CHAPTER III

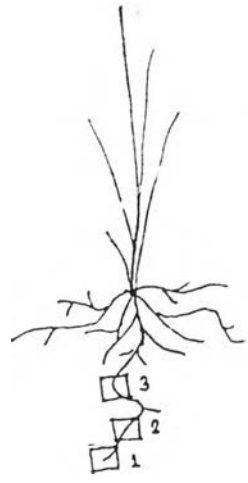
RESULTS

3.1 The comparative study of ultrastructure of non-inoculated and inoculated rice roots and leaves by stereomicroscope.

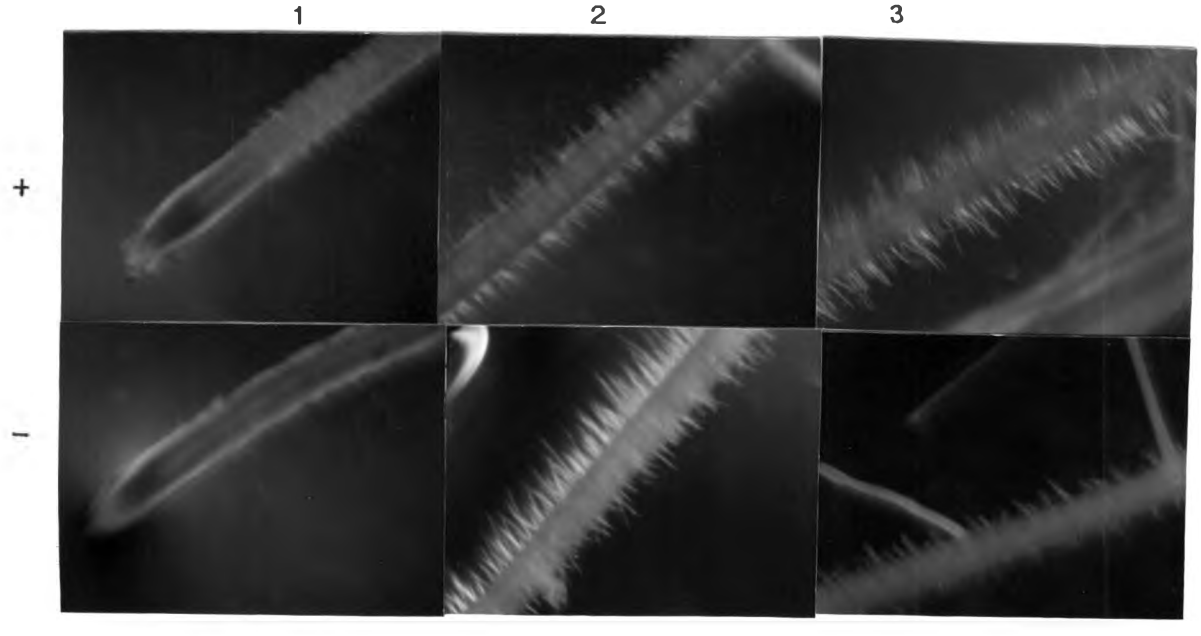
The deformation of morphology of non-inoculated and inoculated roots and leaves were observed, (Figure 3.1, 3.2), under stereomicroscope on day 0, 7, 14 and 21 after inoculation with *K. oxytoca* R15. On day 0 the leaves and roots of non-inoculated and inoculated rice were similar. The root tip was straight, having a cap, with root hair located about 0.05 mm from the root tip. The leaves were like many others monocot leaves, about 2X15 mm at this developmental stage. On day 7, curling structure of the inoculated rice roots was observed, on day 14 and 21, the inoculated rice roots appeared to have more branching, denser and longer than the non-inoculated rice roots. In addition, numerous mucous was associated with root hair and occasionally red root hair formation in observed associative rice roots, which could not be found in non-inoculated rice roots. In the leaves however no difference between non-inoculated and inoculated rice leaves could be observed during the period of the observation.

Figure 3.1 The morphology of root of inoculated (+) and non-inoculated (-) rice, 14.3 X.

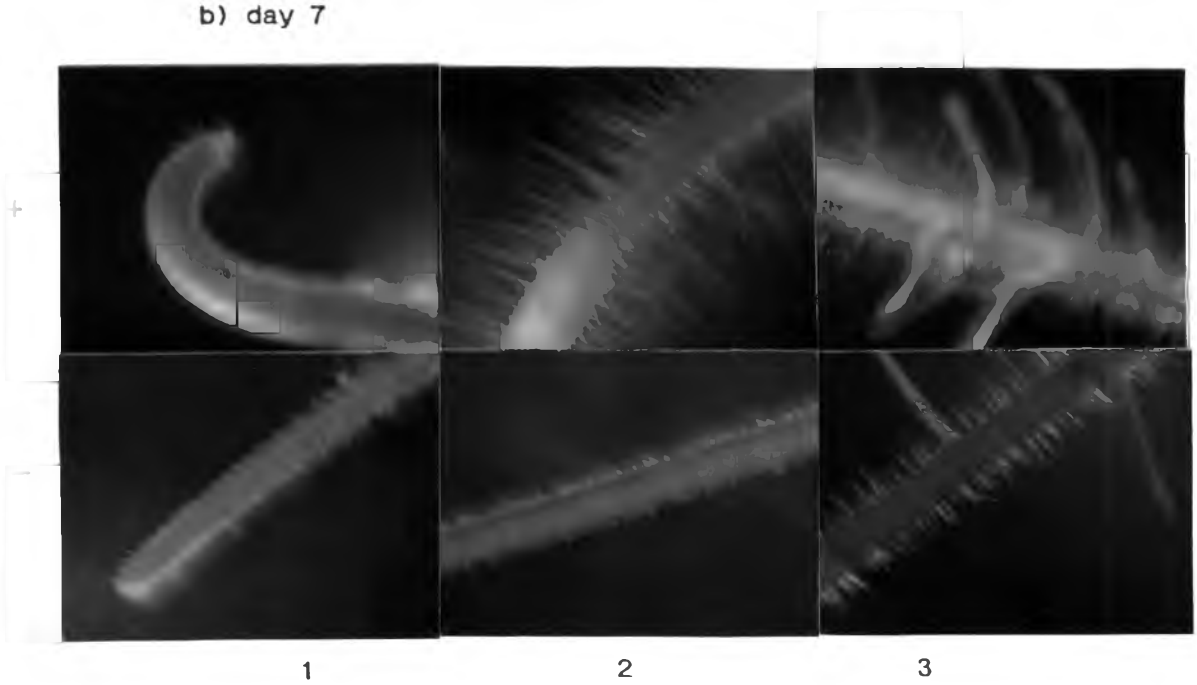
1, 2 and 3 : The numbering system at root sections



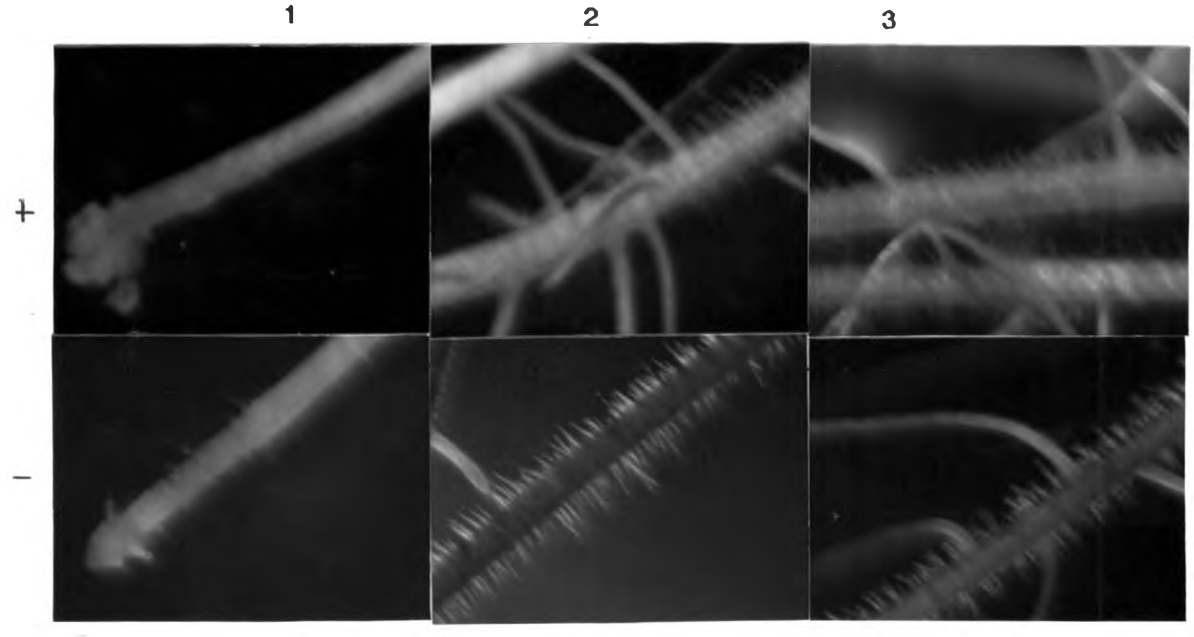
a) day 0



b) day 7



c) day 14



d) day 21

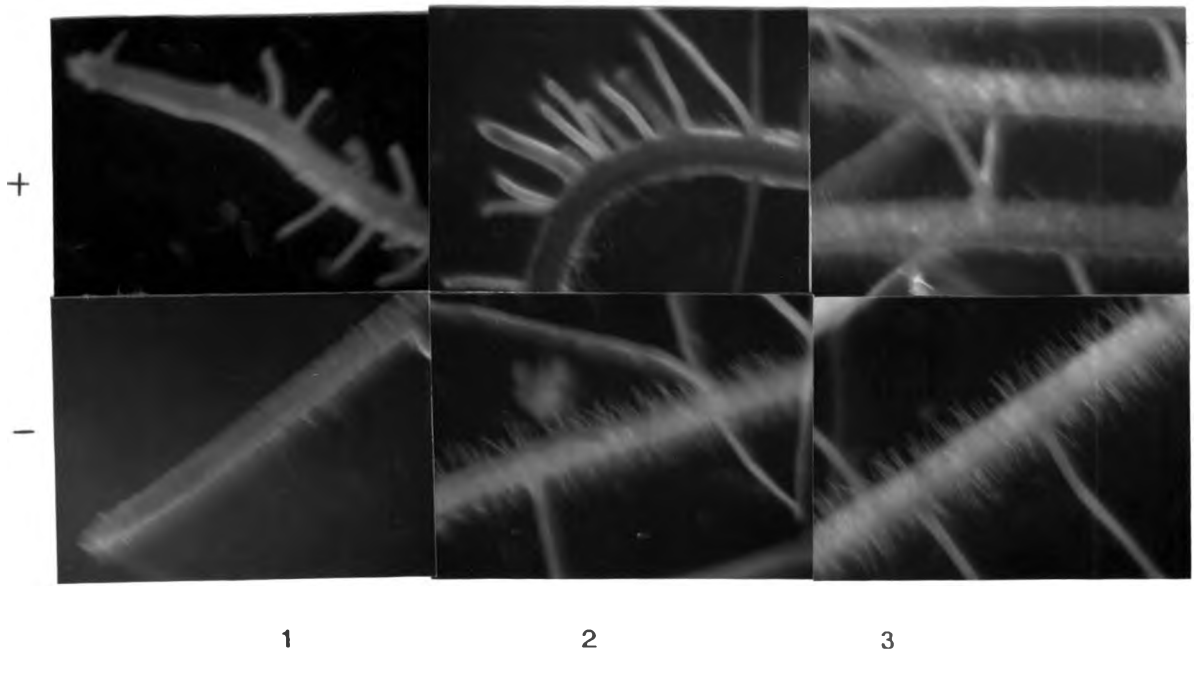
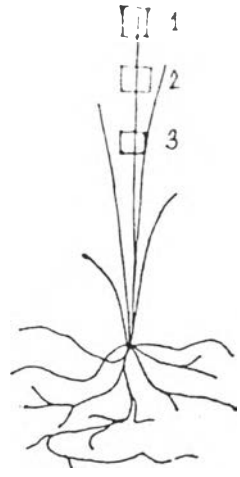
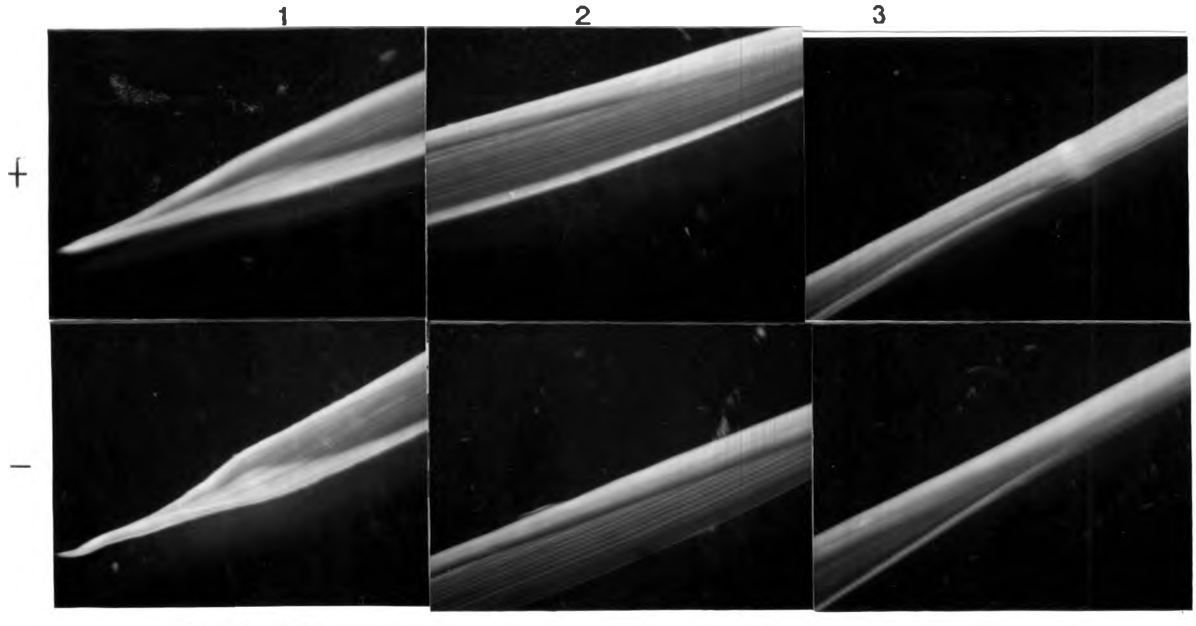


Figure 3.2 The morphology of leaf of inoculated (+) and non-inoculated rice, 14.3 X.

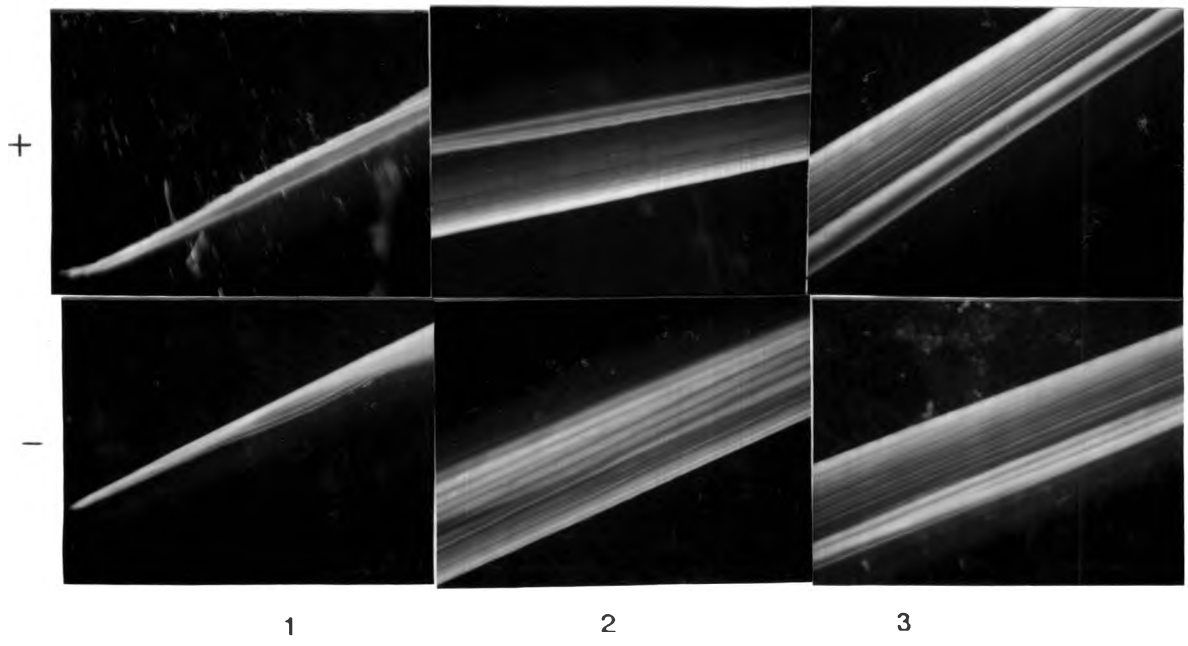
1, 2 and 3 : The numbering system at leaf sections



