CHAPTER 3

RESULTS AND DISCUSSION

3.1 Physical Characteristics of the Selected Plant Leaves

Two types of plants, namely dumb cane (*Dieffenbachia seguine*) and little prayer plant (*Calathea vaginata* Petersen), were selected for testing of their capability on NO_2 absorption from air.

3.1.1 Leaf area

Leaf area was determined by Image J 2X program. Six pots of plants were selected and every single leaf was measured for the area. In total 48 leaves were measured for each plant type. The average leaf area of dumb cane was 4659±1137 mm^2 (Table 3.1) while that of little prayer plant was 78632±1563 mm^2 (Table 3.2). In comparison, leaf of dumb cane was approximately 2 times smaller than the leaf of little prayer plant.

Table 3.1 Leaf area of dumb cane

Pot No.	1	2	3	4	5	6
plant (g)	83.63	69.14	70.24	59.49	59.93	59.84
soil (g)	505.58	504.96	504.31	592.89	600.34	600.86
leaf No.	Leaf area (mm^2)					
1	4063	3420	3189	3241	3185	3240
2	4360	3429	4362	3248	3329	3248
3	5002	3556	4453	4042	3756	3482
4	5129	3757	4844	4163	4132	3810
5	5293	4179	5083	4387	4289	4856
6	5974	4188	5267	4456	5029	5660
7	6073	4546	5378	5067	5293	6256
8	6956	7487	5491	7285	6341	6352
Total area (mm^2)	42851	34563	38069	35889	35356	36904
Average leaf area (n=48)	4659 ± 1137					

Table 3.2 Leaf area of little prayer plant

Pot No.	1	2	3	4	5	6
plant (g)	58.51	56.62	56.14	60.45	58.41	60.38
soil (g)	738.20	554.41	662.28	650.34	595.28	621.33
leaf No.	Leaf area (mm^2)					
1	7129	8149	8048	8430	4188	8048
2	8568	8234	8451	9411	8766	8090
3	8935	9522	8812	9468	8967	9486
4	8952	9686	9046	9781	8989	10358
5	10378	10110	9050	10173	10244	11014
6	10702	10316	10153	10539	10821	11073
7	11261	11246	11479	11114	11213	11281
8	11937	11631	12265	11806	12732	11740
Total area (mm^2)	77863	78894	77305	80723	75921	81090
Average leaf area (n=48)	78632 ± 1563					

3.1.2 Number and Size of Stomata

The density and size of leaf stomata was studied by peeling off epidermis layer method. The stoma size was determined by measuring the size of guard cell through light microscope using an ocular micrometer. The stomatal number was calculated as the average number of stomata in 1 mm^2 area multiplied by the total leaf area (Gupta, 1961). The stoma images and the sizes of stoma and guard cell of dumb cane and little prayer plant are shown in Figure 3.1. The average stomatal sizes of dumb cane and little prayer plant were 6.8 µm x 22.8 µm (Table 3.3) and 5.0 µm x 17.8 µm (Table 3.4), respectively. Sizes of their guard cell were 14.3 µm x48 µm (Table 3.3) and 6.3 µm x 25.5 µm (Table 3.4), respectively. The absolute stomatal number average of leaves of dumb cane and little prayer plant were $8.70x10^5$ and $1.40x10^6$, respectively (Table 3.5).

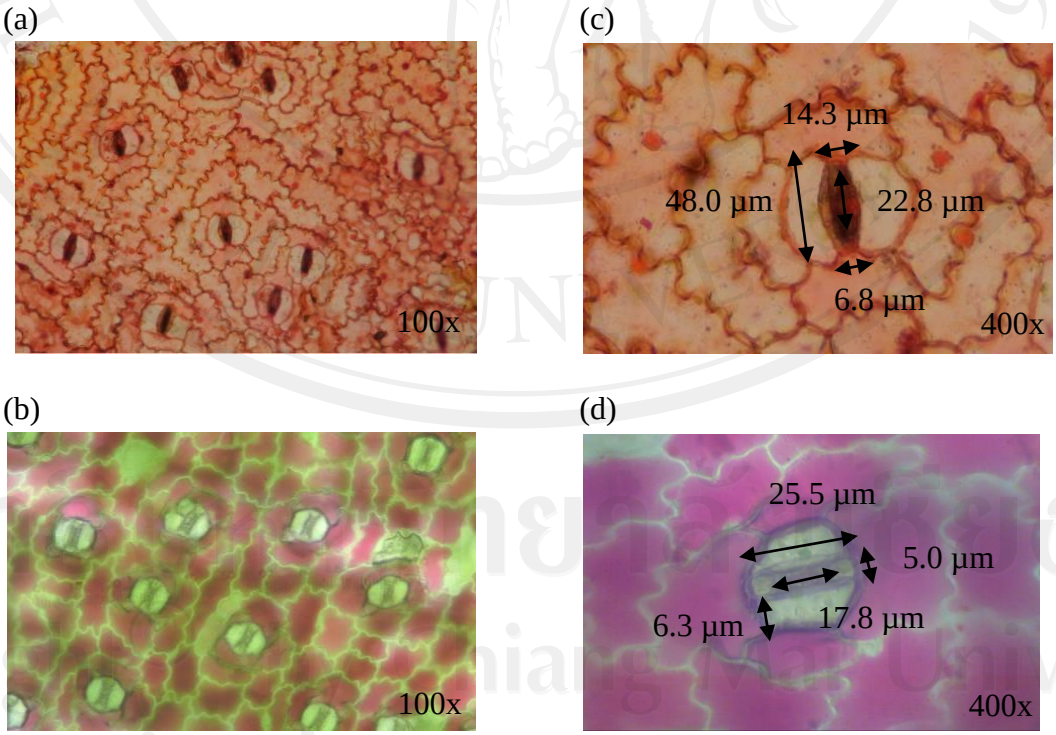


Figure 3.1 Leaf stomata of (a) dumb cane and (b) little prayer plant, and size of guard cell and stoma of (c) dumb cane and (d) little prayer plant

