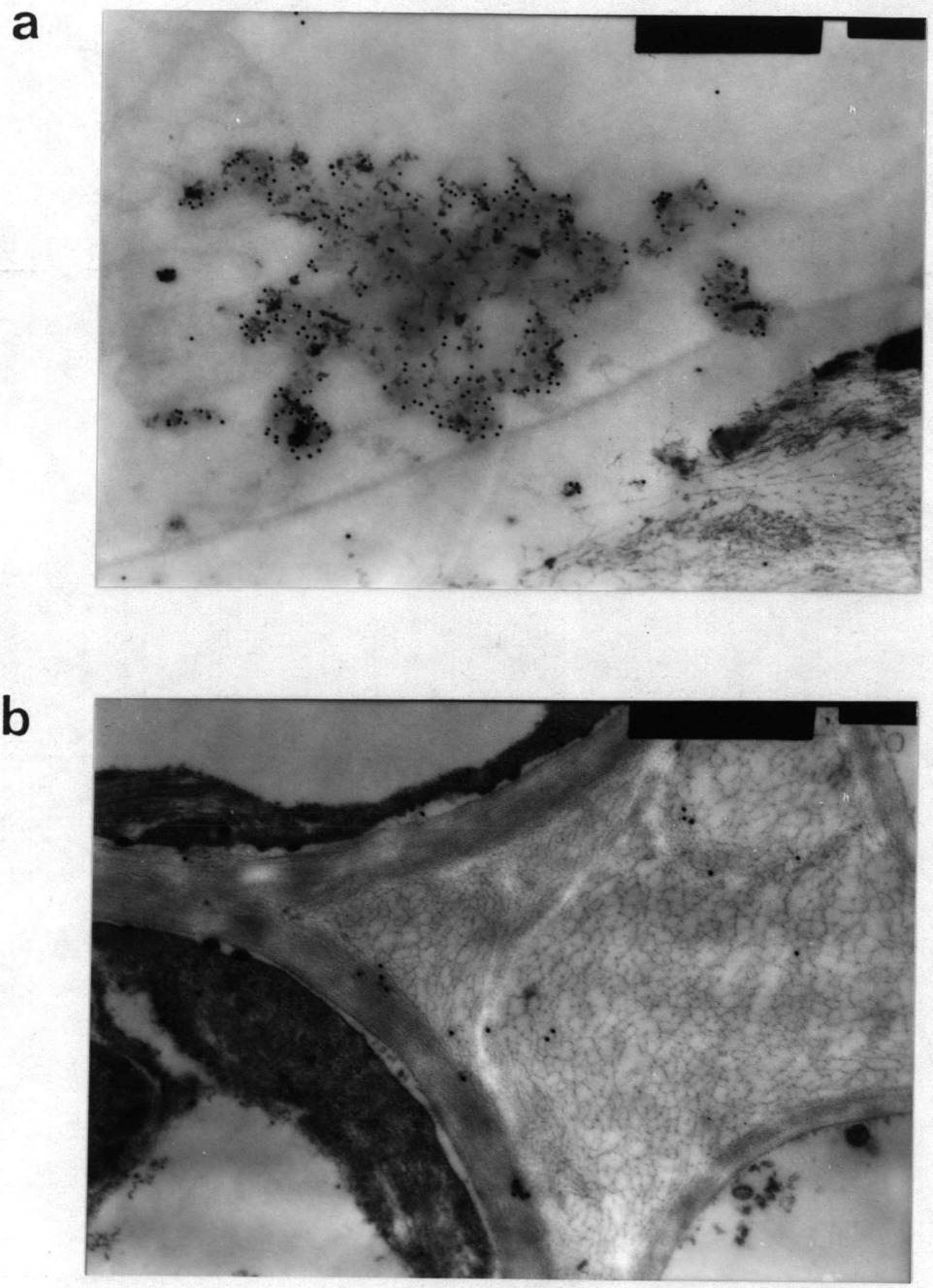


Figure 3.7 Specific localization of lectins in *Klebsiella* inoculated rice root

Detection of gold-labeled lectins in fibrillar-network of glycocalyx on the surface of root epidermal cells (a) x 28,000, and secretory mucin or extracellular polysaccharides on the root tip (b) x 28,000

glycocalyx (Figure 3.8 a, b, c, d, and e). Invasion of bacterial cells into the root exodermis and also in the intercellular space without disrupting the epidermal cells are found labeled with gold particles indicating the function of lectin, firstly as a necessary associative factor which play a role in the adhesion of bacteria to the epidermal cells of rice root, and secondly as carrier in the translocation of invasive bacteria into the inner parts of root.

By focusing on the subcellular localization of lectin in the radicle and branch root cells, Figure 3.9 demonstrates that gold particles are usually present in the vacuoles of epidermal cells. Gold particles are also present in the small vacuoles (v) which are located near the Golgi apparatus (G), or near the cell wall (cw) (Figure 3.9 a and b). In the pericycle area, which is in the middle part of root, those cells next to the pericycle, or "endodermis" show a few gold particles distributed in the vacuoles (v) and occasionally in the electron-densed inclusion bodies (INC) located near the membrane of vacuole (Figure 3.10 a and b). Besides the intracellular distribution in the root cells, lectin are located in the middle lamella among the root cells throughout all the root cells layer.

3.6.1.2 <u>In the absence of bacterial inoculation</u>

Although there is no bacterial inoculation, the labeled lectins are specifically localized in the glycocalyx (Figure 3.11 a and b) on the surface of epidermal root cells. Besides the gold particles are found attached to the small electron-dense particles that are most likely secreting out of the cell (Figure 3.11 a).