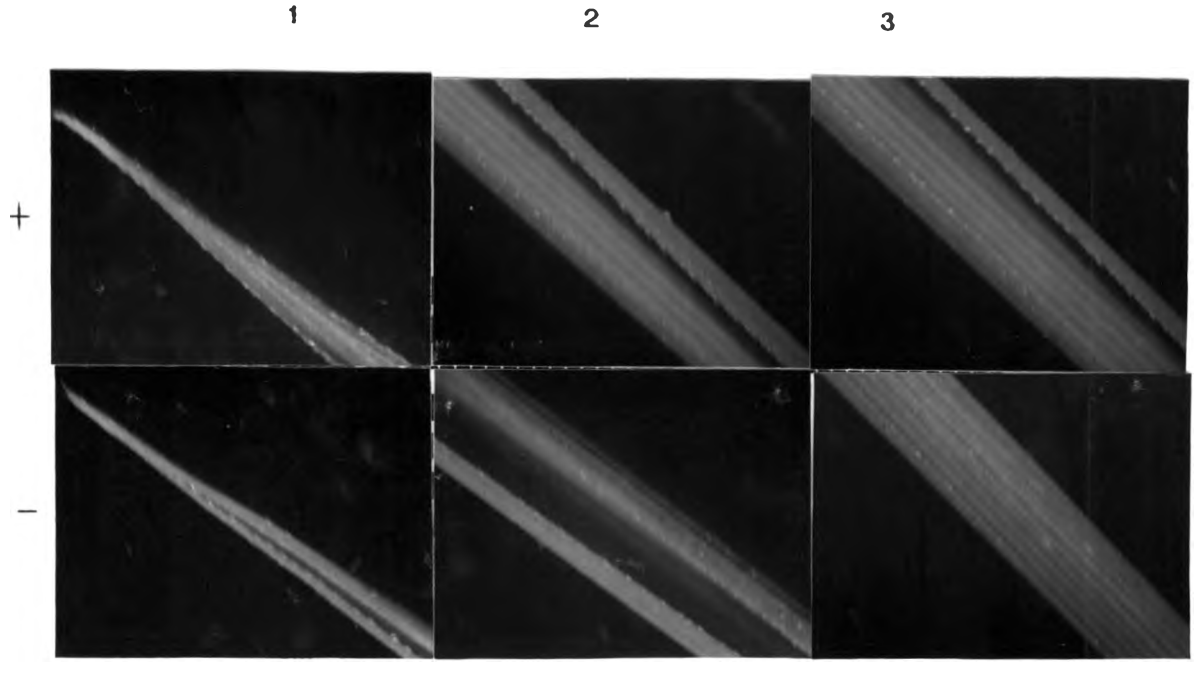
a) day 0



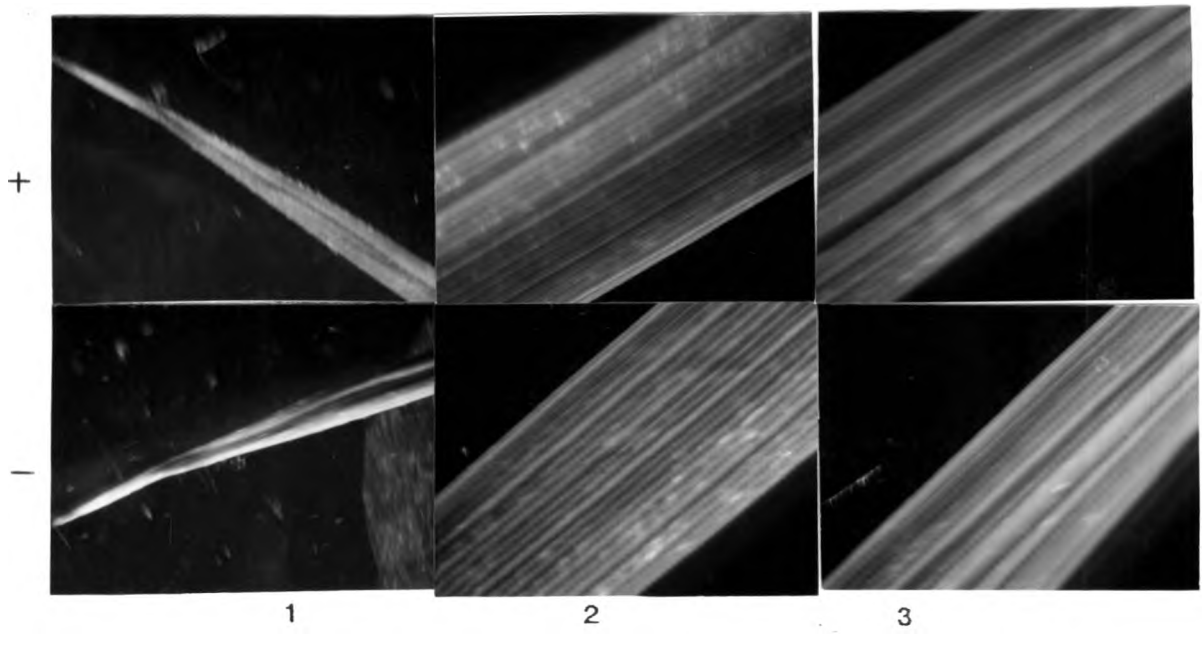
b) day 7



c) day 14



d) day 21

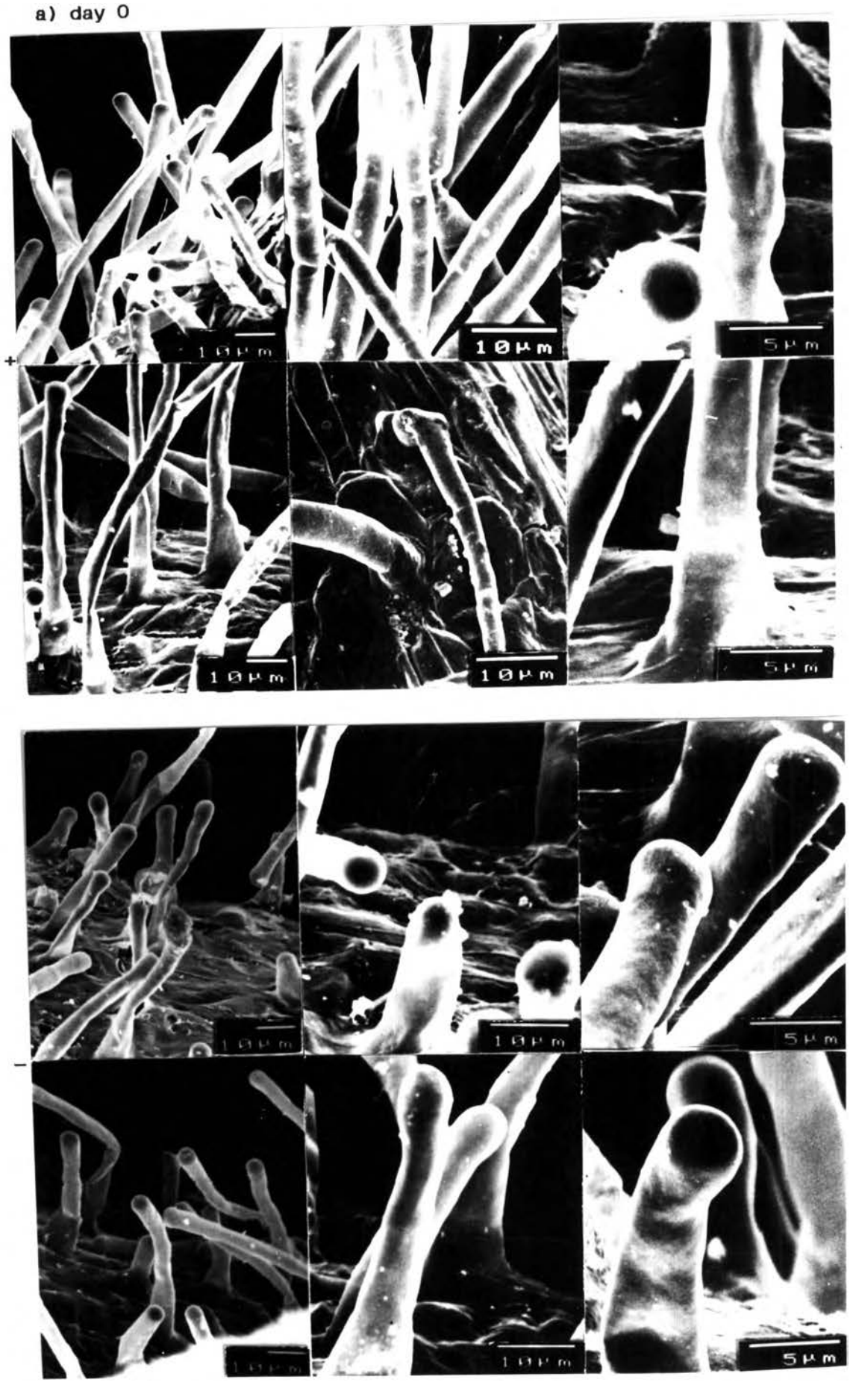


3.2 The comparative study of ultrastructure of non-inoculated and inoculated rice roots and leaves by scanning electron microscope (SEM)

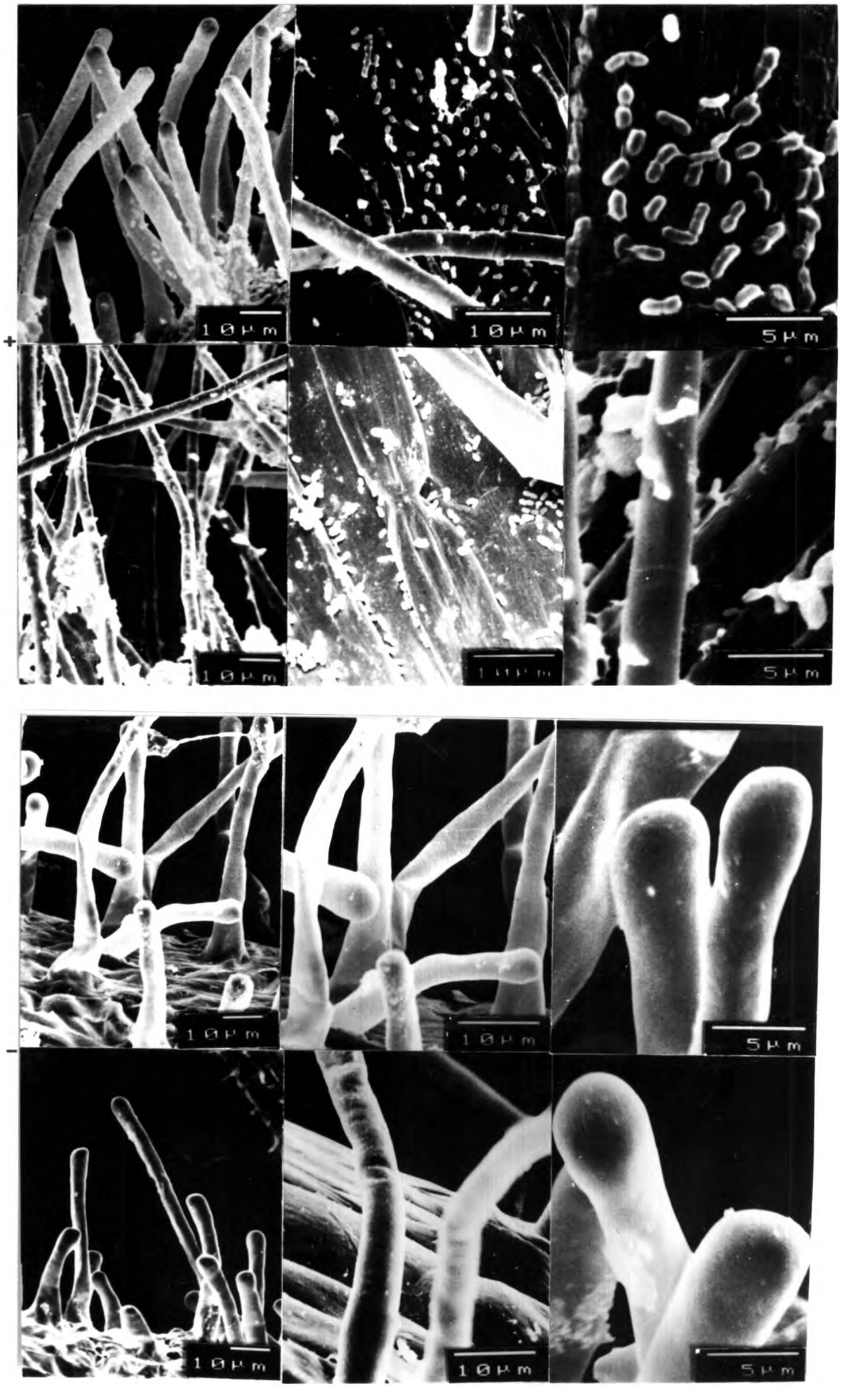
The ultrastructure study of roots and leaves of inoculated and non-inoculated rice plant were performed 0-21 days after inoculation by scanning electron microscope(SEM) and the results were similar to the previous results with stereomicroscope. The significant difference was observed only between the roots of inoculated and non-inoculated rice, beginning from day 7 after inoculation. Figure 3.3 shows many bacteria associated with rice roots and some of them penetrated into the intracellular space of the epidermal cells on the surface of the roots. On day 14 and 21, the roots tips excretory mucous on the surface of inoculated rice roots were much more than in non-inoculated roots. The associative bacteria also contain mucous coating them. In addition the inoculated rice roots contain more branching and longer root hair. The results shown in figure 3.3 and 3.4.

Figure 3.3 The ultrastructure of roots of inoculated (+) and non-inoculated (-) rice.