Table 3.3 leaf area and size of stoma and stomata of dumb cane

leaf no.	leaf area (mm^2)	number of stoma 400x	stoma (μm)		guard cell (μm)	
			wide	long	wide	long
1	4902	30	7.5	27.5	12.5	47.5
2	4856	30	7.5	25.0	15.0	52.5
3	4378	29	7.5	25.0	12.5	47.5
4	5067	28	5.0	25.0	15.0	50.0
5	5129	29	5.0	20.0	15.0	45.0
6	5043	25	5.0	20.0	15.0	50.0
7	4832	24	7.5	22.5	15.0	45.0
8	5267	26	7.5	20.0	15.0	47.5
9	4289	25	7.5	20.0	12.5	50.0
10	4546	24	7.5	22.5	15.0	45.0
average	4831	27	6.8	22.8	14.3	48.0
SD	327.5	2.4	1.2	2.8	1.2	2.6

Table 3.4 leaf area and size of stoma and stomata of little prayer plant

leaf no.	leaf area (mm^2)	number of stoma 400x	stoma (μm)		guard cell (μm)	
			wide	long	wide	long
1	7129	26	5	17.5	7.5	25.0
2	8451	27	5	22.5	5.0	27.5
3	8430	28	5	20.0	7.5	25.0
4	8048	24	5	17.5	5.0	25.0
5	8048	23	5	20.0	7.5	27.5
6	8149	25	5	15.0	5.0	25.0
7	9411	24	5	15.0	7.5	25.0
8	8952	26	5	17.5	5.0	25.0
9	8568	25	5	17.5	5.0	25.0
10	9486	24	5	15.0	7.5	25.0
average	8467	25	5	17.8	6.3	25.5
SD	701	2	0	2.5	1.3	1.1

Table 3.5 Absolute stomatal numbers of dumb cane and little prayer plant

Plant	Average size of stomata*	Average size of guard cell*	Stomatal density (S; mm^{-2})	Leaf area (A; mm^2)	Absolute stomatal number (SxA)
dumb cane	6.8x22.8	14.3x48.0	200	4902	9.80×10^5
			200	4856	9.71×10^5
			193	4378	8.46×10^5
			187	5067	9.46×10^5
			193	5129	9.92×10^5
			167	5043	8.41×10^5
			160	4832	7.73×10^5
			173	5267	9.13×10^5
			167	4289	7.15×10^5
			160	4546	7.27×10^5
average			180	4831	8.70×10^5
SD			16	327	1.05×10^5
little prayer plant	5.0x17.8	6.3x25.5	173	7129	1.24×10^6
			180	8451	1.52×10^6
			180	8430	1.52×10^6
			160	8048	1.29×10^6
			153	8048	1.23×10^6
			167	8149	1.36×10^6
			160	9411	1.51×10^6
			173	8952	1.55×10^6
			167	8568	1.43×10^6
			160	9486	1.52×10^6
average			167	8467	1.42×10^6
SD			9	701	1.20×10^6

* width(μm)xlength (μm)

3.2 NO_2 Absorption by Plants in Chamber

In order to observe NO_2 absorption by plants, the chamber was used for the experiment set up (see detail in subsection 2.6). Two types of plant were selected and tested in the chamber to find their capabilities on NO_2 absorption. Four set up conditions were 1) a control set; the chamber without a plant, 2) soil; a pot containing soil without a plant, 3) plant; a pot containing soil and a plant with a plastic bag covering the pot to prevent gas absorption through the soil media and 4) soil and plant; a pot containing soil and a plant. The temperature inside the chamber was approximately 28 ± 2^oC, while humidity was $67\pm2\%$. Firstly, the NO_2 concentration in the chamber was set at approximately 20 ppm and its concentrations were measured every 30 minutes by a gas analyzer until 8 hours. Dumb cane was first tested. Each condition was repeated for 3 times by using new materials (soil, plant and chamber).

The average NO_2 concentrations (n=3) from each experimental condition were plotted against time (Figure 3.2). The graph patterns of the NO_2 concentrations over an 8-hour period for all conditions were almost the same. For the control set, the NO_2 level was quickly reduced within the first 3 hours of the experiment. After that, it slowly decreased and remained quite constant. This is probably due to the presence of some leaks in the chamber and certain amount of loss through the NO_2 sensor. The final concentration after 8 hours in the chamber was about 5-6 ppm. This means that the NO_2 level in the chamber decreased ~ 4 times over 8 hours.