At the subcellular level, localization of lectins in the middle lamella and inside the vacuoles carrying the electron-dense particles have been observed (Figure 3.12). In some

Figure 3.8 Immunogold staining of lectins on inoculated *Klebsiella* R15 in the rhizosphere of rice root

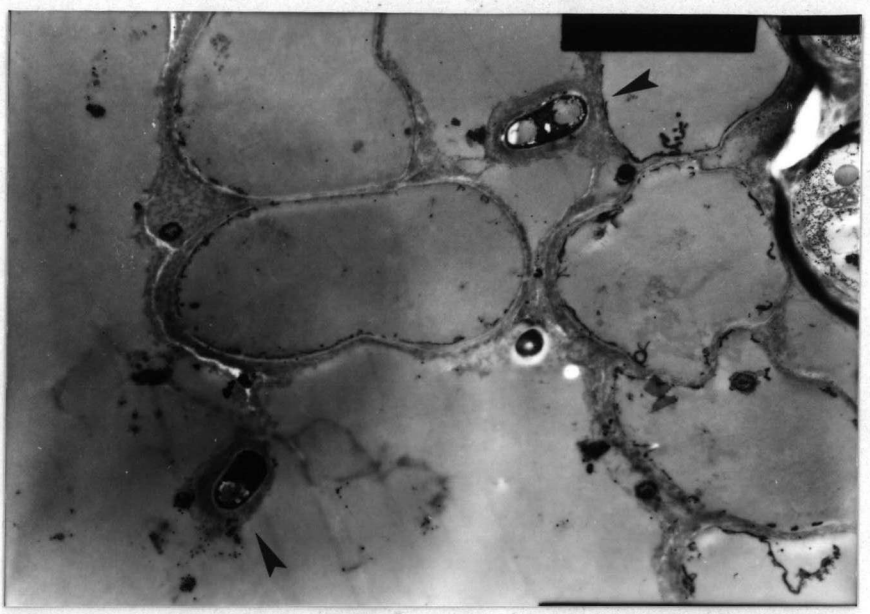
Series of electron micrographs showing:

a) The attachment and penetration of *Klebsiella* R15 into the root epidermal cells mediated by lectin on day 14 after inoculation (x 7,000)

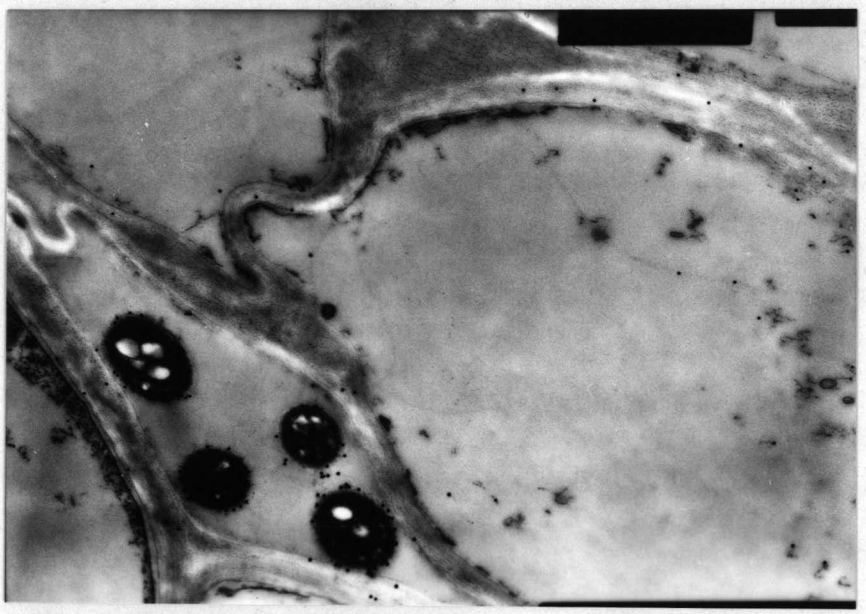
b) Cluster of *Klebsiella* R15 at the intercellular space between epidermal cells (x 21,000)

c), d) and e) Distribution of gold-labeled lectin on the bacterial cells (c 18,000 and d, e x 35,000)

a



b



c

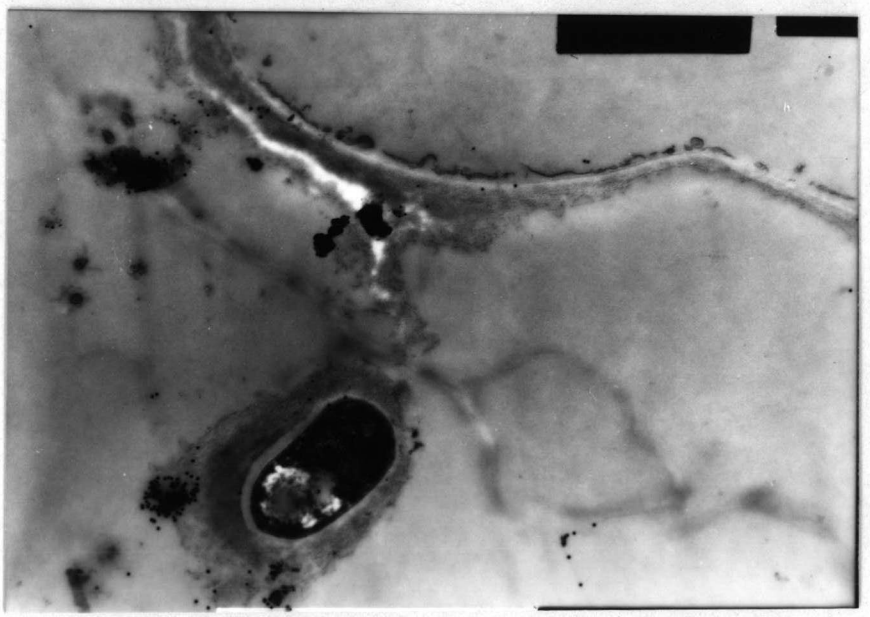
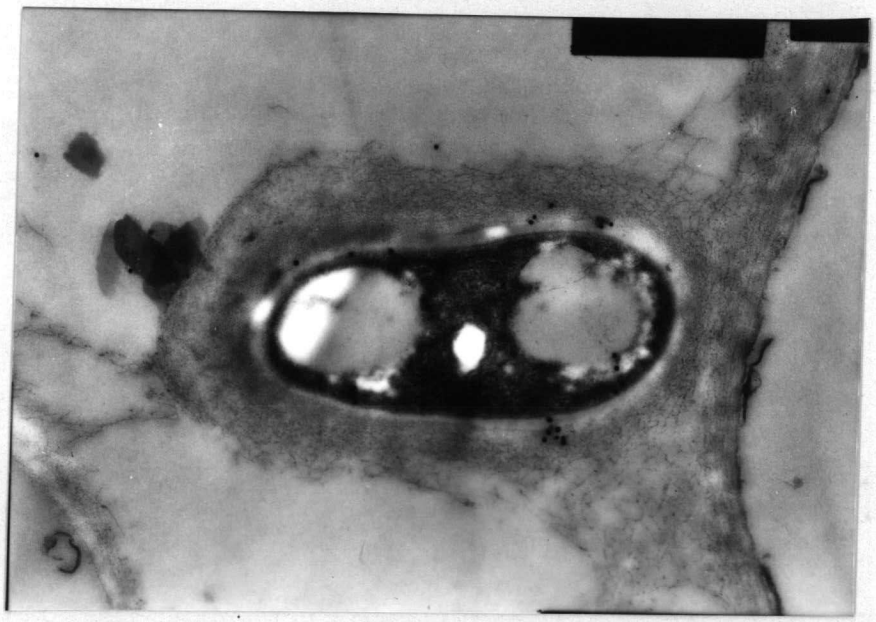
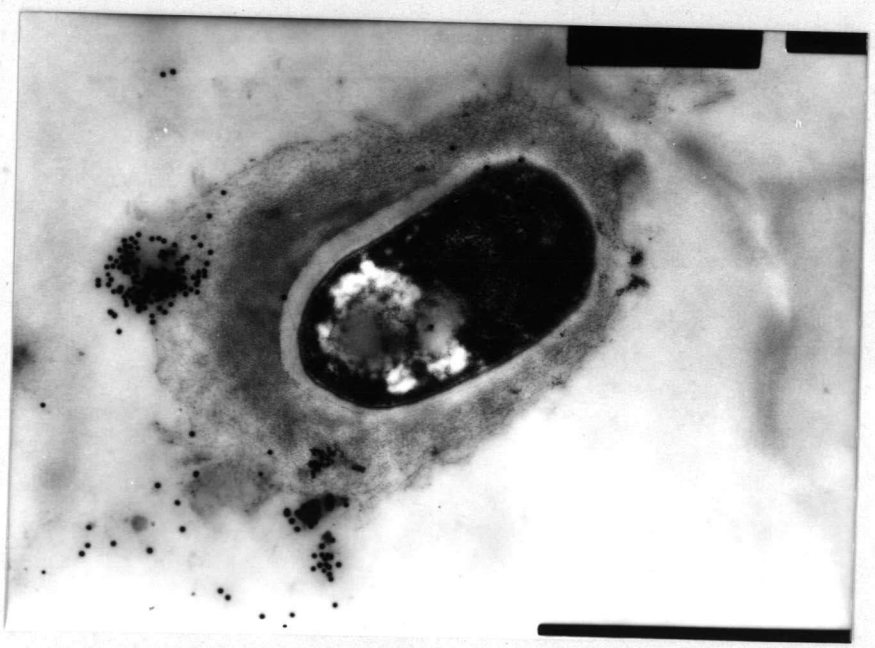