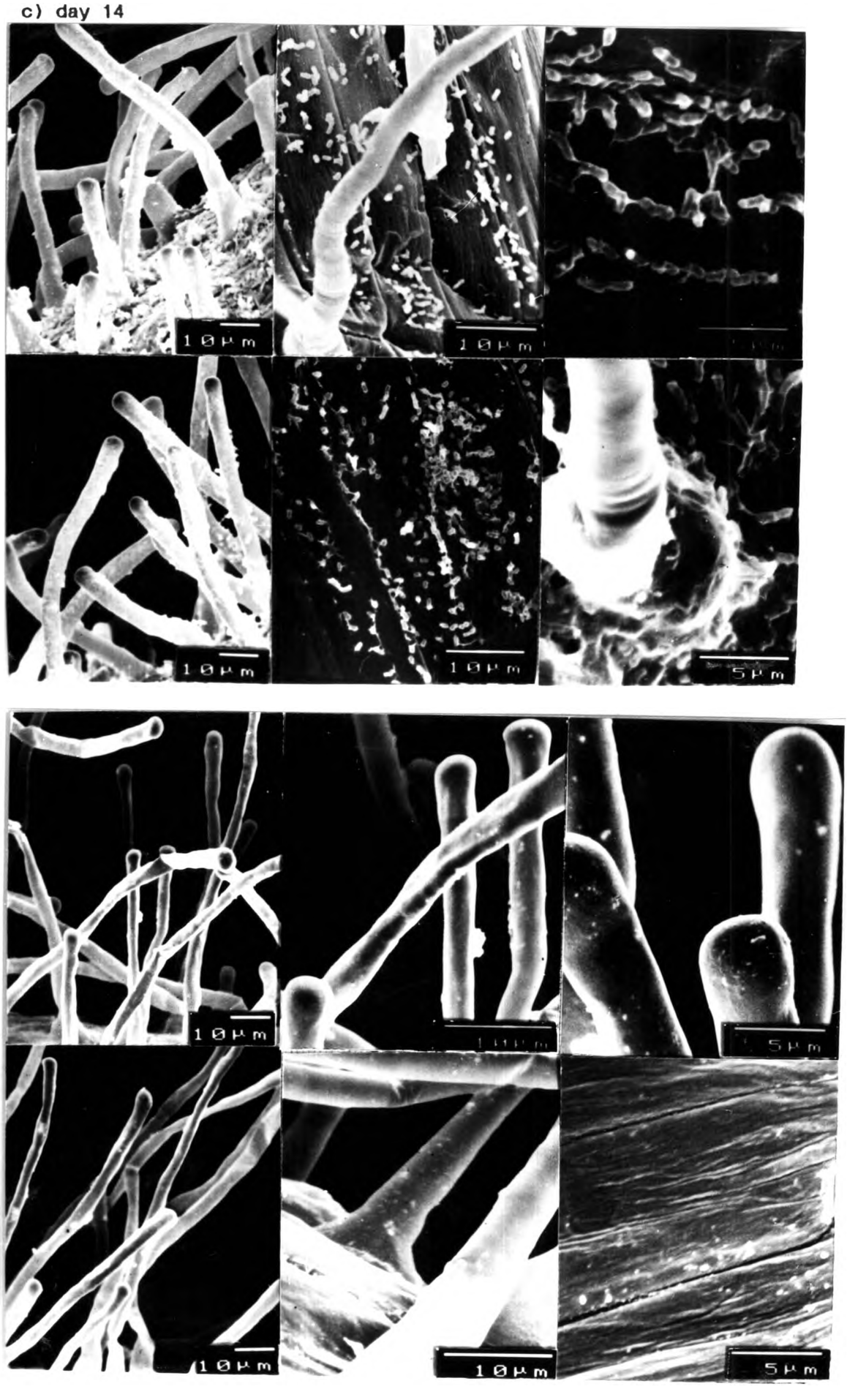
a) day 0



b) day 7



c) day 14



d) day 21

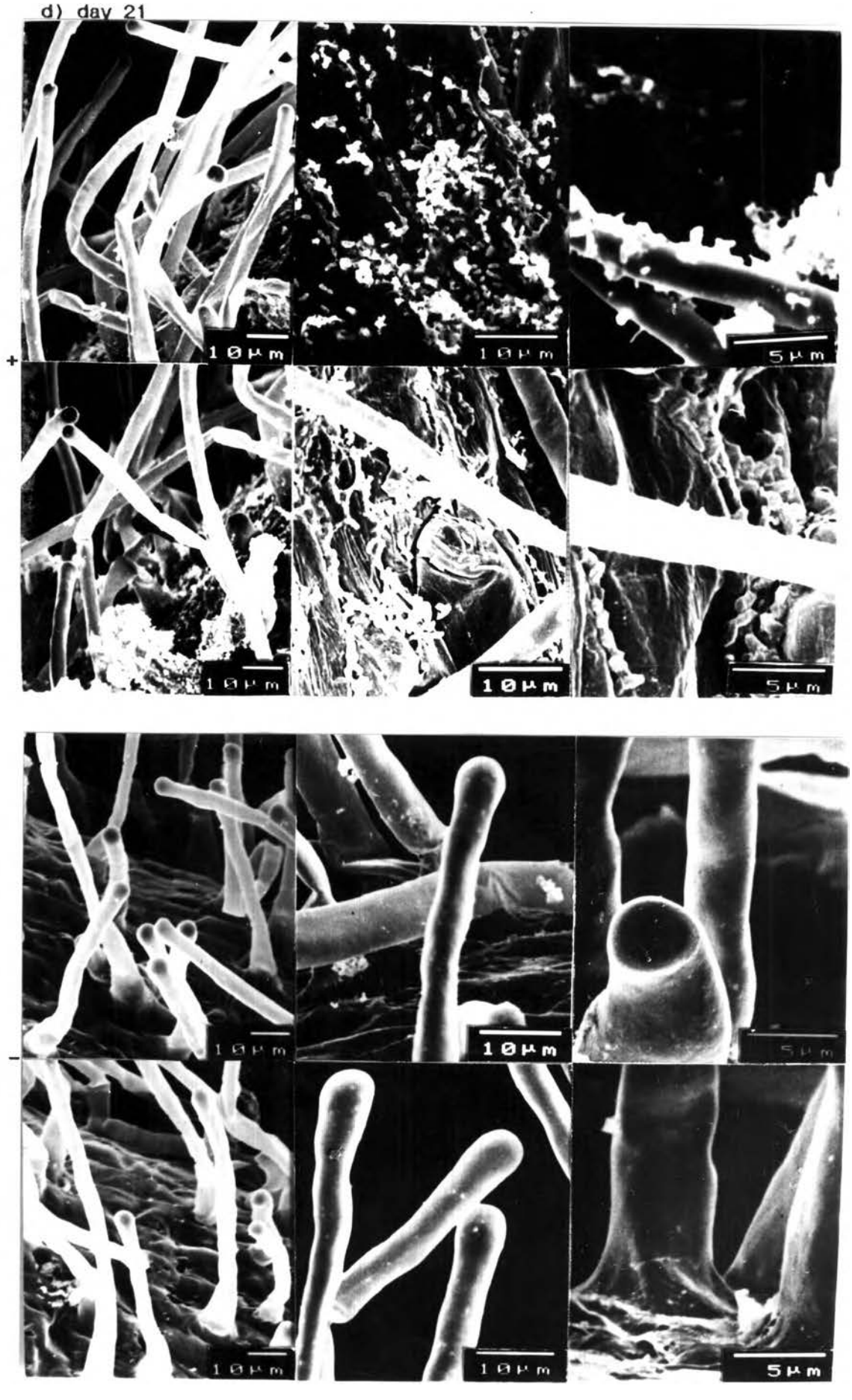
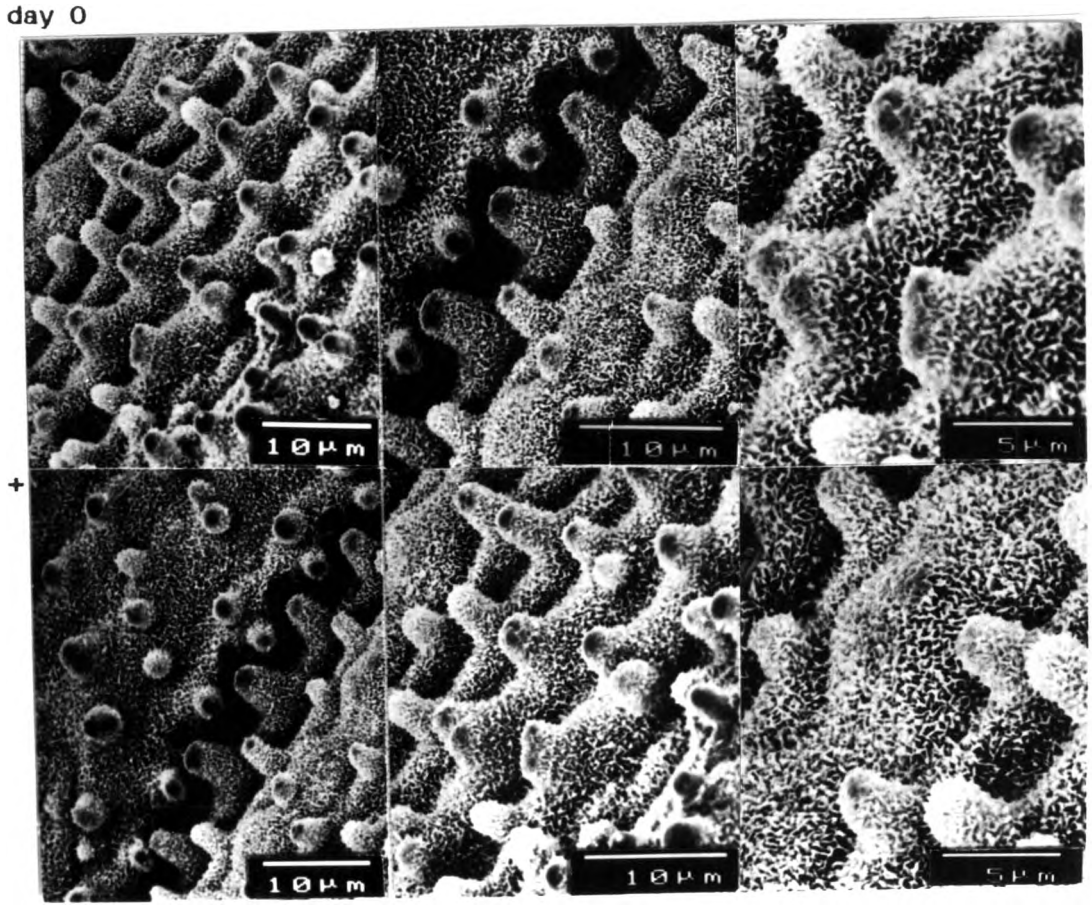
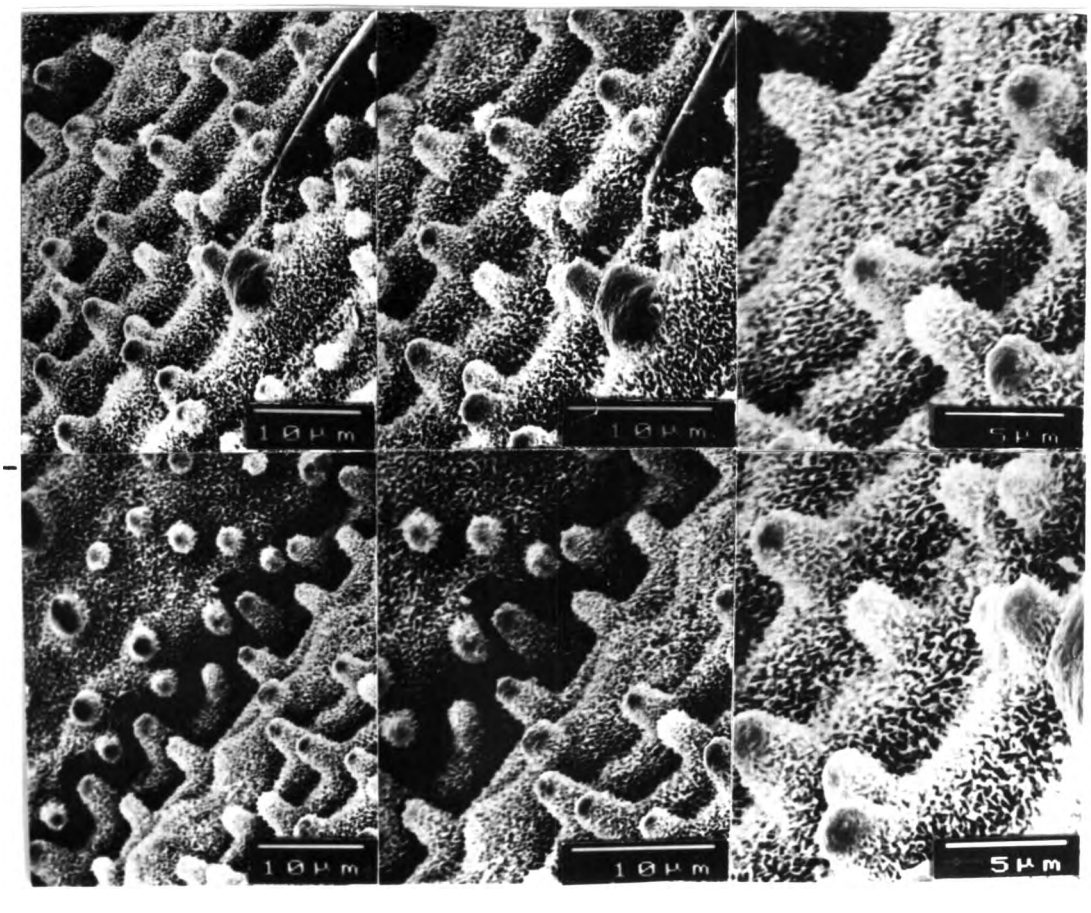


Figure 3.4 The ultrastructure of leaves of inoculated (+) and non-inoculated (-) rice.