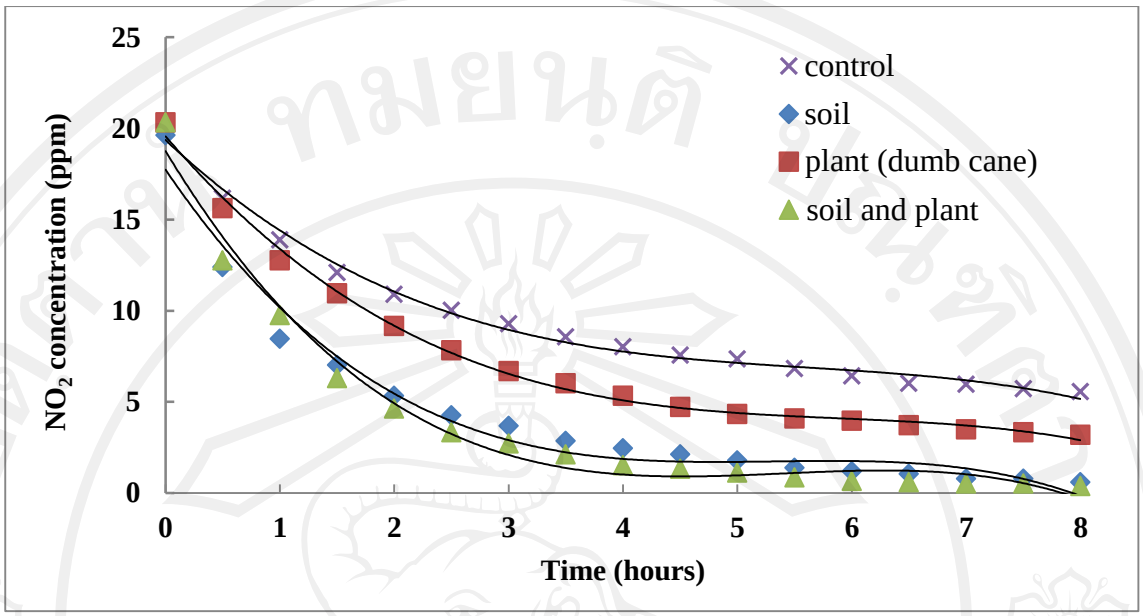
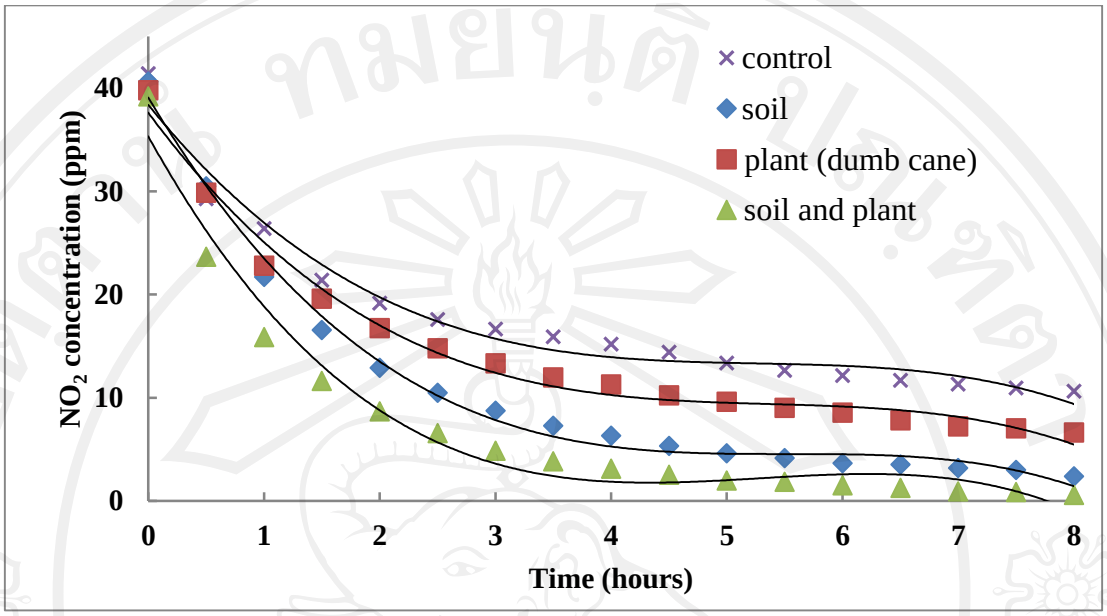


Figure 3.2 Changing of NO_2 concentrations (from 20 ppm) in the chamber for dumb cane experiment

Based on the level detected, it was found that the soil and plant showed a capability to absorb NO_2 from air. However, the decreases of NO_2 concentrations of using soil alone and using soil and plant together at the same time were almost the same. Moreover, their concentrations between 6-8 hours were almost zero. Therefore, the starting NO_2 concentrations were set up for 2 times higher (40 ppm) in order to see more meaningful results of the two afore mentioned conditions.

(a)



(b)