Figure 3.8 Continued

d



e



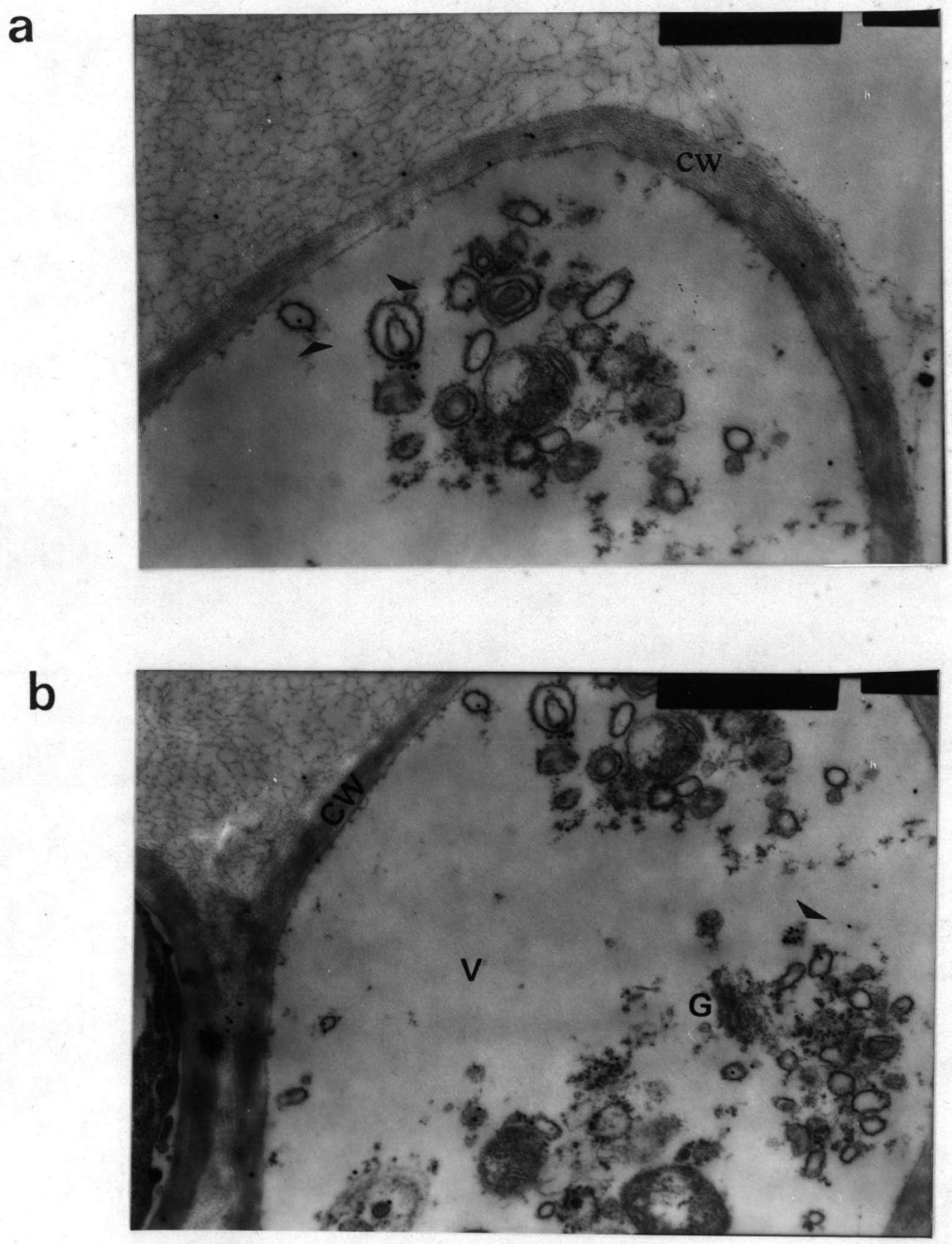


Figure 3.9 Subcellular localization of lectin in the vacuoles adjacent to Golgi apparatus in epidermal cells of inoculated root

Immuno gold labeled lectins in the vacuoles located near the cell membrane (a) and the Golgi body (b) x 36,000