day 0

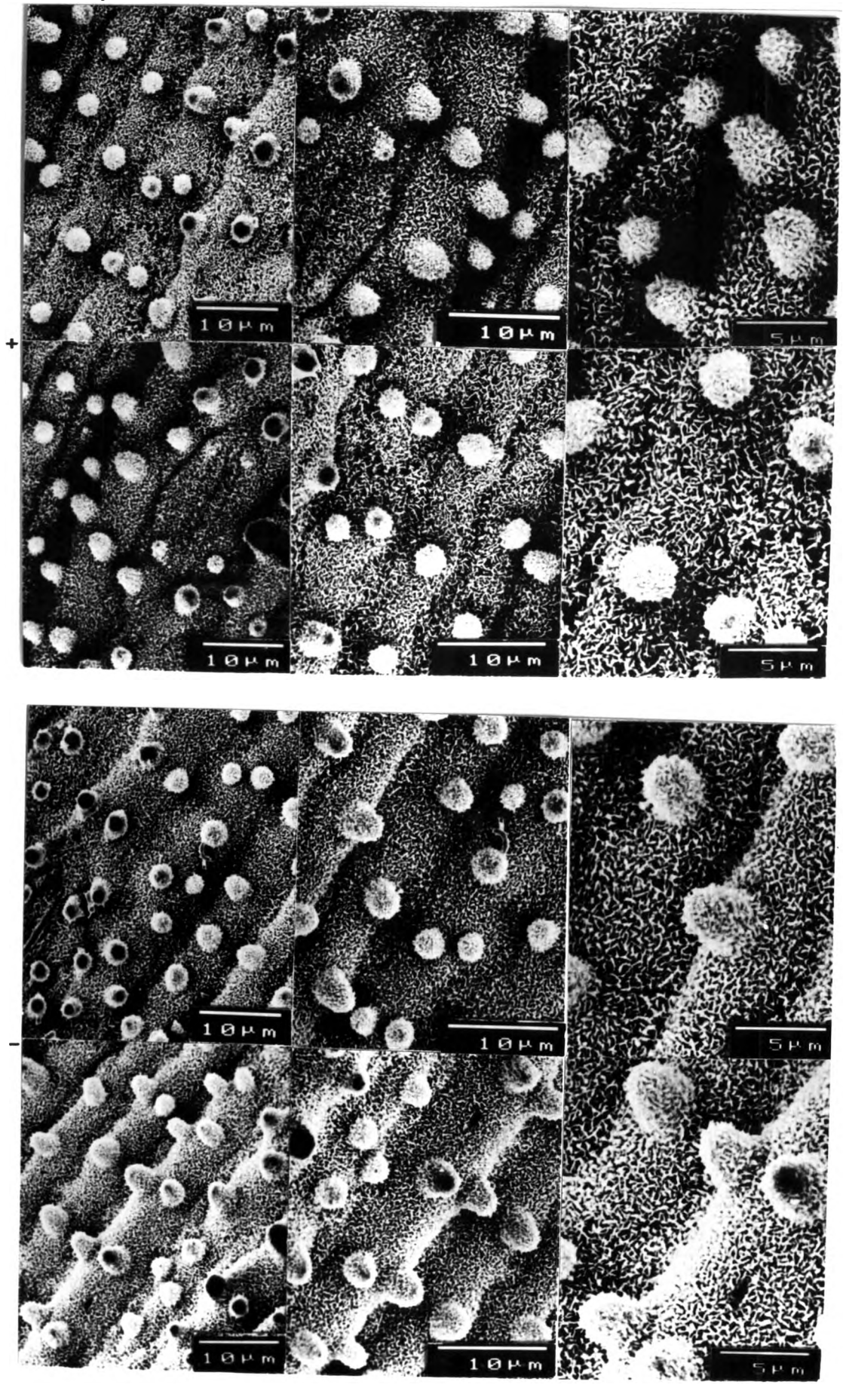


+

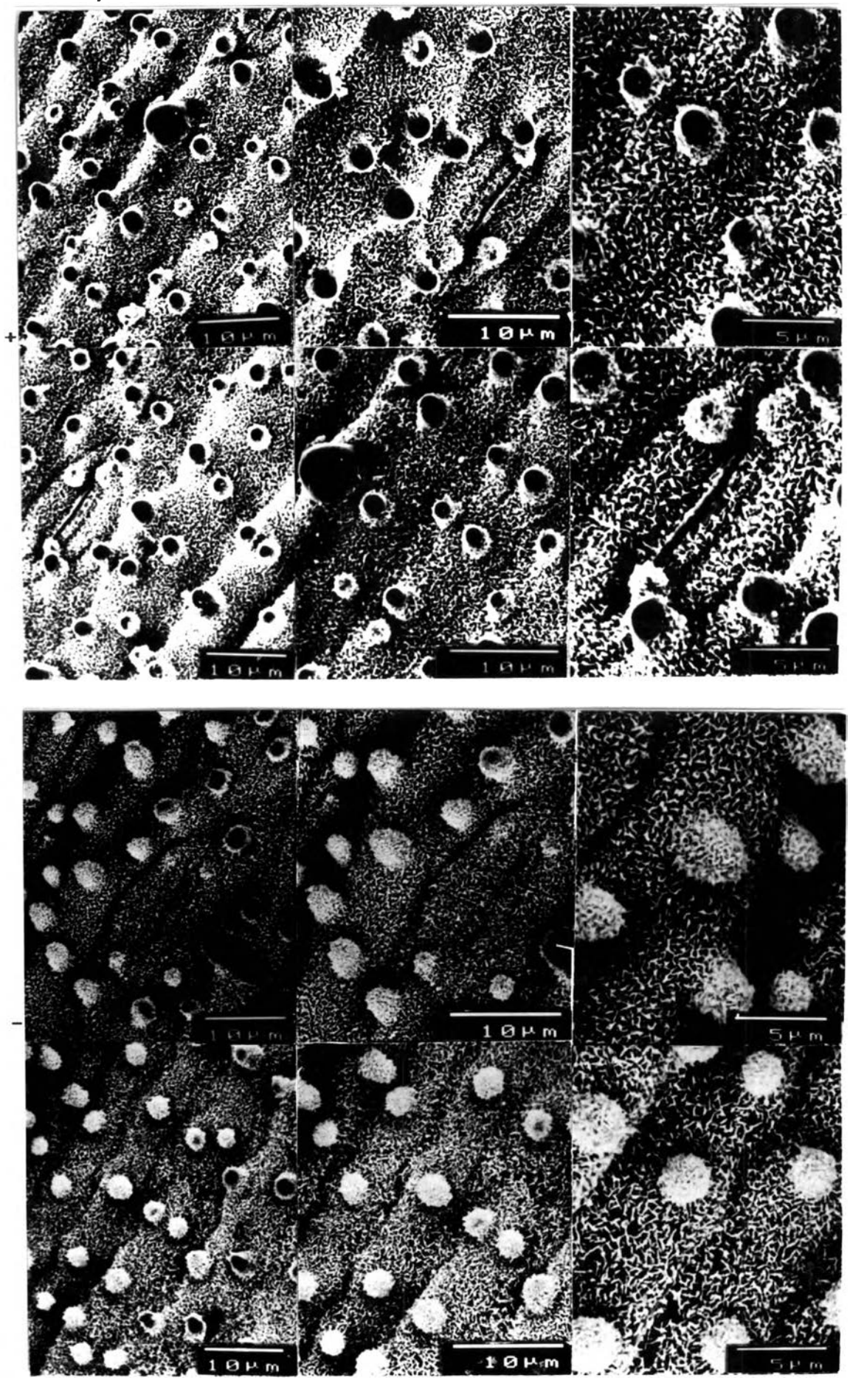


-

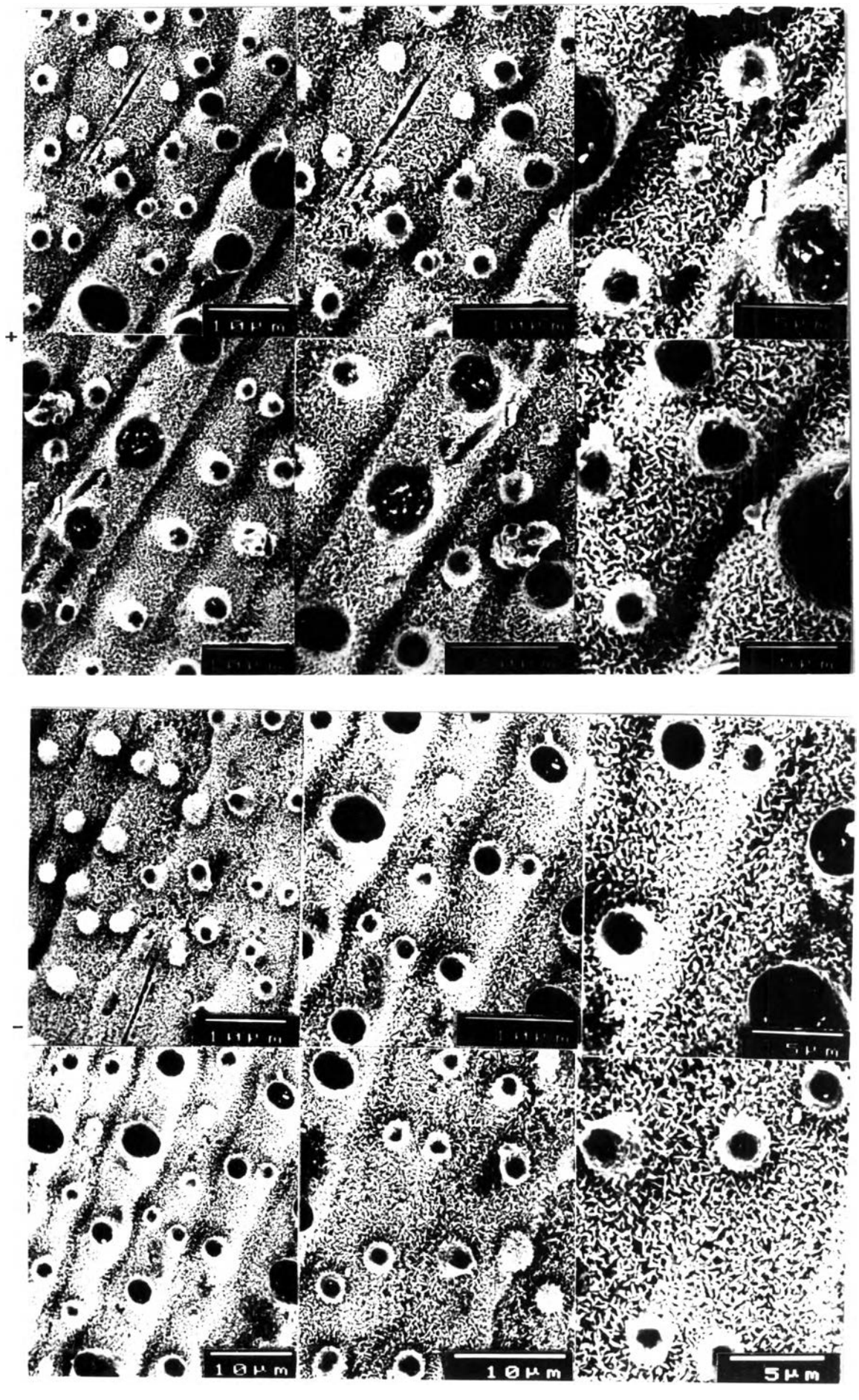
b) day 7



c) day 14



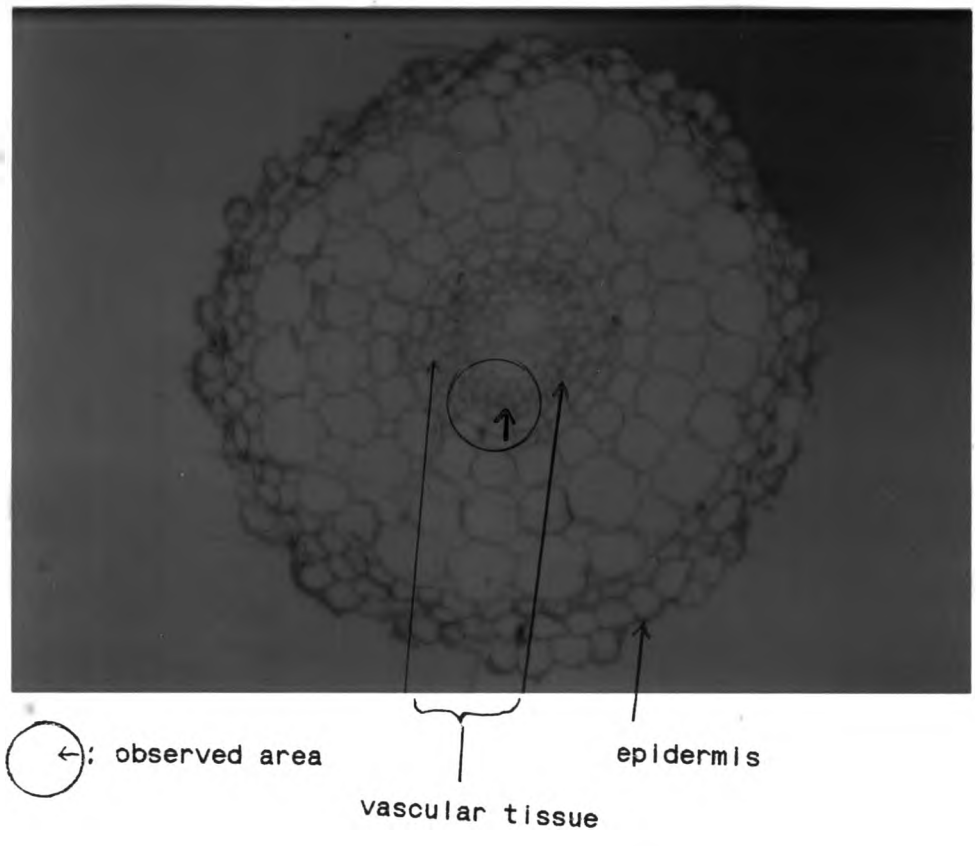
d) day 21



3.3 Distribution of GS in leaf and root of rice with and without R15 inoculation.

The distribution of cytosolic GS in non-inoculated and inoculated rice root and leaf were studied by immunogold-protein A labelling technique, every 7 days interval, from day 0 to day 21 after inoculation. The distribution of gold particle was observed under transmission electron microscope(TEM) from the epidermis to vascular tissue. Having a concentration in this site because recent studies by Yamaya *et al.*, 1992 and Kamachi *et al.*, 1992 show that GS in rice leaf distributed in this site. The significant increasing of a number of gold particle was observed only in the vascular tissue of inoculated rice's leaf on day 7-21 after inoculation. The results shown in table 3.1. and figure 3.7, 3.8. No gold particle was observed in control section, incubated with TBST, substition the anti maize GS (Figure 3.6).

Figure 3.5 Cross-section of root and leaf of rice.



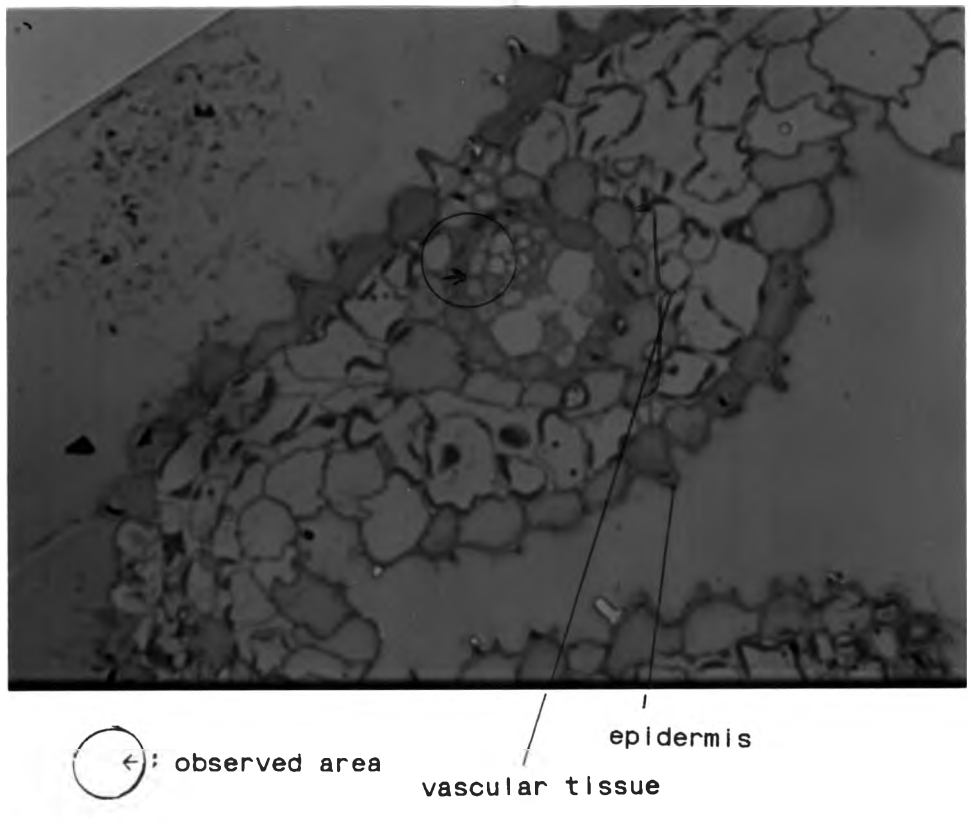
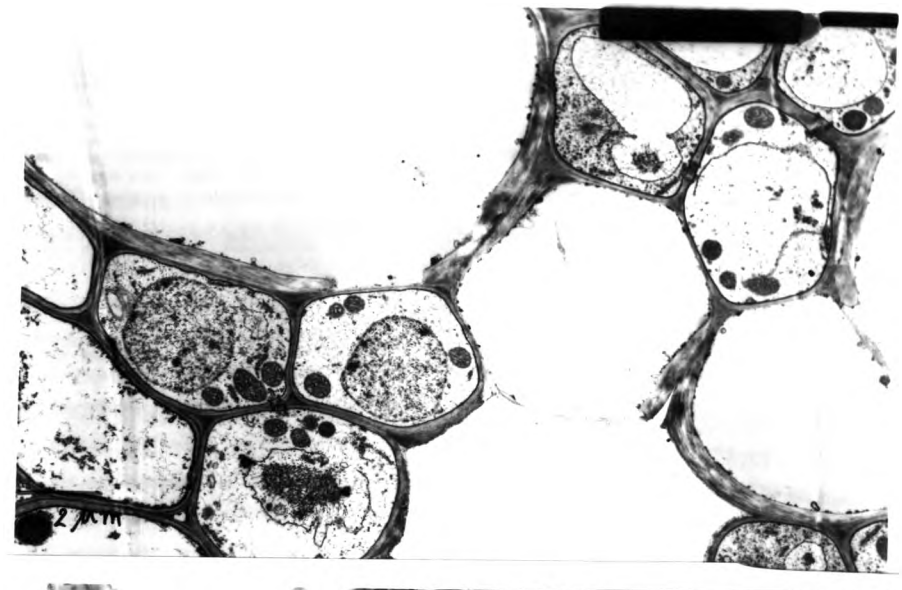
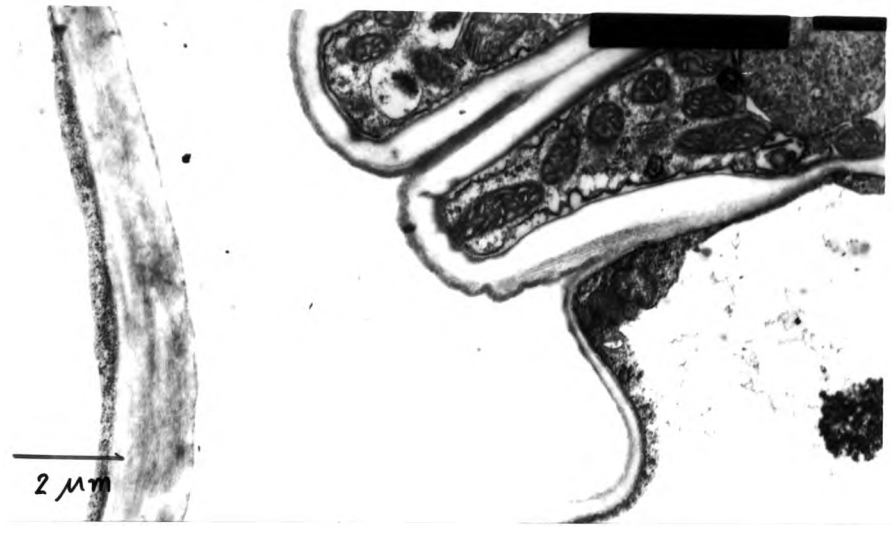
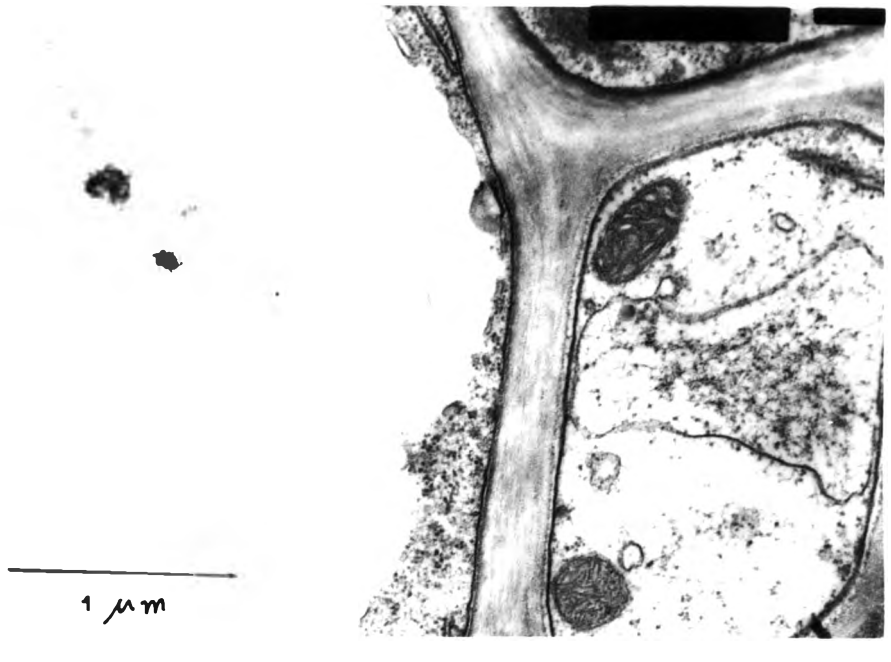


Figure 3.6 The control section of immuonogold-protein A staning

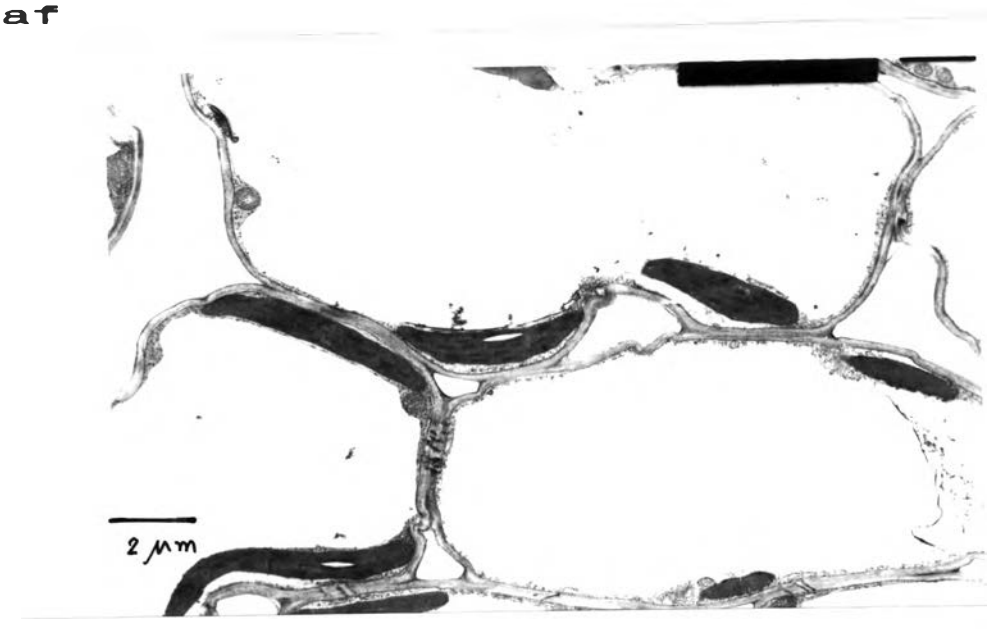
root

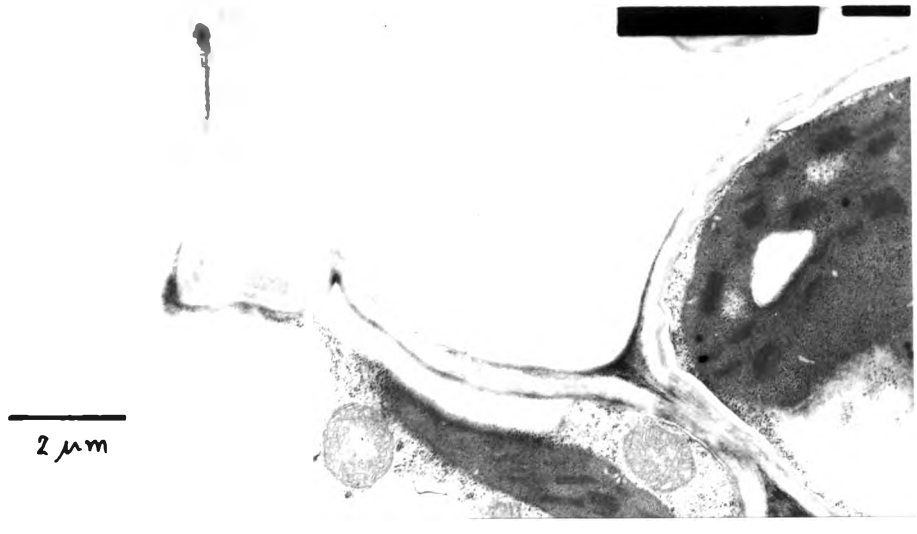


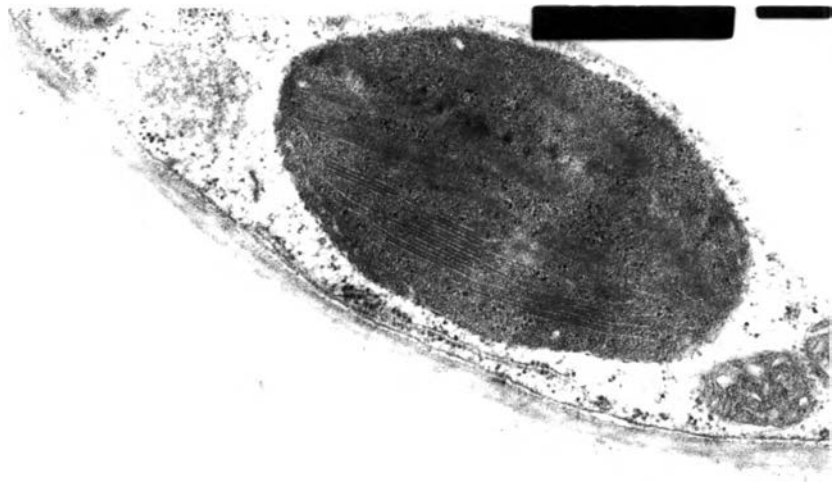




leaf







1 µm

source of GS	day after inoculation	The number of gold particle/cm² X 10²	
		leaf	root
RD7	0	21 ± 6	46 ± 6
RD7+R15		21 ± 6	48 ± 9
RD7	7	24 ± 9	45 ± 9
RD7+R15		73 ± 15	45 ± 6
RD7	14	24 ± 9	34 ± 6
RD7+R15		55 ± 12	31 ± 9
RD7	21	28 ± 6	34 ± 6
RD7+R15		55 ± 12	31 ± 9