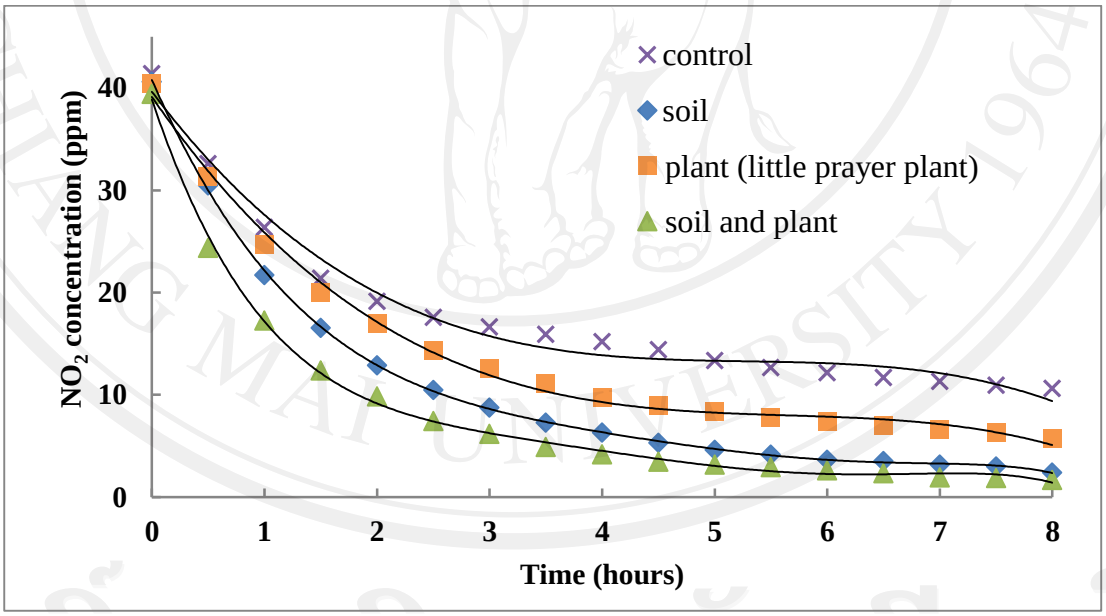


Figure 3.3 Changing of NO_2 concentrations in the chamber with 40 ppm starting concentration: (a) dumb cane and (b) little prayer plant

Figure 3.3 shows the changing of NO_2 concentrations starting from 40 ppm under each condition in the chamber. In the case of the control, the NO_2

concentrations fastly decreased in the first 3 hours and slightly decreased after that until 8 hours. The final concentration was about 10-12 ppm. This trend was the same as in the previous experiment (20 ppm NO_2). The experiments set up for both dumb cane and little prayer plant were almost the same (Figure 3.3(a) and (b)).

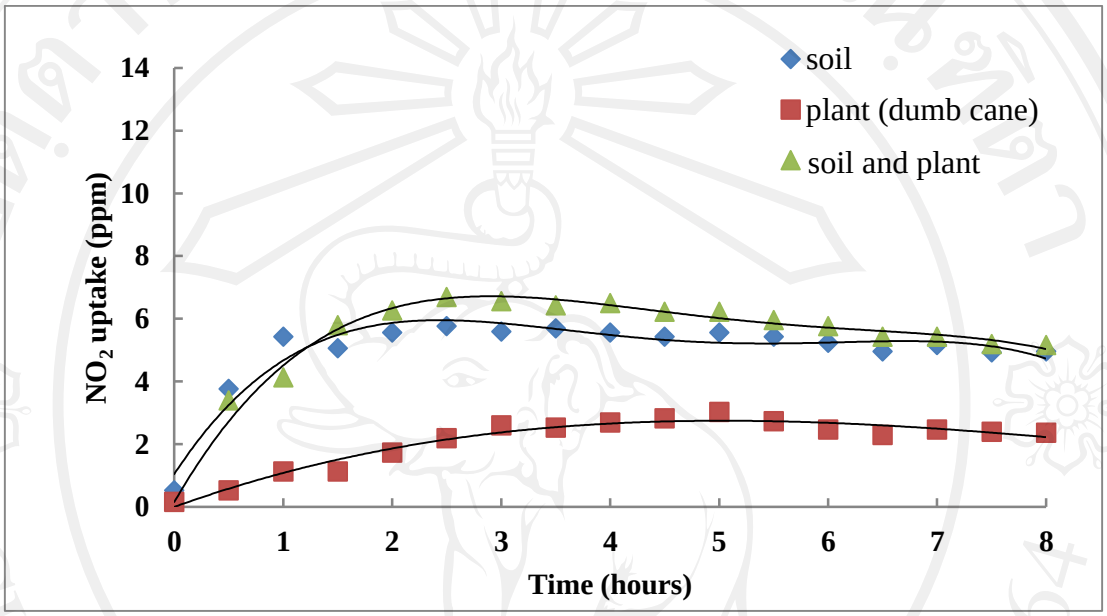
Based on the Kruskal-Wallis test for both of the experiments using dumb cane and little prayer plant, there was no significant difference ($\alpha = 0.05$) of NO_2 absorption by the soil alone or by a combination of plant and the soil. They however differed in NO_2 levels remaining in the control set and the set using the plant alone. This could be because NO_2 absorption through the soil was greater than that by the plant leaves. However, a combination of plant and soil did provide a higher capacity of absorption than did soil without plants and/or an individual plant.

3.3 NO_2 Uptake by Plants

The uptake rates of NO_2 by plants were calculated from the NO_2 concentrations detected under each condition subtracted by those of the control set. The graphs were plotted by using the NO_2 uptake against time. NO_2 uptake by soil or plant alone and a combination of soil and plant are shown in Figure 3.4 (dumb cane experiment) and Figure 3.5 (little prayer plant experiment). In the case of dumb cane, the different starting concentrations of NO_2 showed different uptake of NO_2 by soil alone and by a combination of soil and plant. At lower concentration (20 ppm NO_2) there was almost the same uptake of NO_2 by these two conditions. However, at high concentrations (40 ppm NO_2), the NO_2 uptake by a combination of soil and plant was about 2 times greater than that of soil alone. The same result was also found in little prayer plant. At higher NO_2 concentrations in air, plant seems to absorb more NO_2

than at the lower concentrations. The capacity of pollutant absorption may depend on concentration in the environment.