Key to labels on figures : (V) vacuole; (G) Golgi body; (CW) cell wall

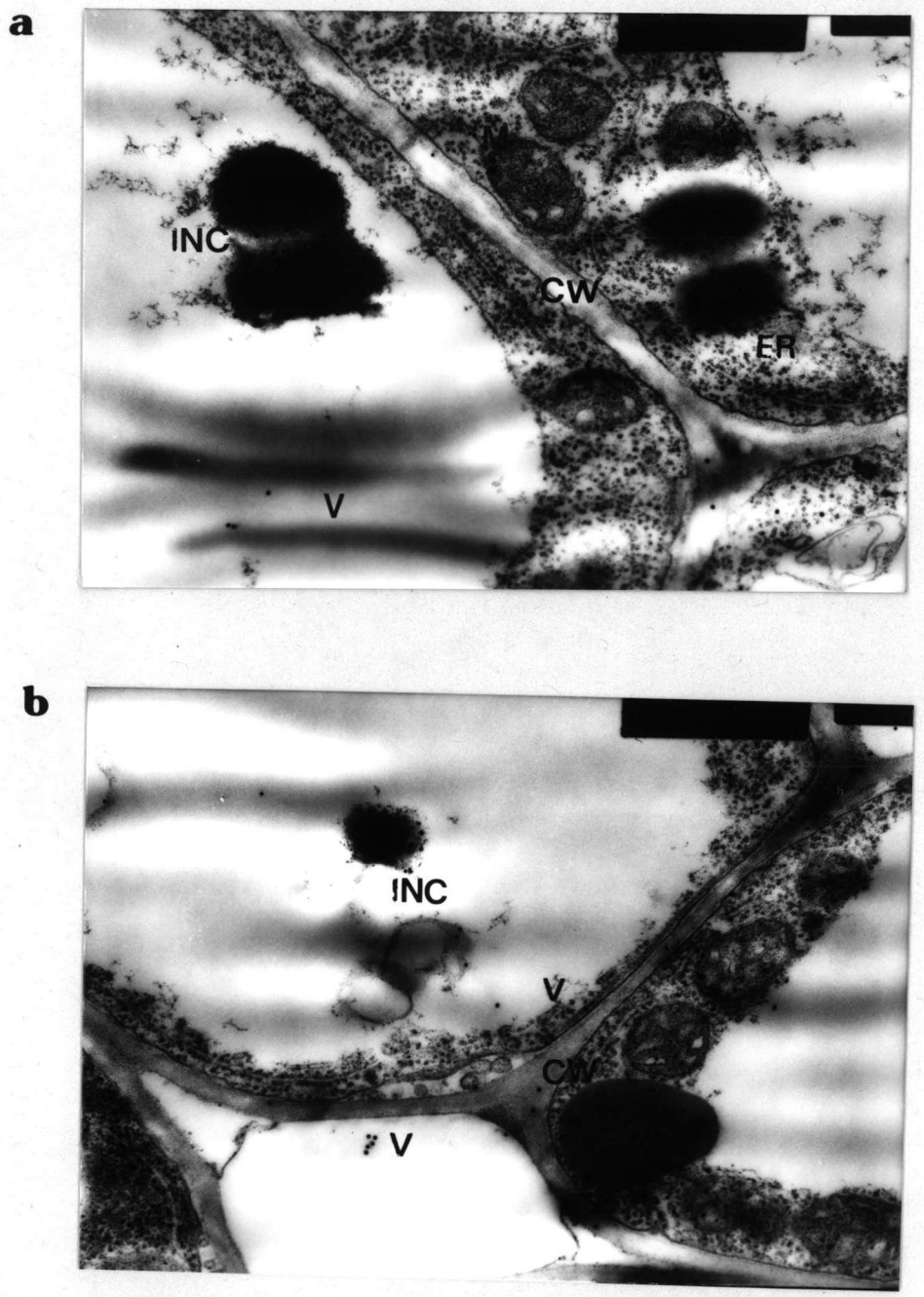
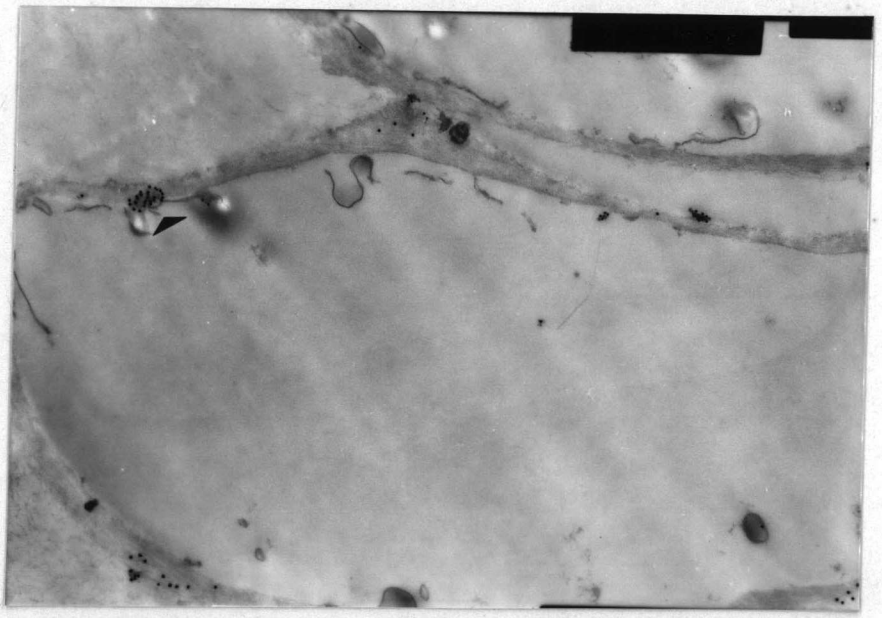


Figure 3.10 Distribution of lectins in vacuoles, inclusion bodies of endodermis of inoculated root