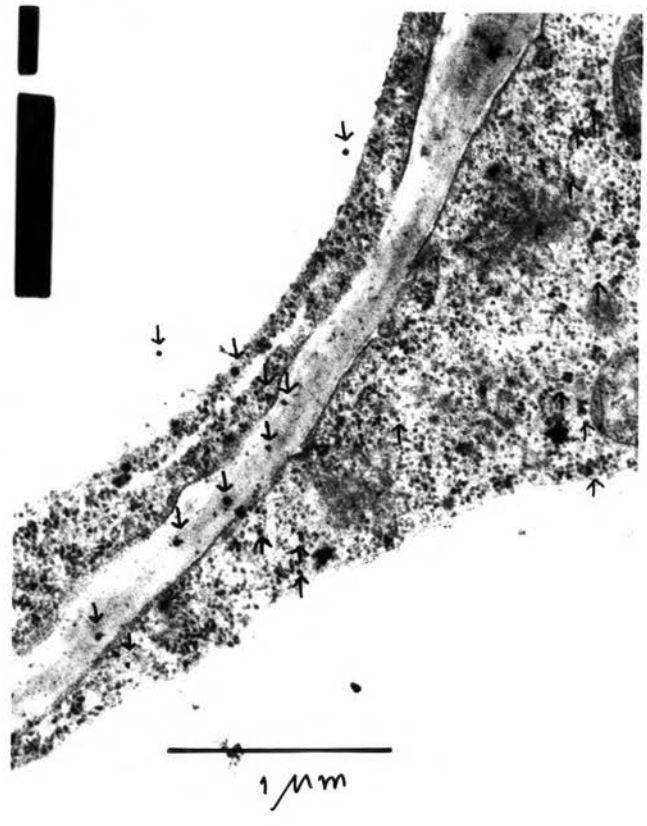
Table 3.1 The number of gold particles/cm², observed by TEM. Values presented are mean ± standard deviation of 50 fields repeated count at 28,000 X.

Figure 3.7 The distribution of GS enzyme in rice root and leaf, study by immunogold-protein A labelling technique.