(a)



(b)

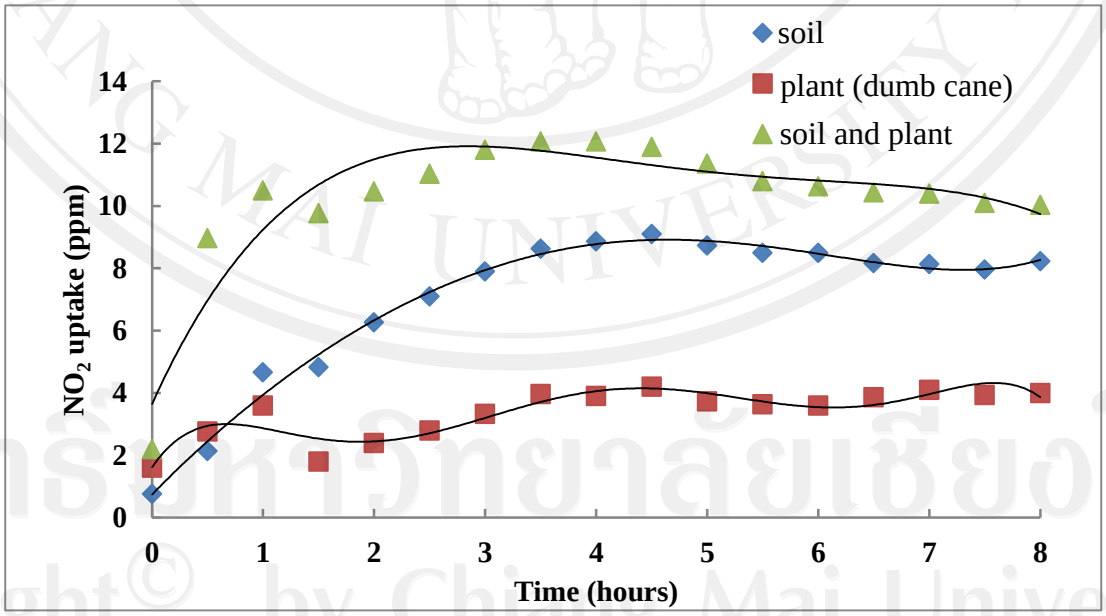


Figure 3.4 NO_2 uptake for dumb cane experiment with NO_2 starting concentrations of (a) 20 ppm and (b) 40 ppm

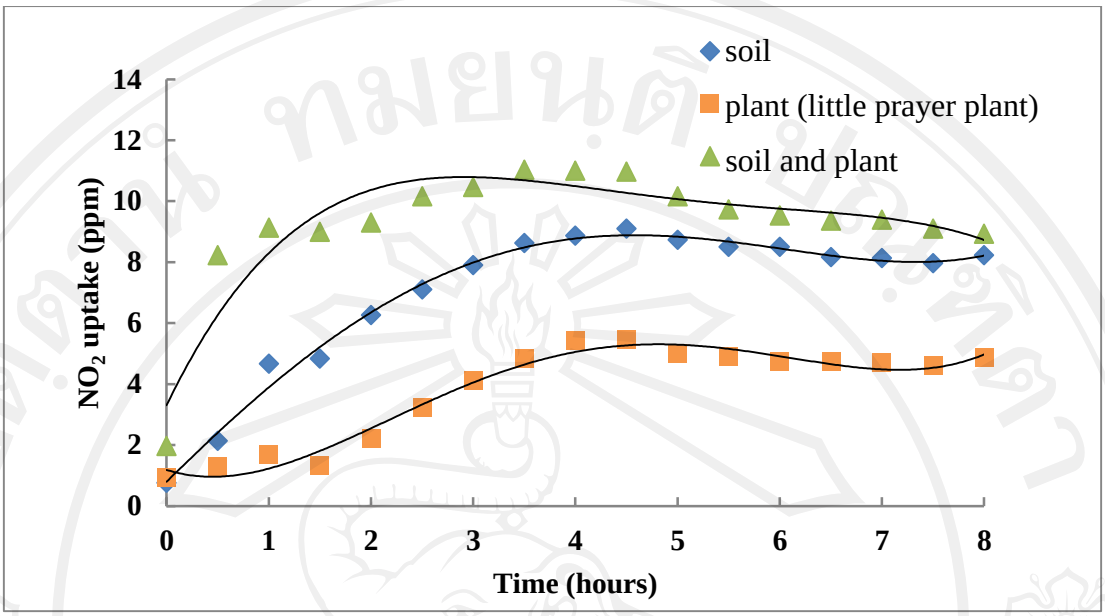


Figure 3.5 NO_2 uptake (ppm) for little prayer plant experiment at 40 ppm NO_2

It can be explained that the plant and soil could efficiently absorb NO_2 from air in the sealed chamber. It was also found that these 3 conditions were significantly different ($p < 0.05$) in terms of NO_2 uptake from the air. The capability of NO_2 removal of the 3 conditions in descending order were plant+soil > soil > plant. The absorption by the surfaces of the plant and the soil was easily reached in a short time. A dramatic change in the removal concentrations could be due to the uptake by the stomata of the plant and the degradation by microorganisms in the soil media.

The NO_2 uptake by the plant and the soil is shown in Table 3.6. It was found that the removal concentrations were almost the same at 20 and 40 ppm either by the soil (1) or by the plant (2). Even though the results from 3 conditions showed obvious different NO_2 uptake nevertheless these values were not significantly different ($p>0.05$). Moreover, the soil showed an NO_2 absorption rate at 2-5 times higher than the plant for dumb cane and 2-3 times for little prayer plant.