Electron micrographs of endodermis showing immuno-gold on electron-dense particles (INC) located in the vacuoles, and a few particles in the middle lamella. (a and b x 28,000)

a



b

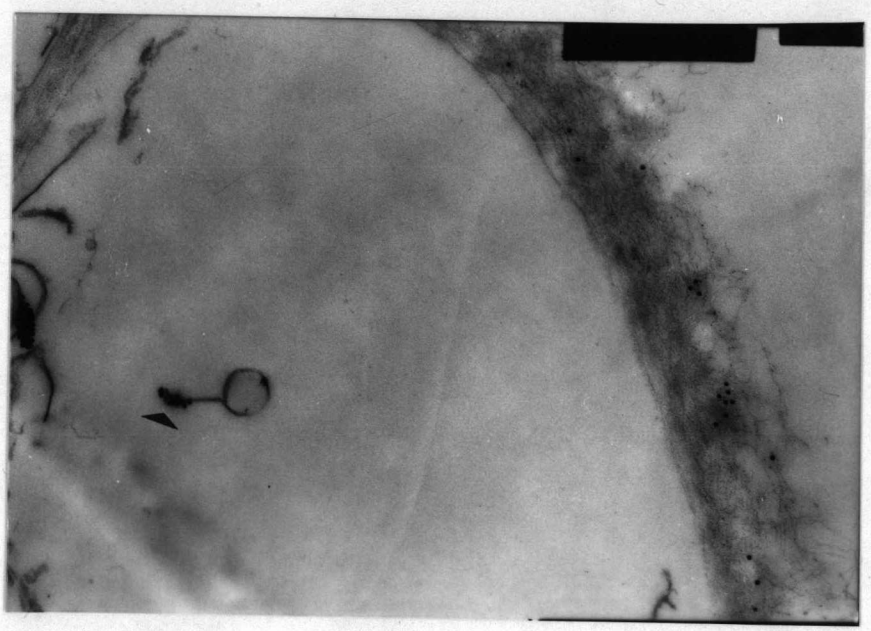


Figure 3.11 Localization of lectins in the glycocalyx on the surface of non-inoculated epidermal cells

Gold particles are present in the glycocalyx, and attached to the small electron-dense particles which are secreting out of the cell (x 21,000). (b) Gold particles stained lectin in the inclusion bodies within the vacuole (x 36,000).

sections, the labelled lectin in the cytoplasm are located on the endoplasmic reticulum (ER) which are the translational sites and directly translocated across cell membrane (Figure 3.12 a, b). And infrequency, gold particles are found labelled on electron-lucent matrix, called whorl (Figure 3.12 c).

3.6.2 Localization of lectin in leaf tissues

The pattern of immunogold-labeled lectins in leaf tissues of both inoculated and non inoculation rice seedling are similar, that is the lectins are mostly observed in the xylem, especially in the cell wall around the xylem and the cells surrounding the xylem (Figure 3.13 a, b, c and d). Interestingly, the cluster of lectins are translocated across the cell membrane and cell wall connected to the vacuole (Figure 3.13 e). Besides the xylem, the phloem are also stained with the gold-labeled lectins but less dense than in xylem (Figure 14 a, b). In addition the immunogold labeled lectins are observed in the stoma and at the interface between cell membrane and cell wall of the bulliform cells or motor cells (Figure 15 a, b and c). Study on the subcellular localization of lectin show that no gold particle is localized in the other organelles inside the cell, except the vacuole.

These results imply that firstly the physiological function of lectins should be involved in the translocation of some metabolites in the root via the xylem or from leaf to root via the phloem. Secondly they are involved in the adhesion between *Klebsiella* R15 and epidermal cells of root. Thirdly, they may be involved in the invasion of *Klebsiella* R15 into the exodermis layer. Besides these since lectins are detected in the stoma cells and in the periphery cells of leaf and root tissues, which are the potential infection sites of plant pathogens, they could play a role in plant defense mechanism.

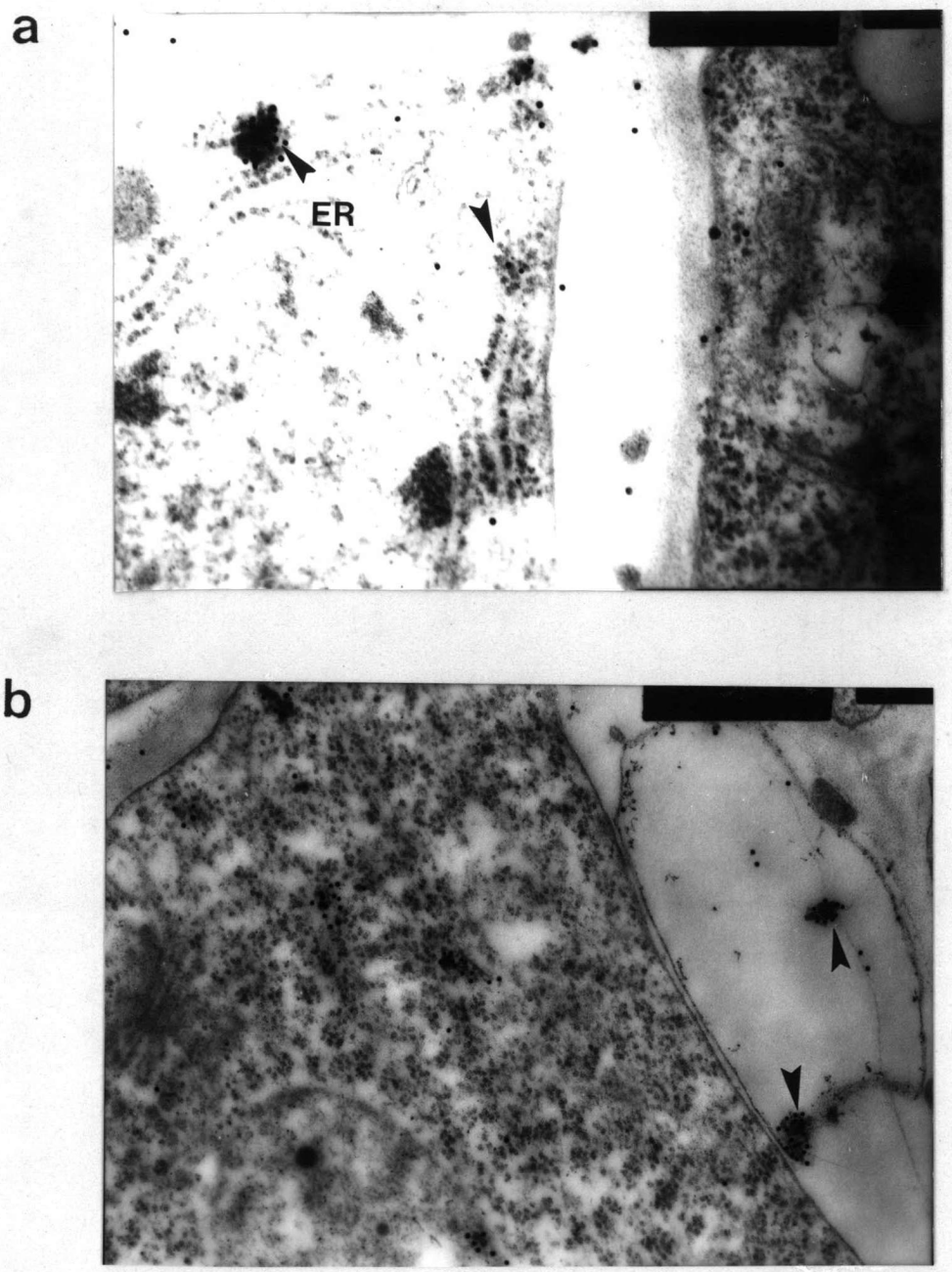
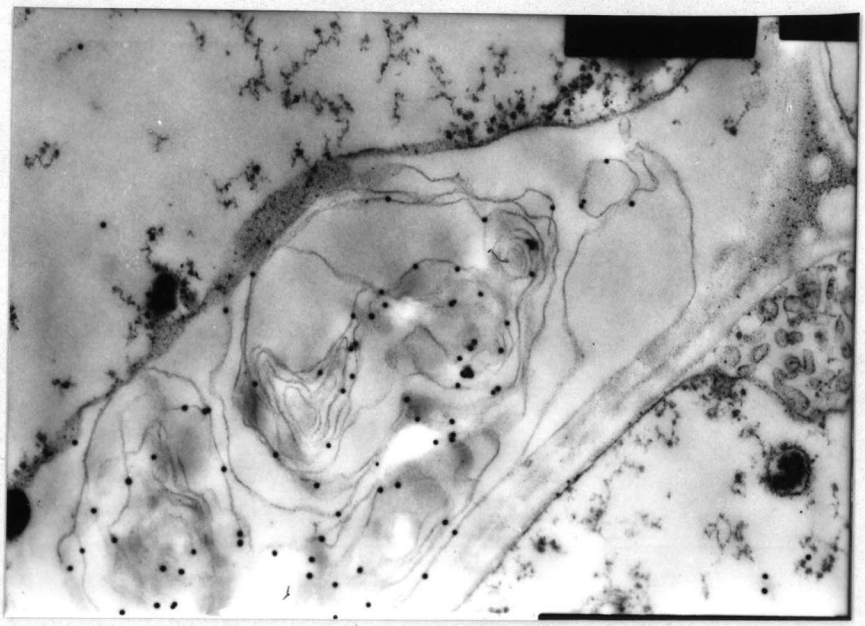