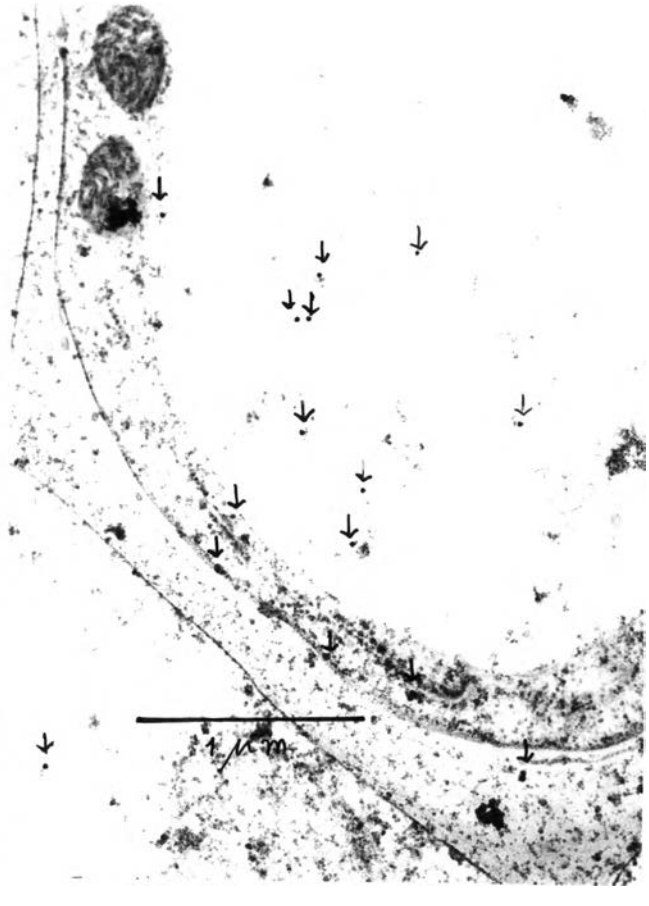
(-) : non-inoculated rice

(+) : inoculated rice

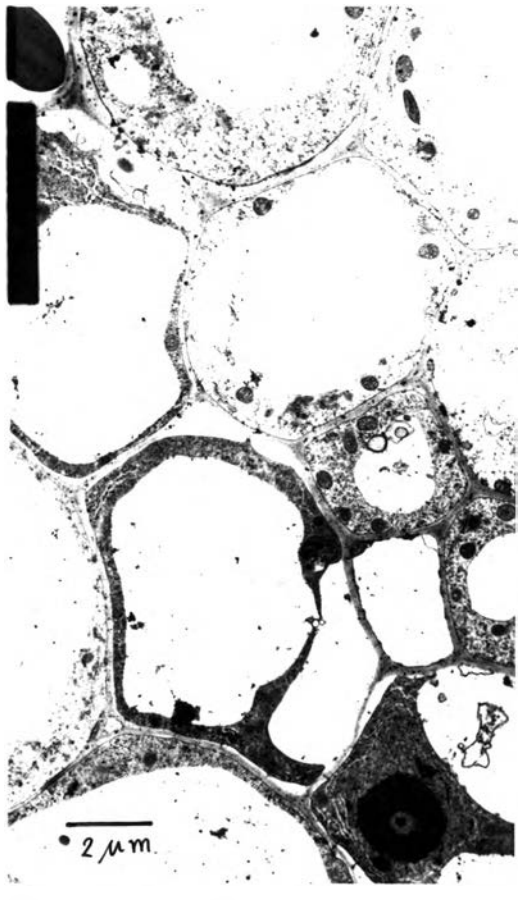
day 0 (+) root

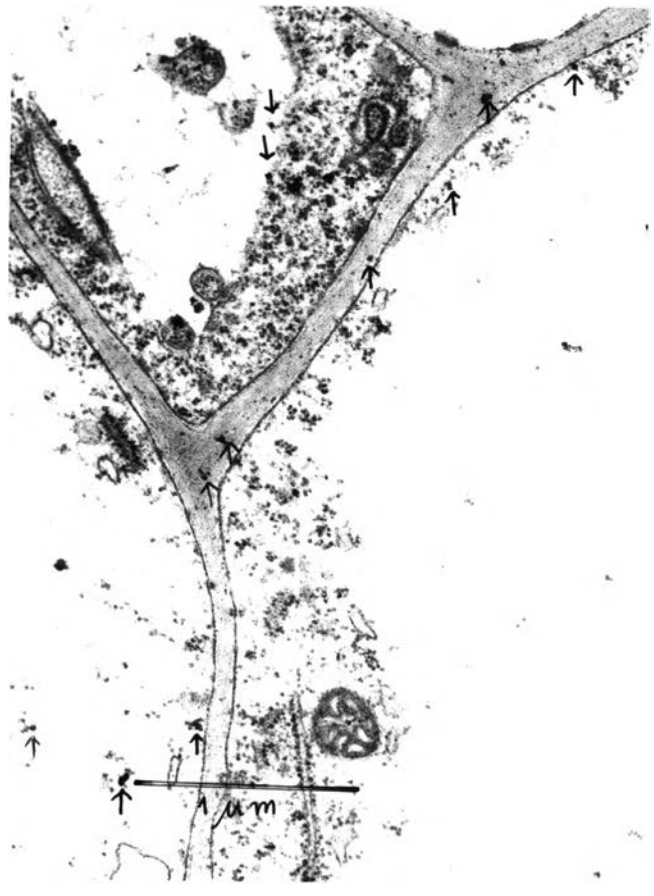


day 0 (-) root

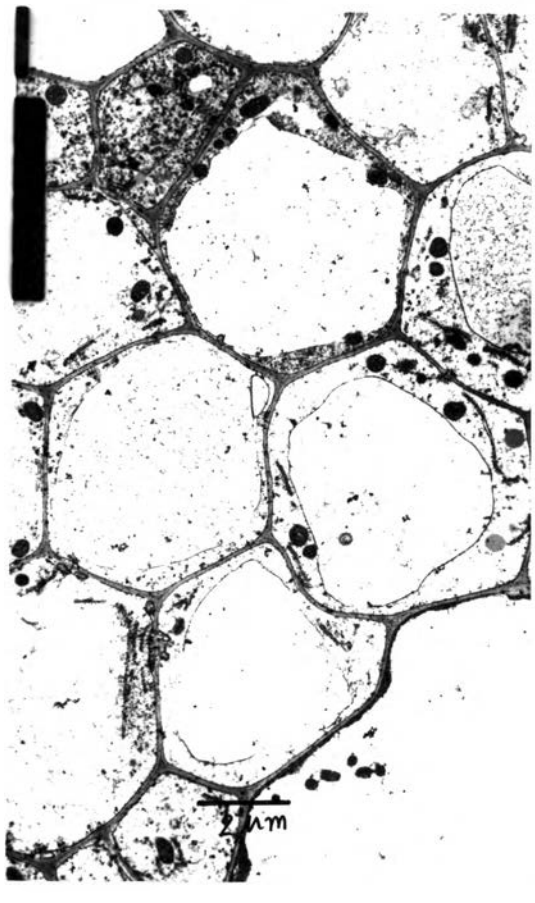


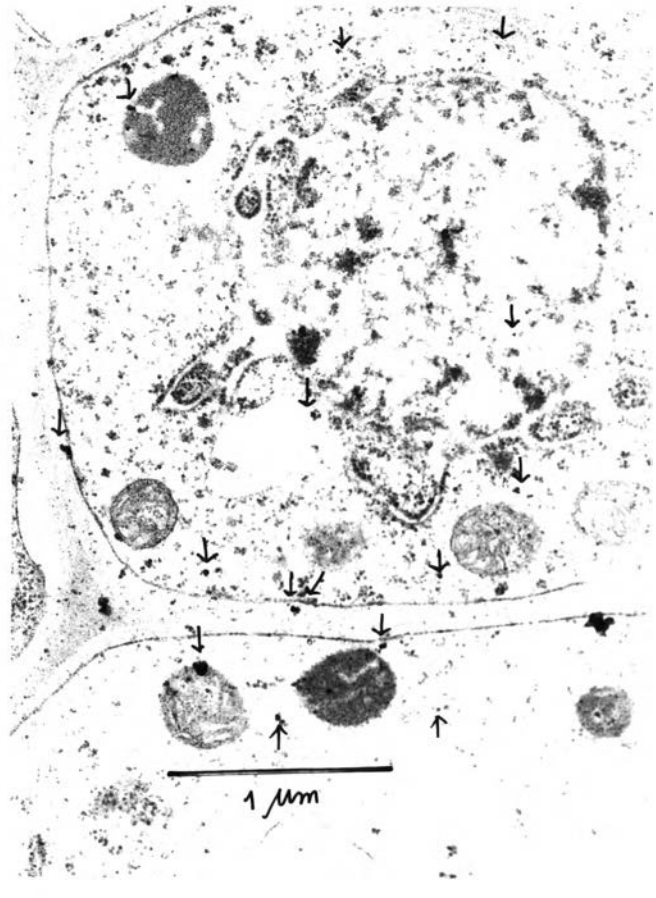
day 7 (+) root



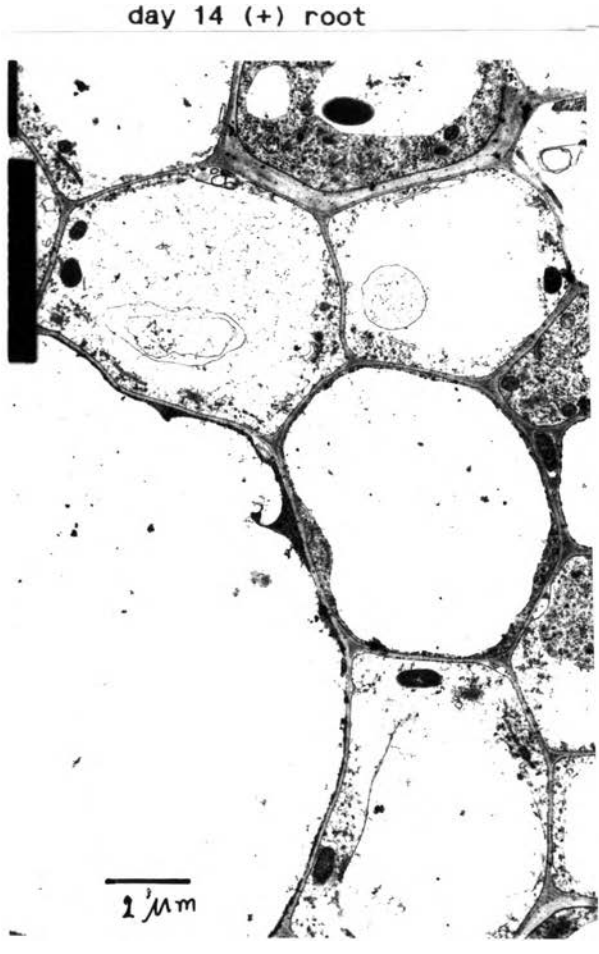


day 7 (-) root

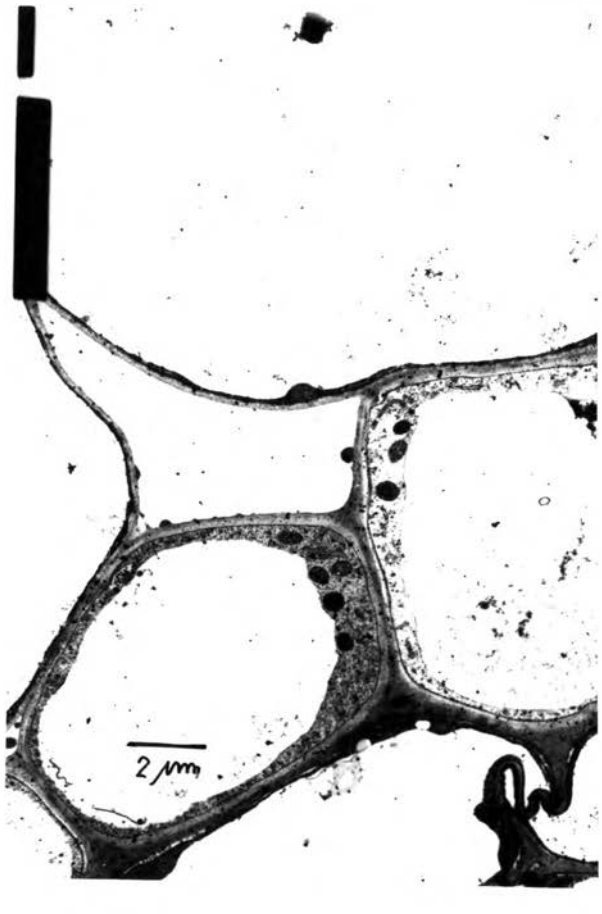


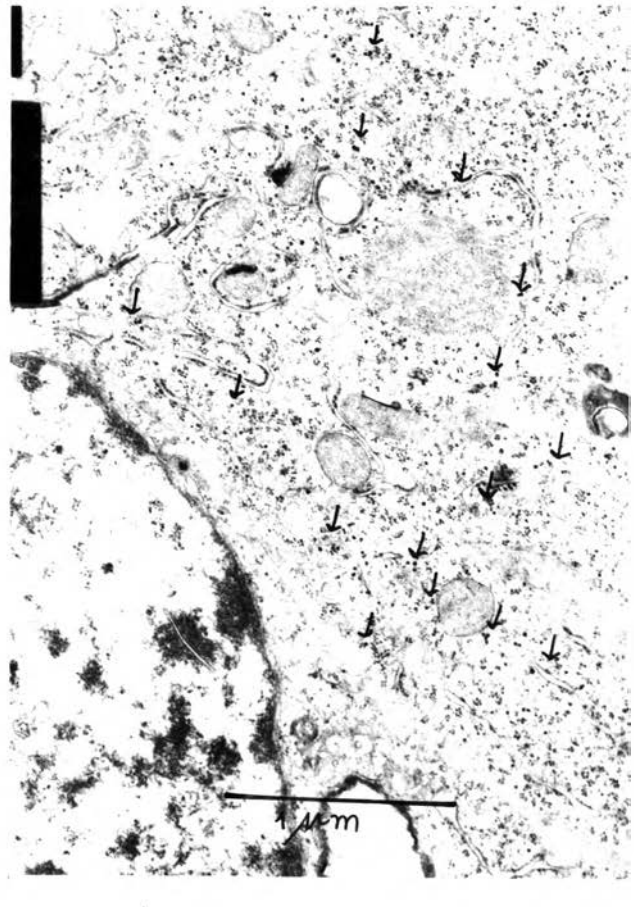


day 14 (+) root

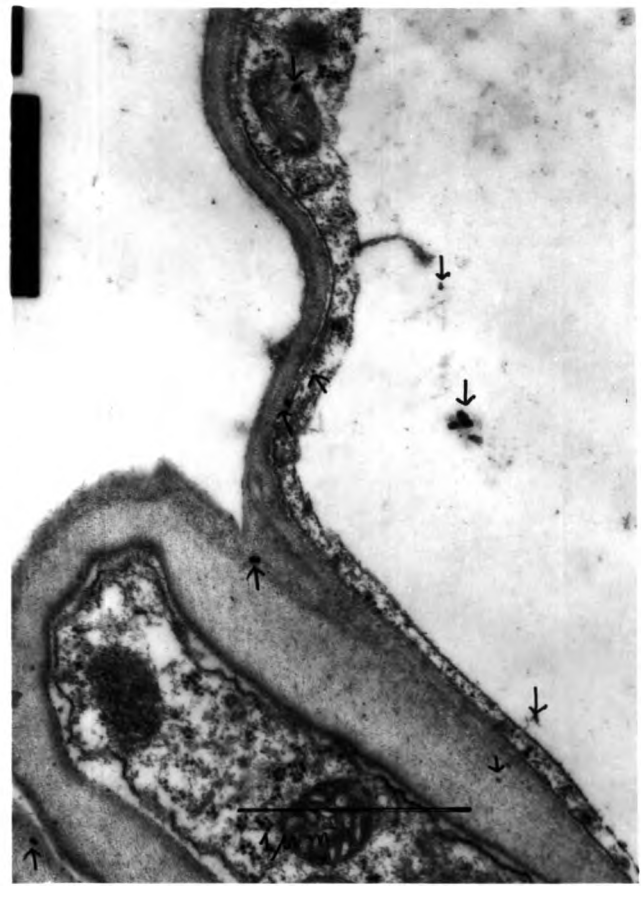


day 14 (-) root

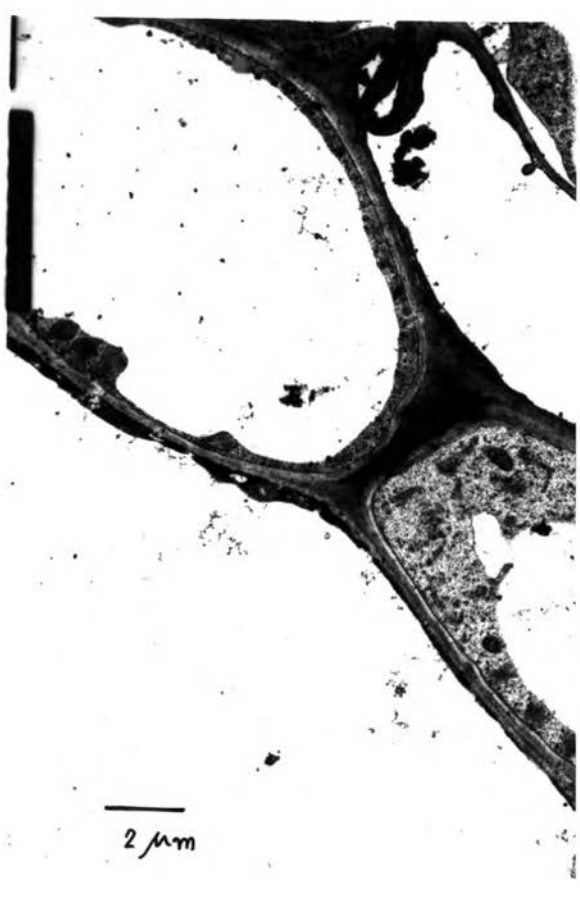




day 21 (+) root



day 21 (-) root



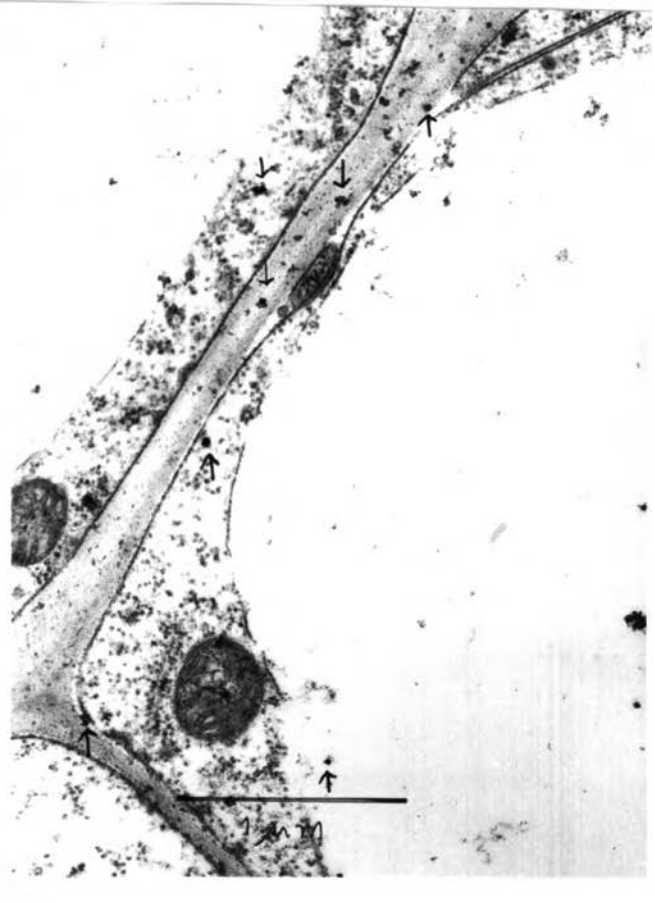
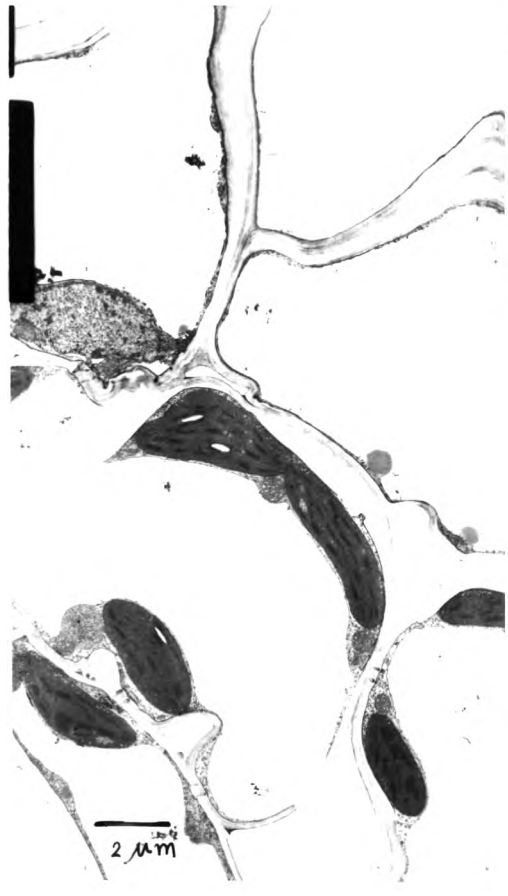


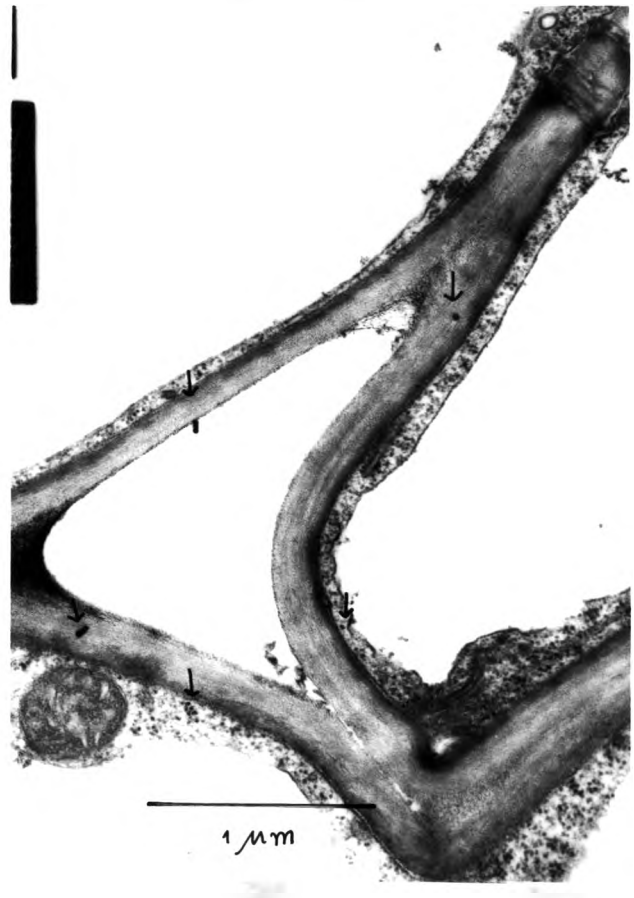
Figure 3.8 The distribution of GS enzyme in rice leaf, study by immunogold-protein A labelling technique.