Table 3.6 NO_2 uptake by plant

Experimental conditions	NO_2 uptake (ppm)		
	dumb cane		little prayer plant
	20 ppm (n=3)	40 ppm (n=3)	40 ppm (n=3)
(1) Soil[a]	5.1-5.8	4.7-9.1*	4.7-9.1*
(2) Plant[b]	1.1-3.0	1.8-4.2	1.7-5.5
(3) Plant & soil[a]	4.1-6.7[x]	9.0-12.1[y]	8.2-11.0

* The values are from the same experiments

[x, y] represent significant difference at 95% confidence ($\alpha = 0.05$) for NO_2 concentration

[a, b] represent significant difference at 95% confidence ($\alpha = 0.05$) for conditions of NO_2 40 ppm

However, when NO_2 concentration was increased from 20 to 40 ppm for dumb cane, NO_2 removal by the plant & soil (3) also increased from 4.1-6.7 ppm to 9.0-12.1 ppm. By a comparison of NO_2 uptake between 20 and 40 ppm, it was found that only the condition of the plant and soil combination showed a significant difference ($p < 0.05$) of NO_2 uptake. At higher concentrations of NO_2 (40 ppm), the plant and soil showed a higher NO_2 uptake than that of lower concentrations of NO_2 (20 ppm). Considering morphology of plants, an average value of stomatal density of dumb cane (180±16 number/mm^2) was slightly higher than that of little prayer plant (167±9 number/mm^2). Moreover, the stomatal size of dumb cane (6.8 µm x22.8 µm) was also slightly larger than that of little prayer plant (5.0 µm x17.8 µm). However, NO_2 uptake levels by both types of plant were not significantly different (*p>0.05*). This finding was related to the nitrate content in the plant leaves.

Similar work has been done in the real environment using trees for ambient NO_2 uptake. Farrelly (2011) reported that bullet wood and queen's flower trees could absorb NO_2 from ambient air in highly polluted areas at a range of 6.2-46.1 and 0.4-18.3 ppbv, respectively, whereas, the absorption levels at low polluted areas were 3.2-10.3 and 0.7-13.3 ppbv, respectively. However, it should be noted that the NO_2 concentration in the real environment is much lower than it was in the experiments carried out in the closed chamber system.

3.4 Ion Analysis

3.4.1. Calibration curve of ion analysis

In each analytical run, 10 concentrations (0.02-1.0 µg/mL) of ions calibration standard was prepared and analyzed by IC. The calibration curve of each ion standard was constructed using concentration of standard solution versus peak area. Concentration rangs must cover at least 95% of ion concentrations of samples, whereas the regression value was controlled at $r^2 \geq 0.9978$. The calibration curves for determination of nitrite and nitrate are show in Figure 3.6

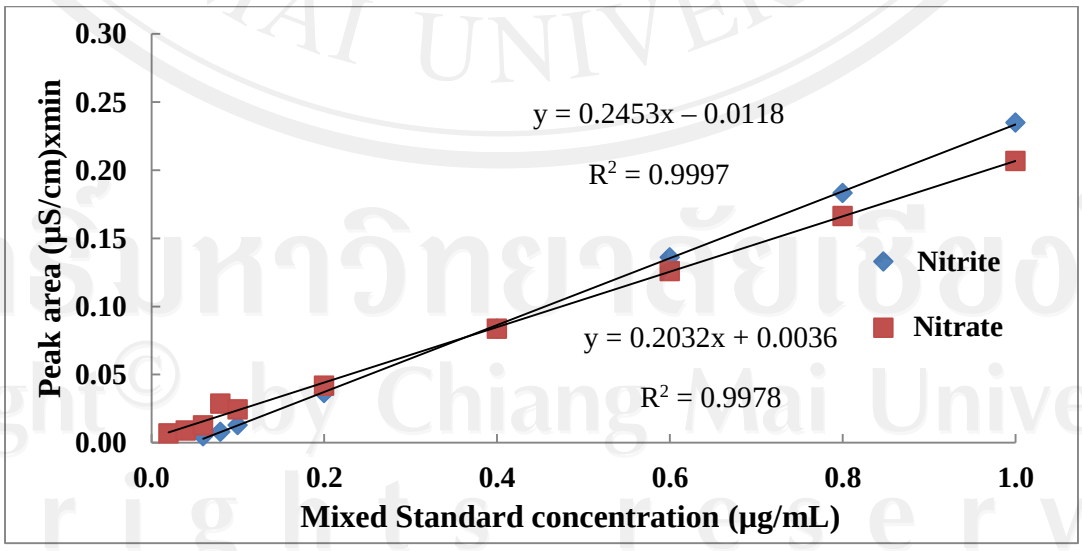


Figure 3.6 Calibration curves for determination of mixed standard (nitrite and nitrate)

3.4.2. Limit of detection

The limit of detection (LOD) is the lower amount of analyte which can be detected with an acceptable statistical significance. According to Taylor (1987) LOD was calculated as three times of standard deviation (SD) of the noise at zero solution. Their concentrations were calculated from the calibration curve in ranges of 0.02 to 1.0 µg/mL for mixed standard. Limit of detection values of all analytes are shown in Table 3.7.