Figure 3.12 Localization of lectin in the endoplasmic reticulum of root endodermis

a. Labeled lectin on endoplasmic reticulum (ER) and translocation of lectin across cell membrane x 36,000

b. The presence of lectin. In vacuole, attached to the electron-dense particle, and some single gold particles are distributed in the middle lamella x 36,000

C

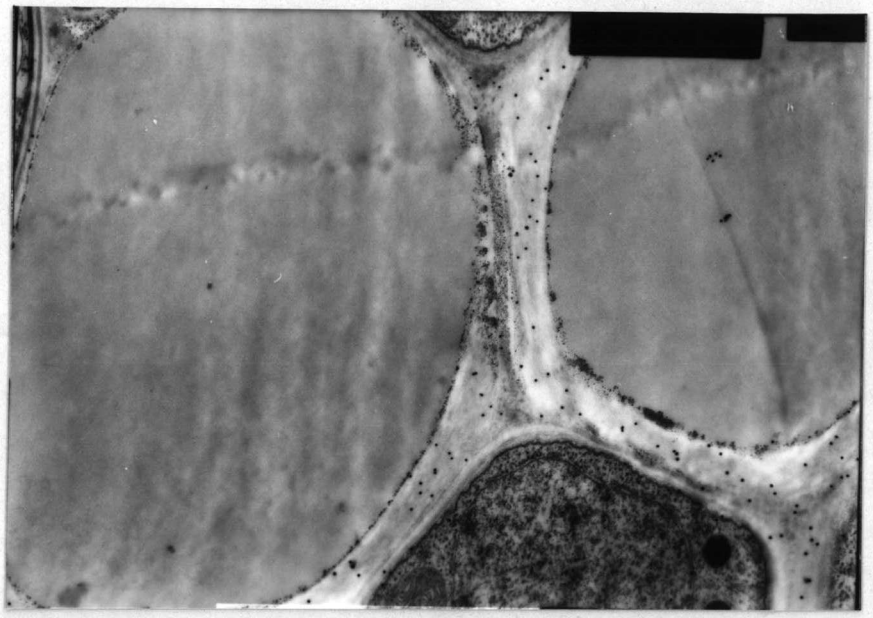


c. Distribution of lectins over the electron-translucent matrix located in the vacuole of endodermis (x 35,000)

Figure 3.13 Localization of lectin in and around the xylem of rice leaf

(A), (B) and (C) show largely distribution of gold particles at the cell wall of xylem throughout the intercellular space of the adjacent cell (D) The cell located between two xylem cells are also stained with gold particles (E) showing the exocytic of clusters of gold at the cell membrane of cell located above xylem (a x 18,000, b x 18,000, c x 18,000, d x 16,000 , e x 56,000)

a



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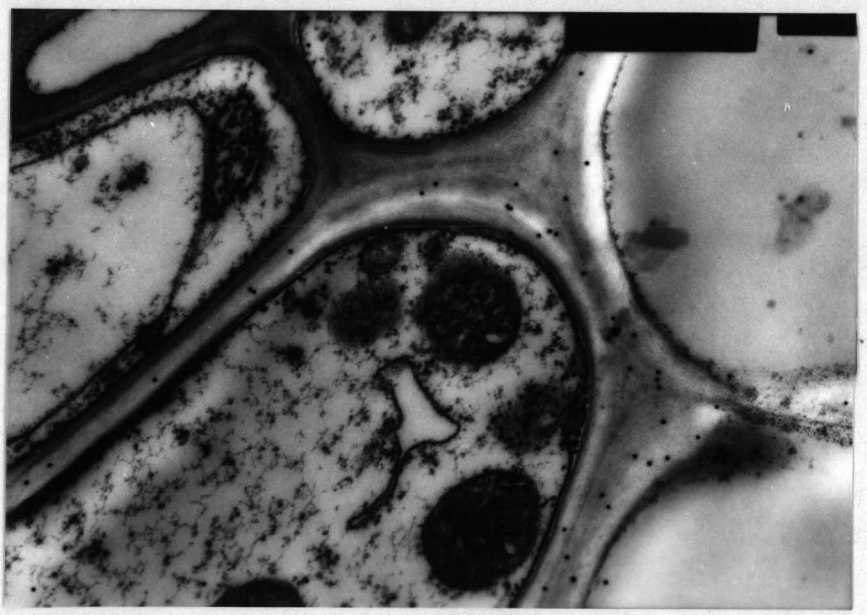