(-) : non-inoculated rice

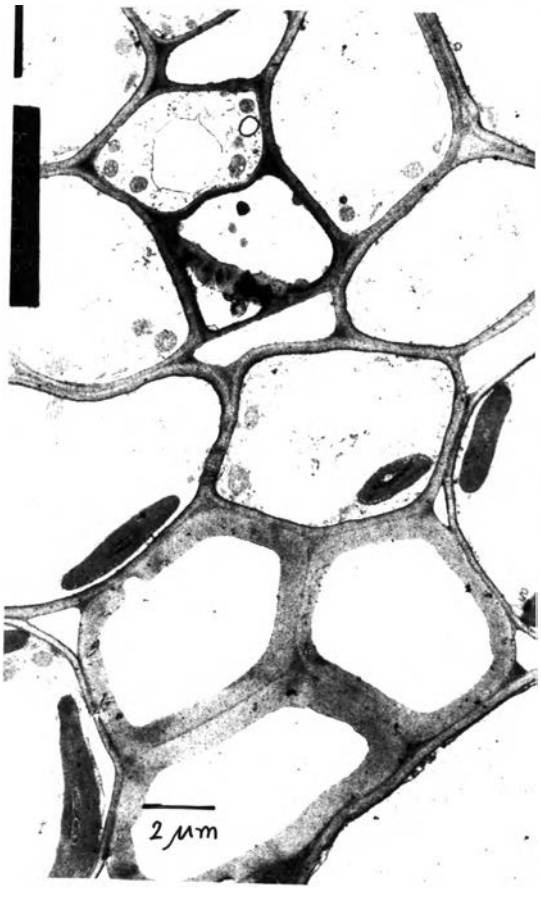
(+) : inoculated rice

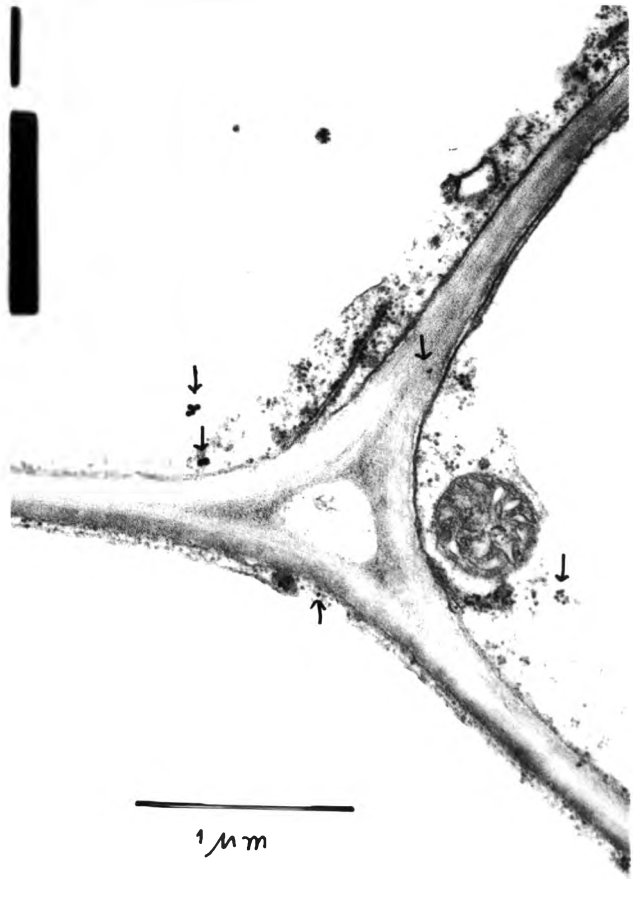
day 0 (+) leaf



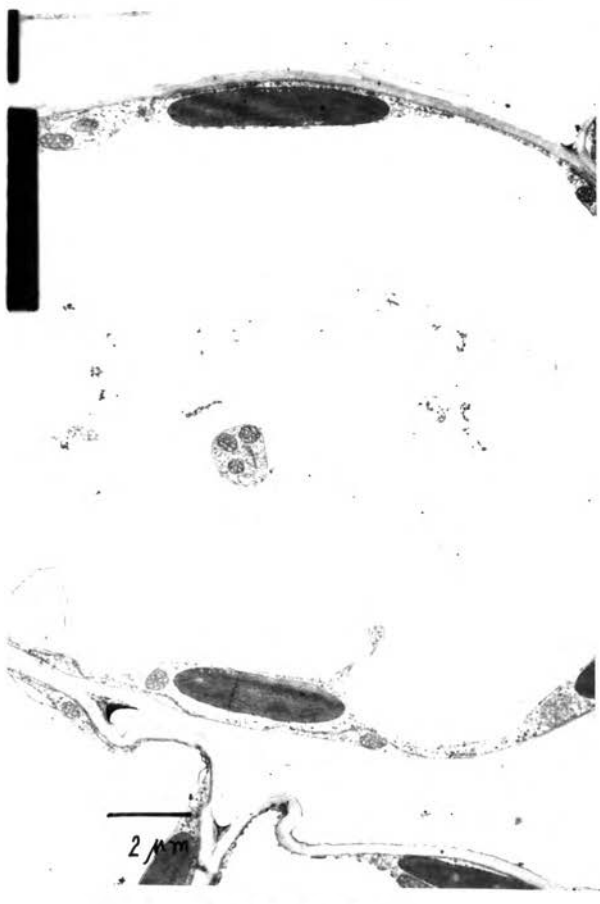


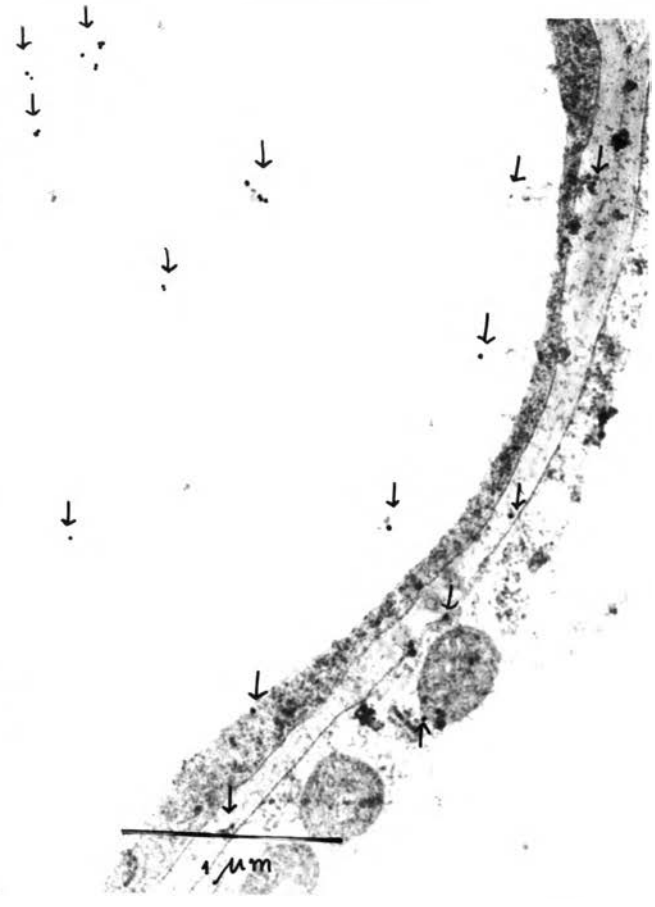
day 0 (-) leaf



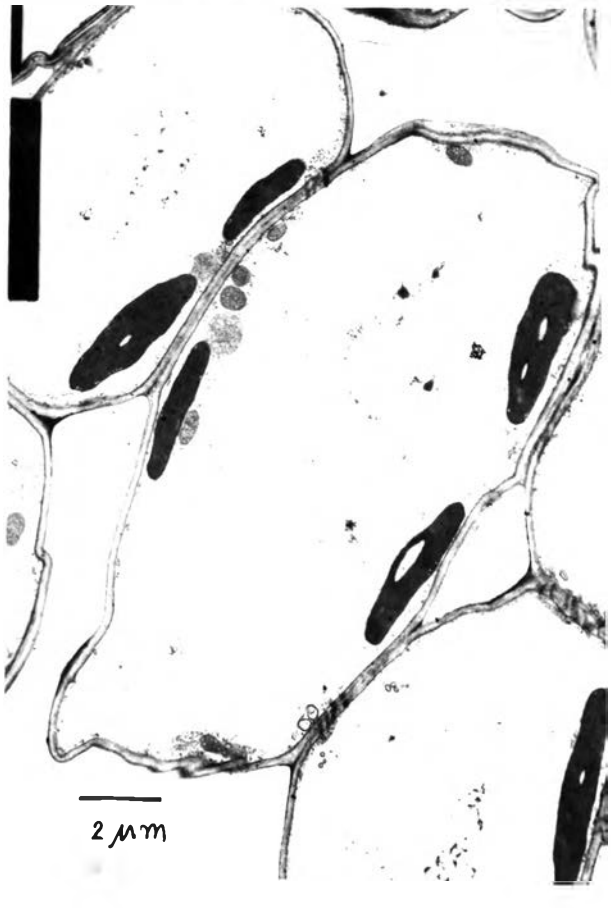


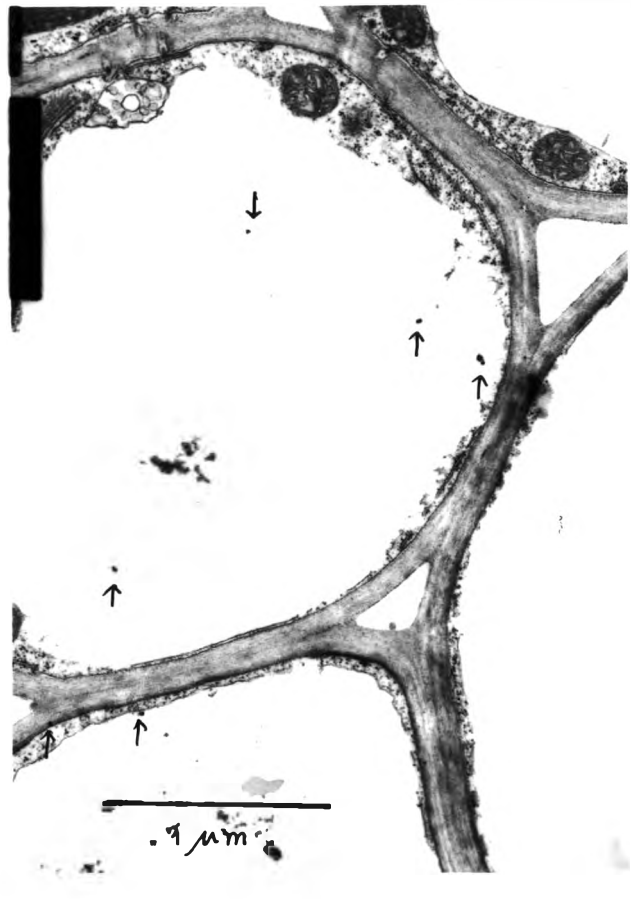
day 7 (+) leaf



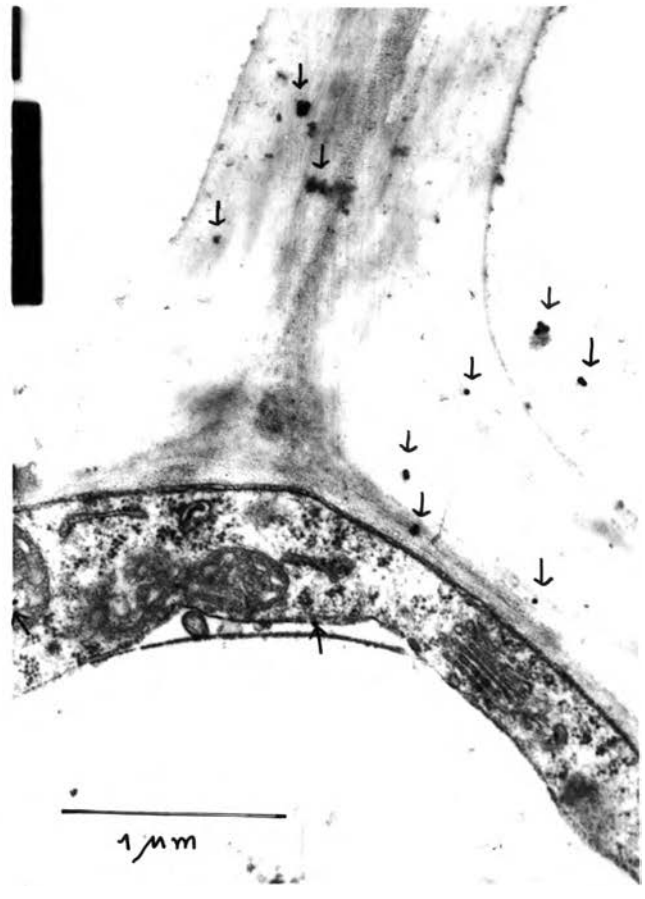


day 7 (-) leaf

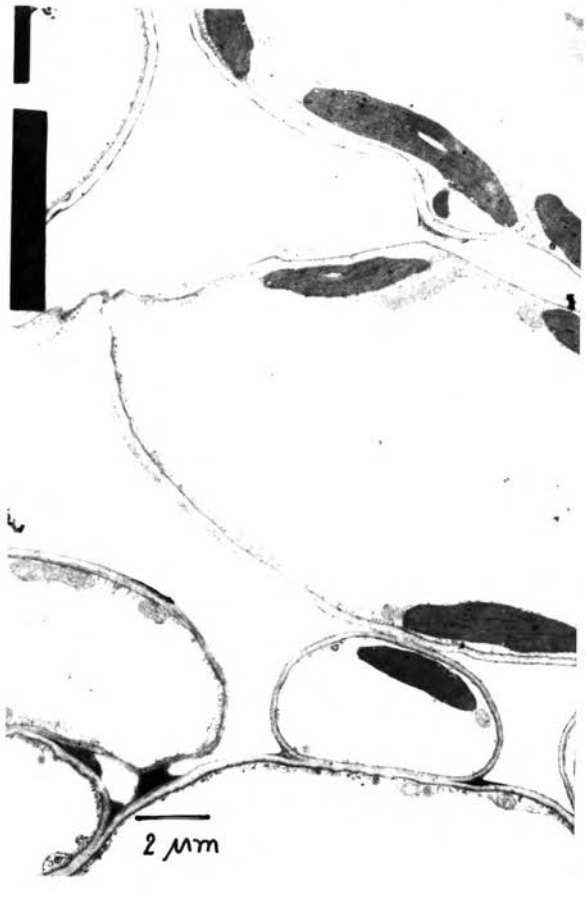


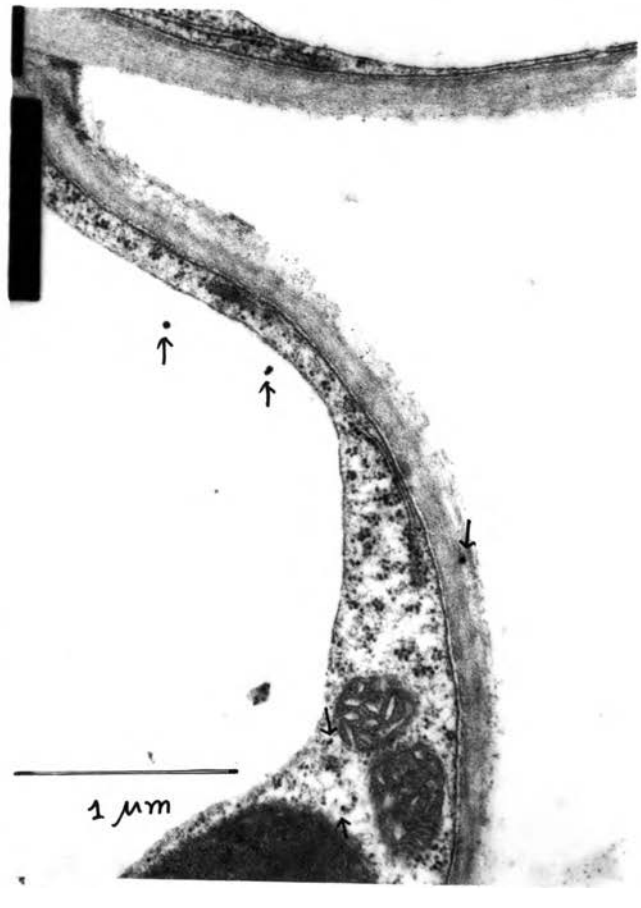


day 14 (+) leaf

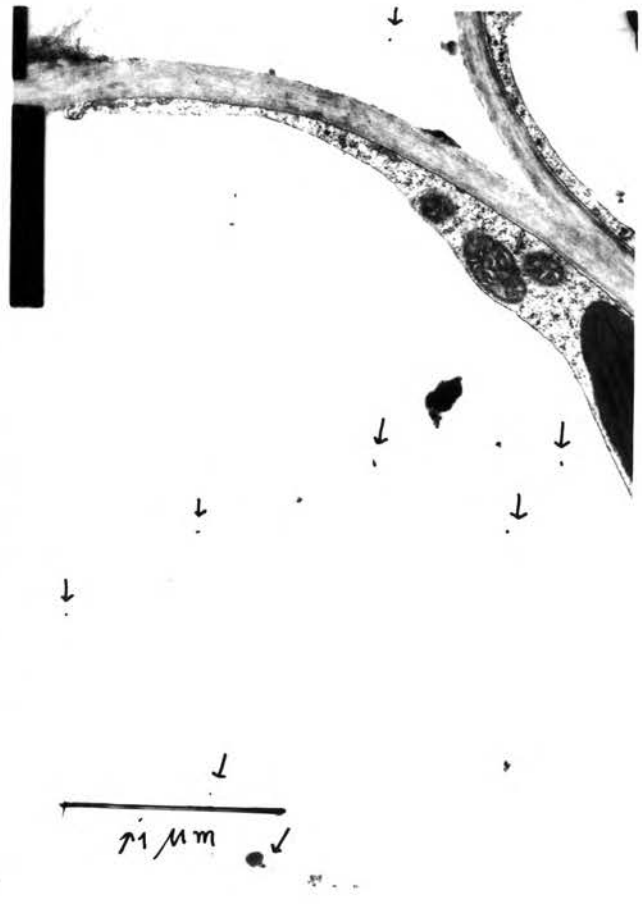


day 14 (-) leaf

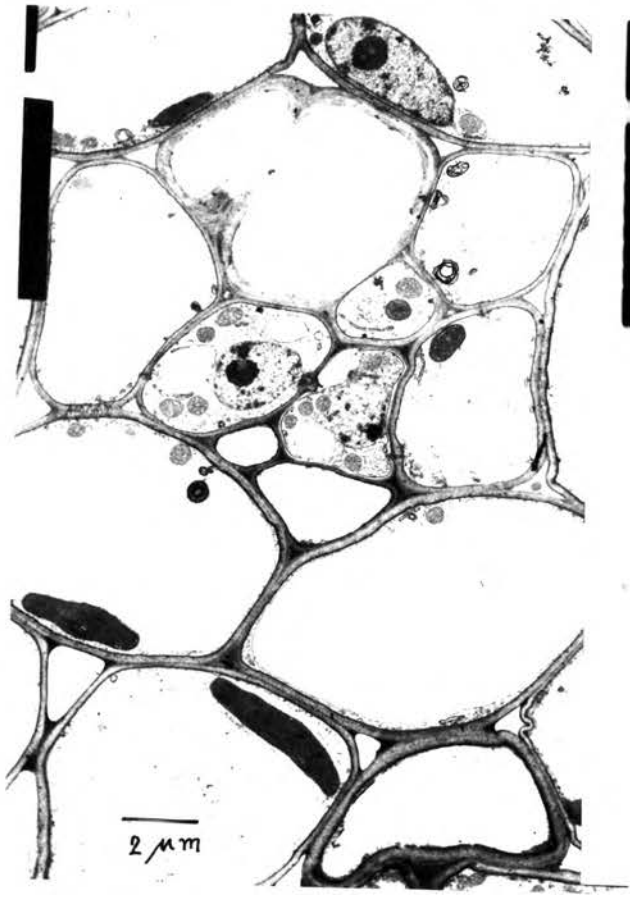


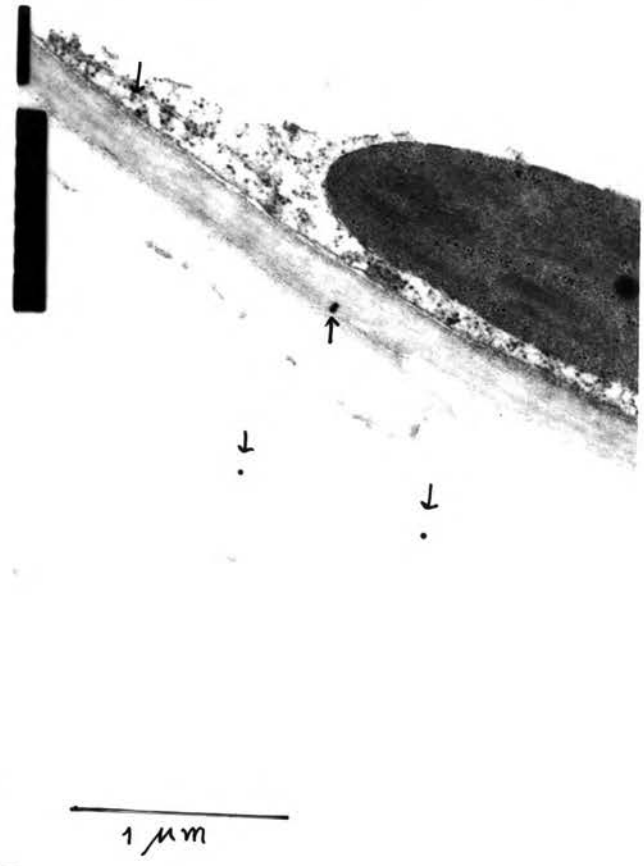


day 21 (+) leaf



day 21 (-) leaf





3.4 The effect of association on nitrogen-fixing activity and cell numbers of *K. oxytoca* R15

The nitrogen-fixing activity was measured by acetylene reduction assay(ARA) on day 0, 2, 4, 6, 8 and 10 after inoculation, using 4 sets of 10 replications 13 ml tube with 6 ml solution (5 ml Weaver medium + 1 ml of 0.85% NaCl or suspension of bacteria). Each set contained

(a) 0.85% NaCl

(b) non-inoculated rice plants (3 rice plants.tube^{-1})

(c) associative condition between rice-R15 (rice seedlings with *K. oxytoca* R15 10^8 cell.ml^{-1}.

(d) free-living *K. oxytoca* R15 (10^8 cell.tube^{-1}).

In free-living *K. oxytoca* R15 (d), the nitrogen-fixing activity on day 0 to day 10 was slightly increased from 7 nmole. tube^{-1}. day^{-1} to 15 nmole. tube^{-1}. day^{-1} on day 10. That similar to (b) the control non-inoculated rice that ethylene was increased from 6 nmole. tube^{-1}. day^{-1} to 18 nmole. tube^{-1}. day^{-1}. In associative *K. oxytoca* R15 and rice (c), the activity increased from 7 nmol on day 0 to 380 nmol on day 10 (about 50 fold) as shown in and figure 3.9.

By serial dilution and counting number of bacterial colonies in free-living and associative condition set (a) and (c), Figure

3.10 shown that in free-living condition the number of the bacteria remained rather constant ($4.8X10^{8}$ CFU.ml^{-1}) from day 0 to day 10, whereas, in associative condition, the number of the bacteria were significantly increased, about 60 fold on day 10 to $33.3X10^{7}$ CFU.ml^{-1}.

Figure 3.9 The nitrogen-fixing activity in various conditions.

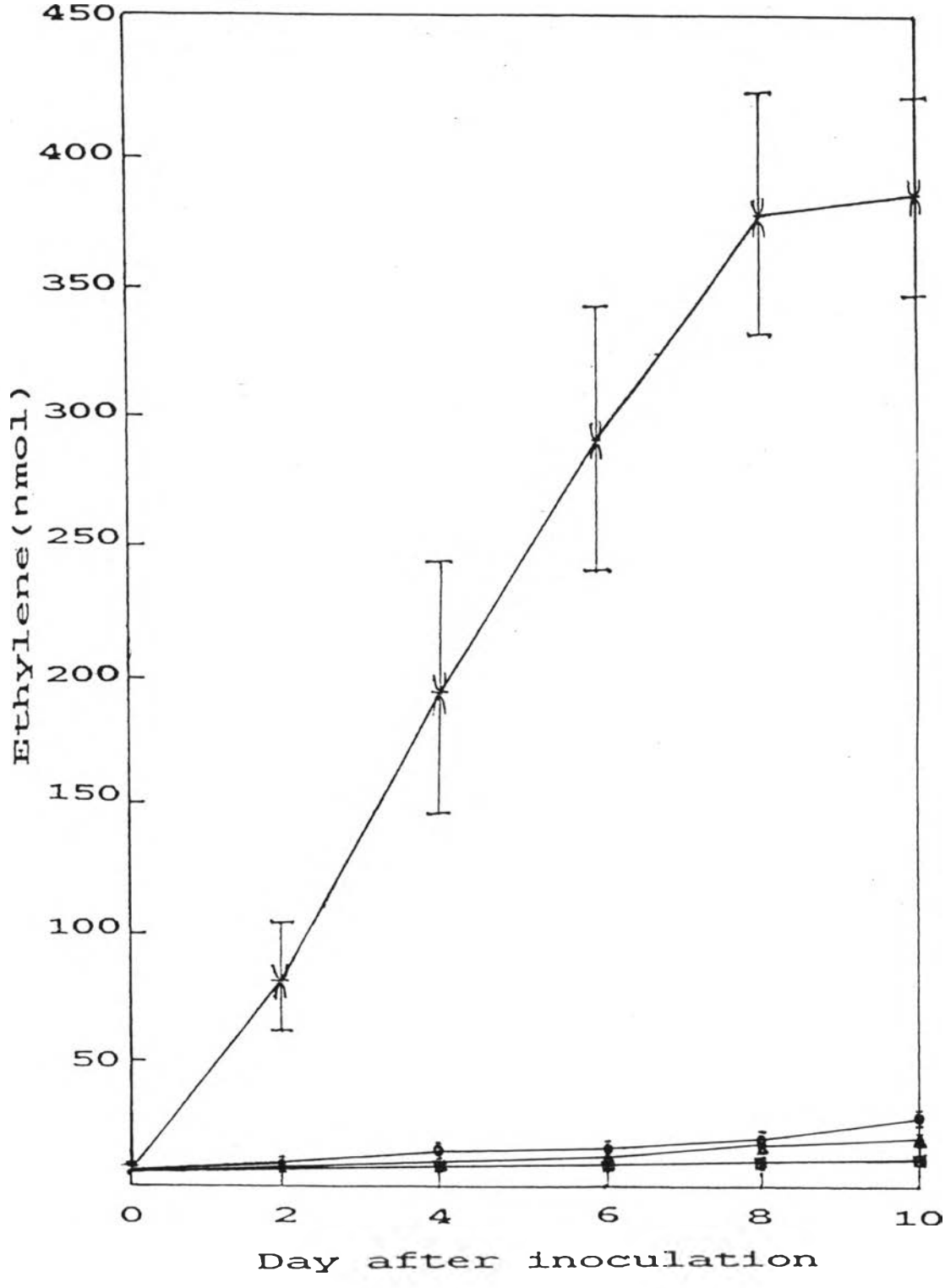
⊕ : control 0.85 NaCl

▲ : non-inoculated rice plants (3 rice plant.tube^{-1})

✱ : associative condition between rice and R15, 10^8 cell.ml^{-1}

● : free-living *Klebsiella oxytoca* R15

Values presented are mean ± standard deviation of 9 repeated experiments.