Table 3.7 Limit of detection of IC (Metrohm) for nitrite and nitrate ion

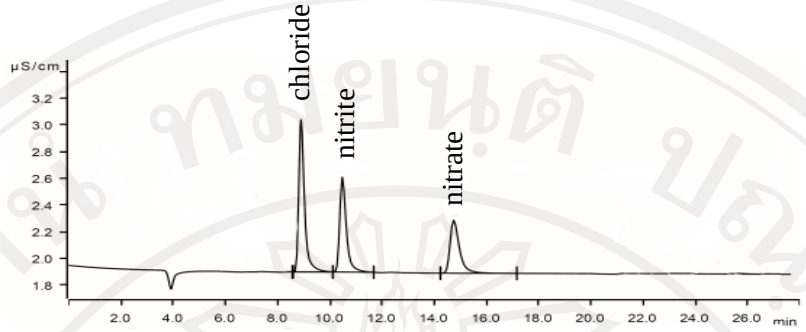
No. of injection	**Concentration (µg/mL)**	
	NO_3^-	**NO_2^-**
1	0.0162	0.0715
2	0.0176	0.0744
3	0.0181	0.0723
4	0.0172	0.0731
5	0.0167	0.0744
6	0.0162	0.0748
7	0.0157	0.0735
8	0.0167	0.0719
9	0.0186	0.0748
10	0.0176	0.0756
Average	0.0171	0.0736
Standard Deviation (SD)	0.0009	0.0014
Limit of Detection (3*SD)	**0.0028**	**0.0041**
Limit of Quantification (10*SD)	**0.0095**	**0.0138**

3.4.3 Chemical Composition Changes in Plant Leaves After NO_2 Exposure

According to the exposure of plants to NO_2 in the chamber, ion contents (NO_2^- and NO_3^-) accumulated on and in plant leaves were analyzed by IC. The solutions from the wash method (adsorption) and the extraction method (absorption) were analyzed for determination of anions contained in the exposed- and unexposed plant leaves. Figure 3.7 shows chromatograms of anions found in sample solutions in comparison with mixed standards. Only two major ions including chloride and nitrate were detected. It has been reported that chloride is generally essential for the growth of higher plants and it accumulates in tissue of plant. (Plants require chloride for enzyme synthesis, cofactor in photosynthetic O_2 and it is a composition of amino acid etc. Therefore, its peak was detected from both preparation methods. However, only nitrate peaks were taken into consideration in this study.

Table 3.8 shows the exposed and unexposed leaves. For adsorption mechanism (wash method), the difference of nitrate concentrations in the leaves of little prayer plant prepared from two methods (14.5 ng/mm^2) was slightly higher than that in dumb cane (10.9 ng/mm^2). In the case of absorption (based on the extraction method), the different values from both plant types were almost the same. This result well agrees with the NO_2 uptake (Table 3.6).

(a)



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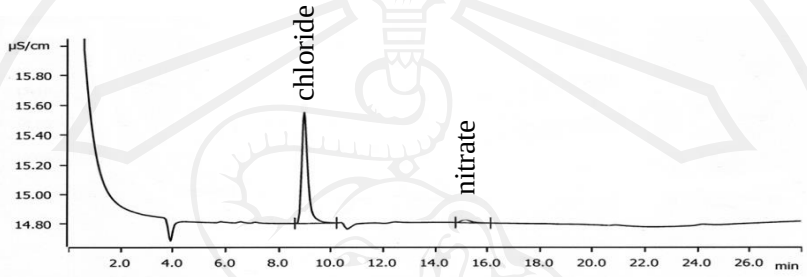

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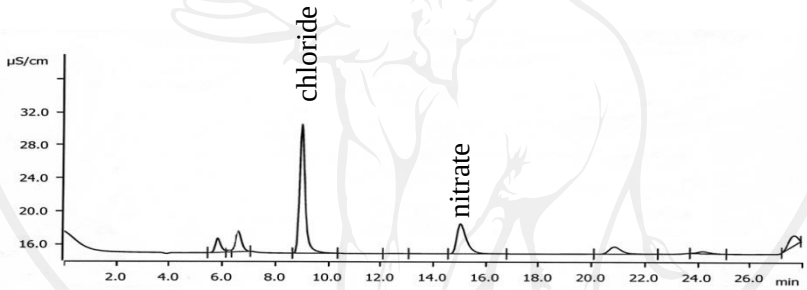

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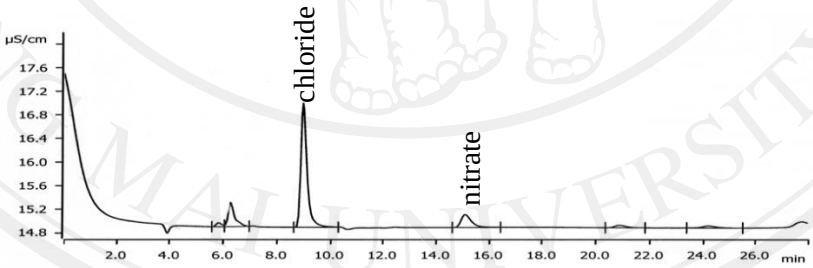


(e)

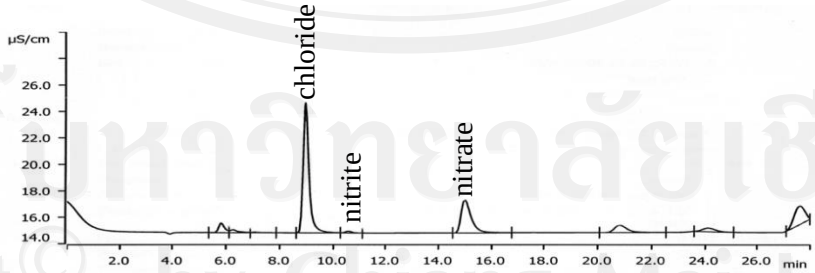


Figure 3.7 Chromatograms of (a) 0.5 µg/ml mixed anion standards (b) wash method from unexposed sample (c) wash method from exposed sample (d) extraction method from unexposed sample (e) extraction method from exposed sample