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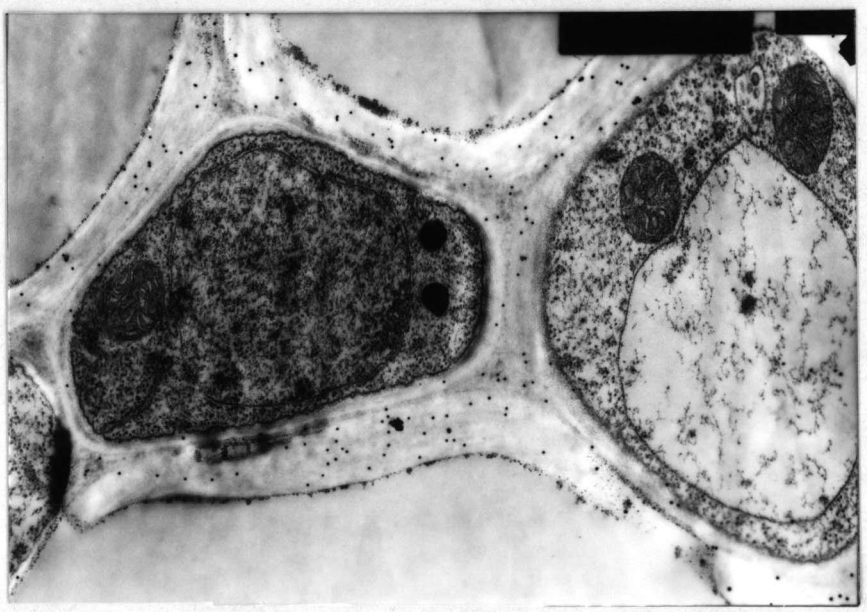

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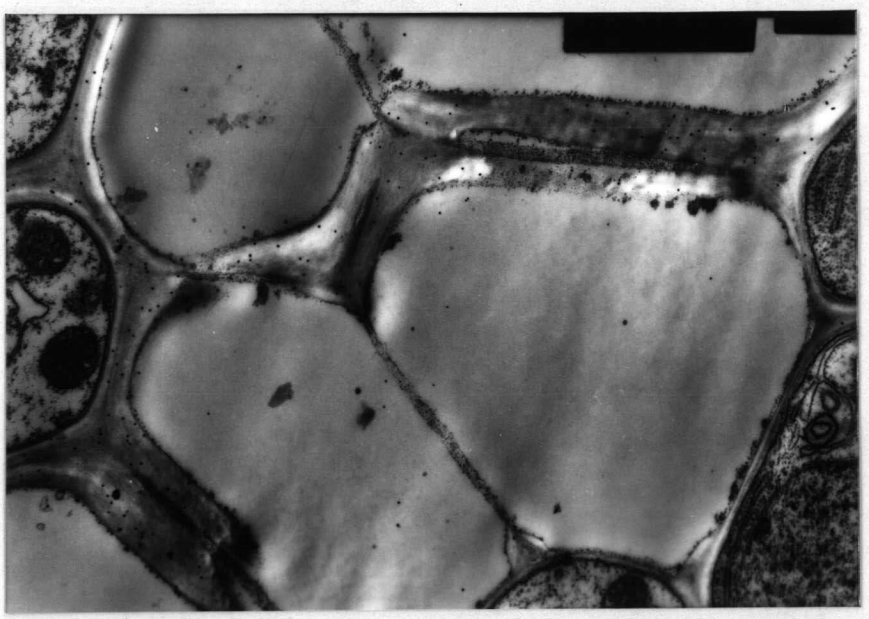
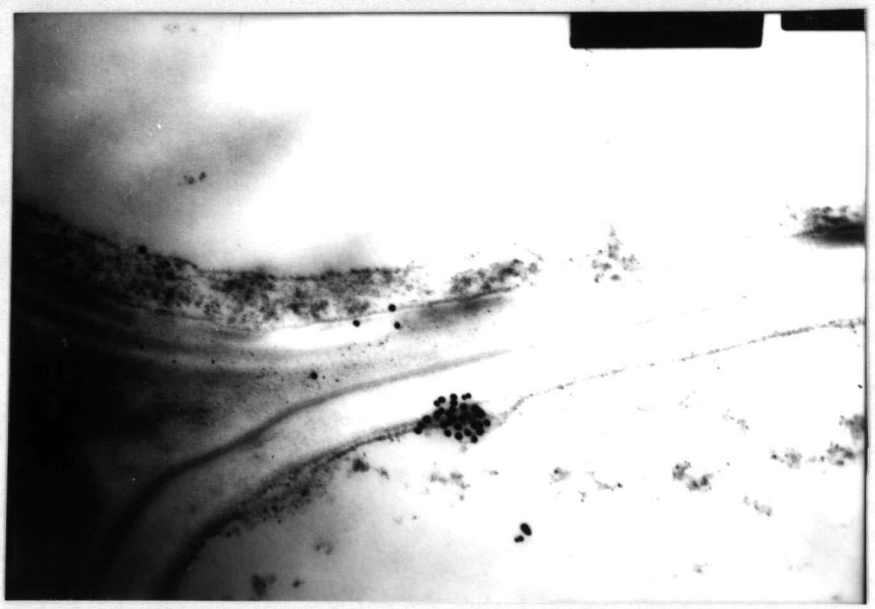