450
400
350
300
250
200
150
100
50
Ethylene (nmol)
0
2
4
6
8
10
Day after inoculation

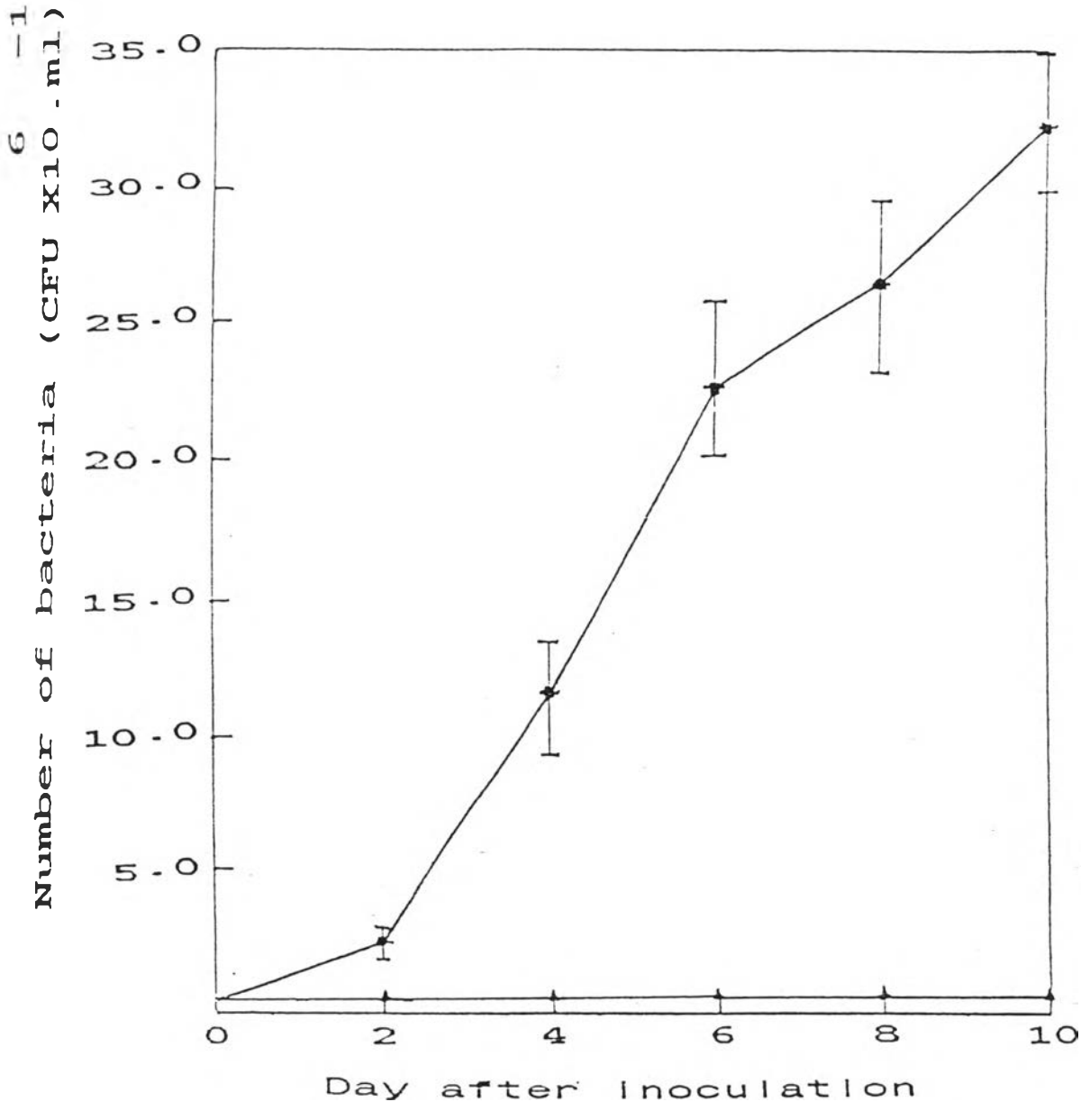


Figure 3.10 The number of bacteria (CFU X 10^6. ml^{-1})

✱ associative condition

▲ free-living condition

Values presented are mean ± standard deviation of 9 repeated experiments.

3.5 Effect of *K oxytoca* R15 inoculation on root and leaf GS specific activity and total proteins

The effect of *K. oxytoca* R15 inoculation on roots and leaves GS specific activity, determined according to Farnden and Robertson (1980), and total proteins, determined according to Bradford (1976), were studied. GS determination was performed interval from day 0 until day 21 after inoculation, when the rice plant were 28 days old. The inoculation of rice seedling with *K. oxytoca* R15 resulted in an increase of the GS specific activity of rice leaves, about 30%, since day 7 after inoculation but not in the roots and not for the total proteins of both rice roots and leaves. In associative condition, GS specific activity of rice leaves was sharply increased between day 0 to day 7 (0.11 to 0.24 μmol. mg.protein^{-1}. min^{-1}) and day 7 to day 14 (0.24 to 0.29 μmol. mg.protein^{-1} .min^{-1}). The activity on day 21 slightly decreased comparing with day 14 (0.29 to 0.26 μmol.mg.protein^{-1}. min^{-1}). The increasing pattern of total proteins of rice roots and leaves were similar to the increasing pattern of GS specific activity but there was no significant difference between the amount of total proteins in non-inoculated and associative rice plants (Table 3.2 and figure 3.11.

In addition, the study of GS activity by activity staining on

native gel electrophoresis shows similar results to the quantitative determination of GS specific activity, at all time intervals after R15 inoculation, the band of GS activity of associative rice leaf were more intense than those of non-inoculated bands except on day 0 after inoculation. In contrast, the GS activity stained bands of inoculated and non-inoculated rice roots show the same intensity (lane 5,6 and 7,8 of figure 3.12).

Source of GS	Day	Specific activity*		GS Ratio		Total cytosolic protein(mg)		Protein Ratio	
		Leaves (L)	Roots(R)	L	R	L	R	L	R
RD7	0	0.108± 0.024	0.093± 0.022	-	-	8.13± 1.13	5.40± 1.04	-	-
RD7+R15		0.109± 0.013	0.092± 0.024	-	-	8.21±1.27	5.35± 0.95	-	-
RD7	7	0.189± 0.019	0.205± 0.031	1.00	1.00	12.00± 0.98	7.70± 1.03	1.00	1.00
RD7+R15		0.242± 0.014	0.201± 0.026	1.28★	0.98	12.77± 1.00	7.89± 1.02	1.06	1.02
RD7	14	0.218± 0.037	0.189± 0.032	1.00	1.00	12.80± 2.64	7.39± 1.13	1.00	1.00
RD7+R15		0.291± 0.049	0.188± 0.025	1.33★	0.99	13.61± 2.44	7.33± 0.79	1.00	1.10
RD7	21	0.198± 0.030	0.203± 0.035	1.00	1.00	12.79± 3.57	6.91±0.16	1.00	1.00
RD7+R15		0.264± 0.022	0.193± 0.035	1.33★	0.95	13.49± 3.62	7.10± 1.26	1.05	1.03

Table 3.2 The average valves of GS specific activity versus total protein.

Values presented are mean ± standard deviation of 14 repeated experiments.

RD7 : non-inoculated rice

RD7+R15 : inoculated rice

* : (umol γ-Glutamyl hydroxamate).(mg protein)$^{-1}$.min^{-1}

★ : By t-test at 95% confident, significantly increase

Figure 3.11 The average value of GS specific activity comparing to cytosolic protein content.

**(glutamyl hydroxamate. mg protein^{-1}.min^{-1})

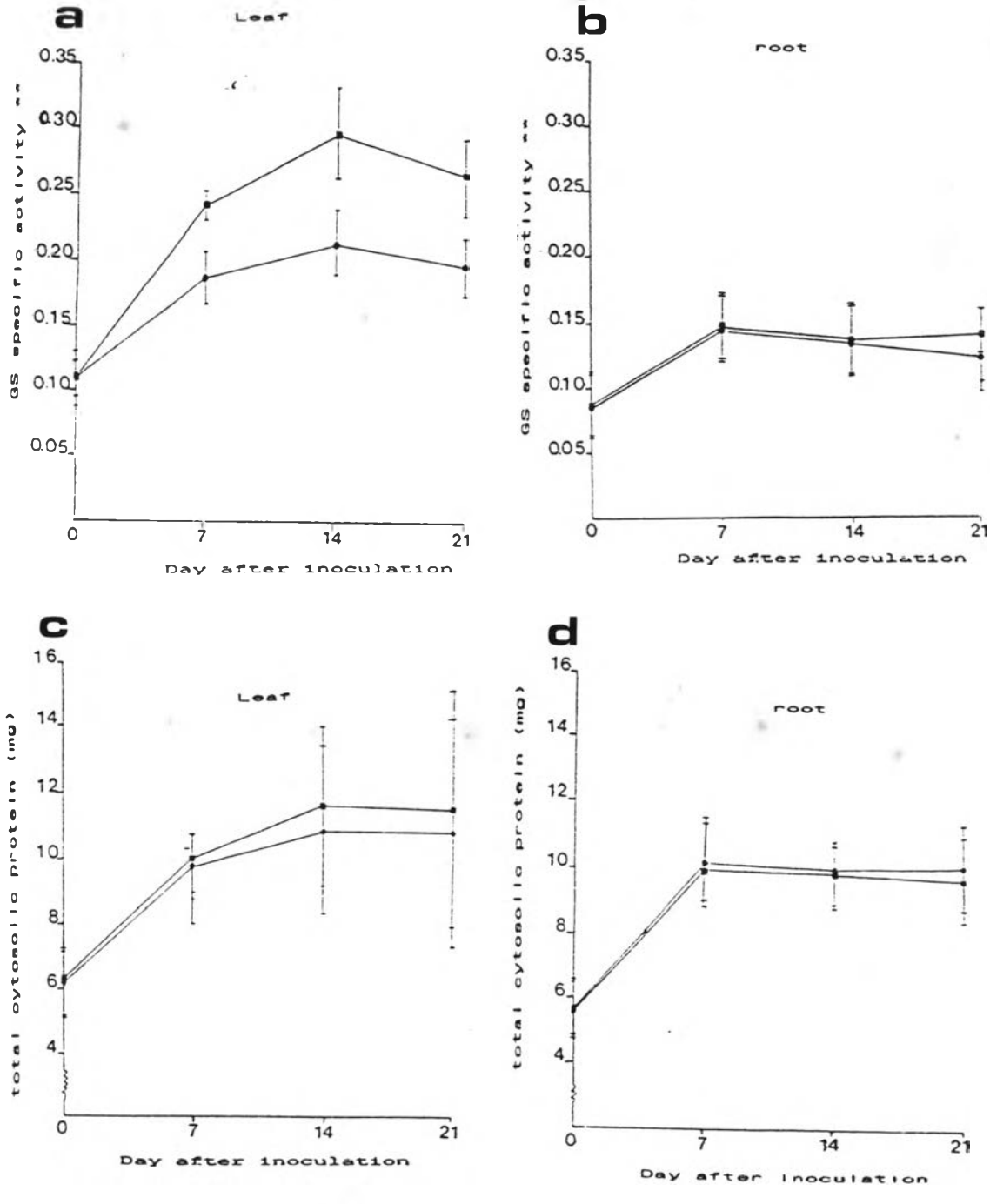
a,b : GS specific activity

c,d : total cytosolic protein (mg/100 plants)

■ : inoculated rice extract

● : non-inoculated rice extract

Values presented are mean ± standard deviation of 14 repeated experiments.


a
Leaf
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
7
14
21
Day after inoculation
b
root
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
7
14
21
Day after inoculation
c
Leaf
16
14
12
10
8
6
4
0
7
14
21
Day after inoculation
d
root
16
14
12
10
8
6
4
0
7
14
21
Day after inoculation

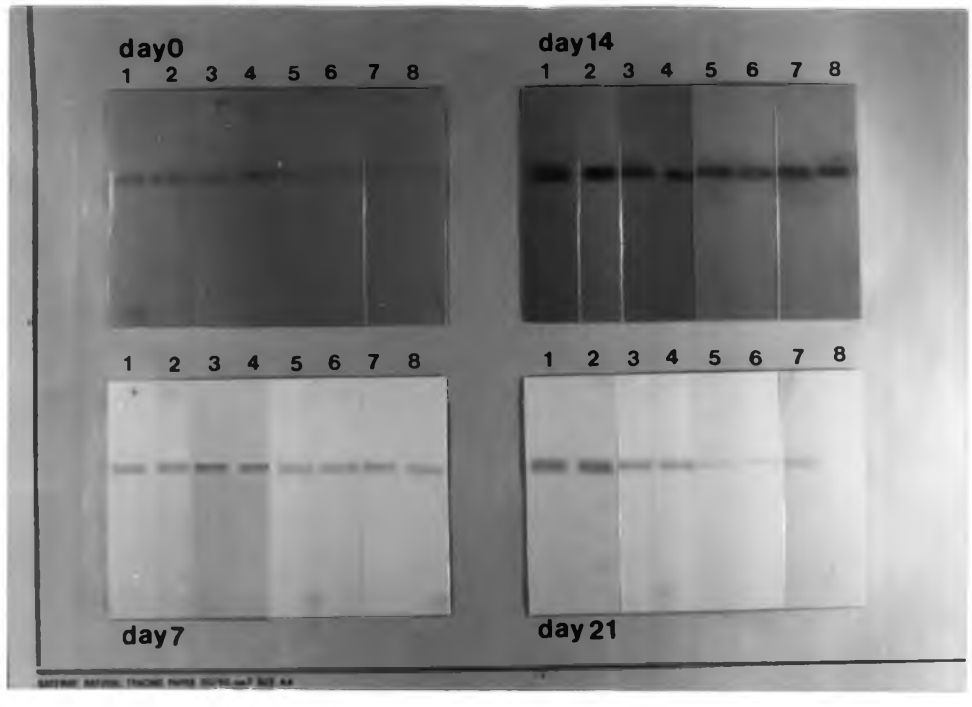


Figure 3.12 The GS activity staining of non-inoculated and inoculated rice root and leaf extract.

Lane 1,2 : inoculated rice leaf

Lane 3,4 : non-inoculated rice leaf

lane 5,6 : inoculated rice root

lane 7,8 : non-inoculated rice root

Day 0, 6 μg of rice crude extract.

Day 7-21, 7 μg of rice crude extract.

3.6 Molecular form of root and leaf GS characterized by Western blot analysis

Comparative study of the molecular forms of roots and leaves GS protein of the non-inoculated and inoculated rice plants by Western blot analysis by anti maize's GS, on day 0, 7, 14 and 21 after inoculation showed that subunit molecular weight of GS enzyme in root and leaf were ,48 kDa (Figure 3.13). The band intensity of GS protein in inoculated rice leaf were more intense than in non-inoculated condition but there were more or less comparable to the non-inoculated and inoculated rice root, at the same interval (the anti maize's GS was not cross-react with bacterial GS). These finding supported the results obtained from the quantitative determination of GS specific activity that inoculation of rice seedling with *K. oxytoca* R15, induced only the leaf GS activity but not in the root, as evident by the 48 kDa band of associative rice leaf were more intense than the 48 kDa bands of the non-inoculated leaf, where similar bands intensity were observed in non-inoculated and associative rice root.

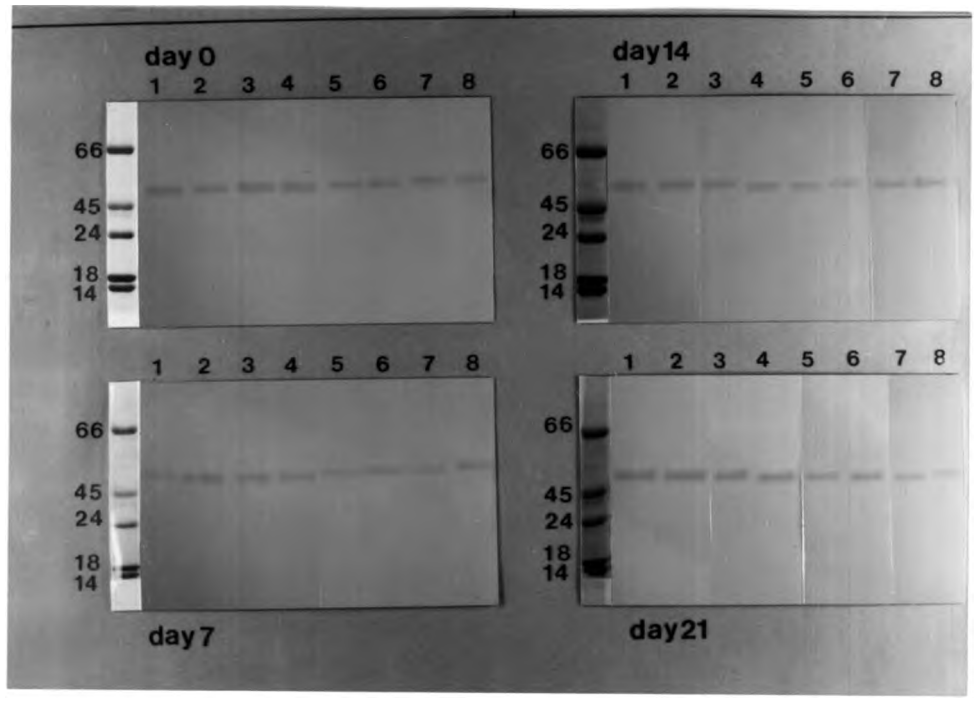


Figure 3.13 Western blot analysis of root and leaf of 4 µg of non-inoculated and inoculated rice extract.

Lane 1,2 : inoculated rice leaf

Lane 3,4 : non-inoculated rice leaf

lane 5,6 : inoculated rice root

lane 7,8 : non-inoculated rice root