e



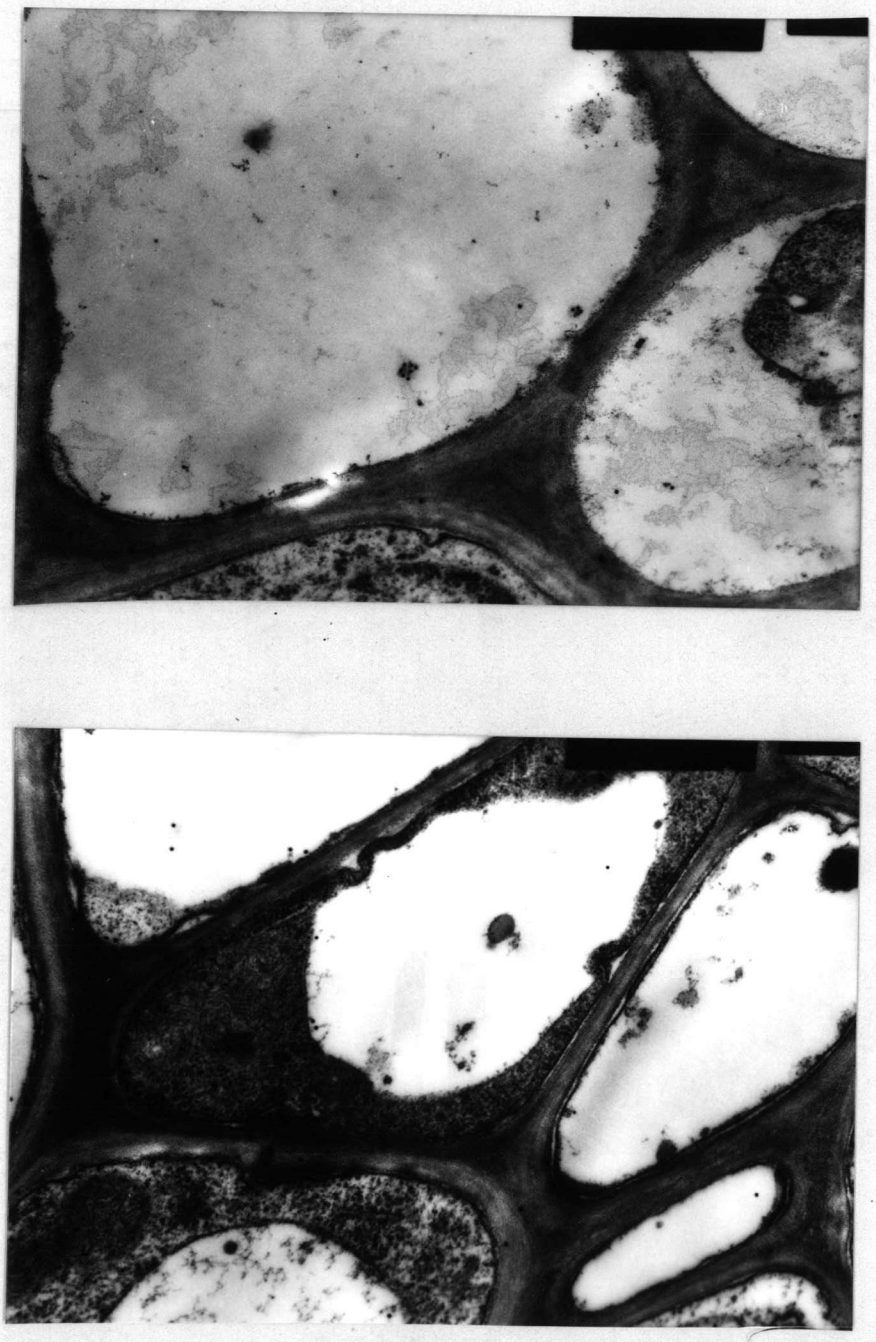


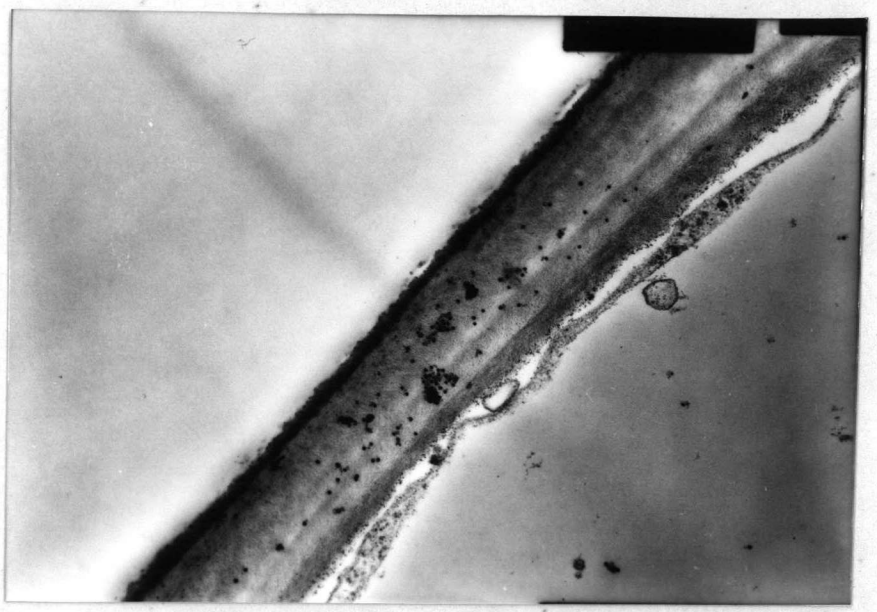
Figure 3.14 Leaf lectins are sporadically present in the vacuoles phloem and intercellular spaces x 28,000

Figure 3.15 Localization of lectins in the bulliform cells and stoma of rice leaf

a) and b) Lectins in bulliform cells are located in the interface between cell membrane and cell wall (x 28,000),

c) Lectins are localized in the stoma (x 21,000)

a



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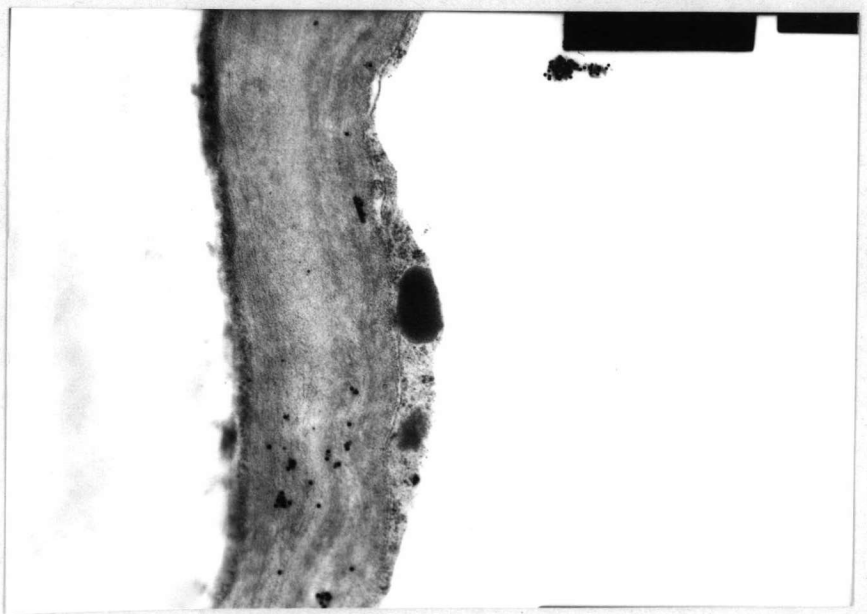

c