Table 3.8 Comparison of nitrate concentration on and in plant leaves of exposed and unexposed plants

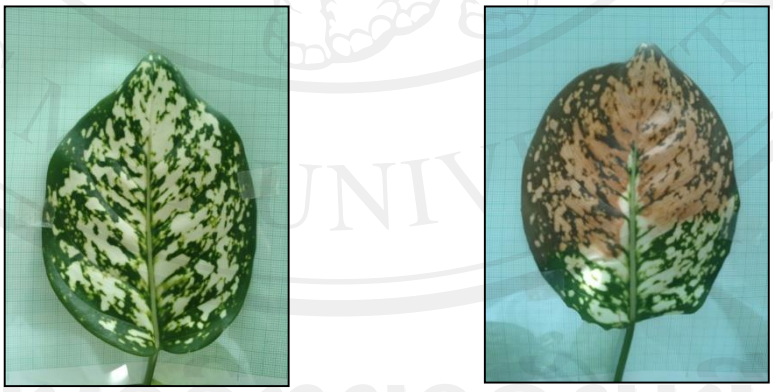
Sample	Condition		NO_3^- (µg/ml)	NO_3^- (µg)	Average leaf area (mm^2)	(ng/mm^2)	Differences between exposed and unexposed leaves (ng/mm^2)
dumb cane	wash method (adsorption)	unexposed leaves (n=3)	0.18	9.1	4880	1.9	10.9
		exposed leaves (n=3)	2.16	108.1	8616	12.6	
	extraction (absorption)	unexposed leaves (n=3)	1.31	78.4	5154	15.2	6.9
		exposed leaves (n=3)	2.72	163	7364	22.1	
little prayer plant	wash method (adsorption)	unexposed leaves (n=3)	0.37	18.7	8692	2.1	14.5
		exposed leaves (n=3)	3.03	151.4	9109	16.6	
	extraction (absorption)	unexposed leaves (n=3)	0.52	26.1	9210	2.8	6.8
		exposed leaves (n=3)	2.08	104.0	10840	9.6	

The average ratio values between NO_2 absorption measured every 30 minutes over 8 hours and leaf area of dumb cane were 0.438 ppb/m^2 (20 ppm NO_2) (see detail in Table B-4; Appendix B) and 0.920 ppb/m^2 (40 ppm NO_2) (see detail in Table B-5, Appendix B). This means the ability of dumb cane to absorb NO_2 from air increases with the NO_2 concentration in the environment. In case of little prayer plant at 40 ppm, the ratio (0.441 ppb/m^2; see detail in Table B-6, Appendix B) was slightly less than dumb cane experiment at 20 ppm and only half of the 40 ppm. This may be can explain in terms of morphology of plant leaves.

3.5 Morphological Changes

1) Dumb cane

Leaves of dumb canes are coated by cutin on the upper epidermis leaves. After NO_2 exposure, it was found that peduncle and leaves of dumb cane were damaged. Peduncle was broken and died after NO_2 exposure. Morphological changes of dumb cane leaves after exposure to high concentrations of NO_2 were observed (Figure 3.8). It was obvious that NO_2 affected and damaged the leaf of dumb cane after one day in ambient air. The external appearance of the healthy leaf (Figure 3.8 (a)) changed to appear as a burnt leaf (Figure 3.8 (b)) after exposure to 20 and 40 ppm of NO_2 for 8 hours. About 60% of leaves necrosis was observed after one day of exposure. However not every leaf was affected; about half of the total leaves in one plant were damaged and ultimately died. This is probably due to the short-term exposure. Therefore, plants can still recover and continue to grow after the exposure.



(a) (b)

Figure 3.8 leaf of dumb cane: (a) before and (b) after being exposed to 40 ppm NO_2 for 8 hours

2) Little prayer plant

Leaves of little prayer plant is a light and dark green feathered pattern with red veins, and are often red underneath. After NO_2 exposure (40 ppm, 8 hours), the leaves color was changed to brownish, which most prominent along the leaf margins. More specifically, necrosis appeared first on the older leaves as nitrogen is translocated through their stomata to the younger leaves which their color were change from green to dark brown. The most severely injuried leaves may develop necrotic area along the margins. Dark spots appeared on leave, less than 1 cm in diameter, and affecting only a few cells, with the stipple appearing in a zone between necrotic and healthy tissue (Figure 3.9 (b)). Nevertheless after spot occurred 8 hours, leaf begins to shrink and wrap on 24 hours. The damage leaves will die in the end after exposure 72 hours.

There are many effects depend on different kinds of plants but is particularly common on ornamental plant and foliage species are suddenly exposed to intentse NO_2 concentration. All the results are well agreed with Tingey *et al.* (1971). They used tobacco, radish, oats and alfalfa plants for NO_2 exposure at a concentration of 8 ppm for 4 hours and found that leaf injuries from NO_2 occurred as marginal and/or as interveinal necrosis on each leaf surface (bifacial) and may result in yield losses for plants grown under field conditions.

(a) (b)

Figure 3.9 leaf of little prayer plant: (a) before and (b) after being exposed to 40 ppm NO_2 for 8 hours

3.6 Anatomical Changes

The epidermis of leaves of different plants varies in the structure, arrangement of the stoma, and number of stoma. Because of the usually flat structure of the leaf, a distinction is made between the epidermal tissue of the two surface of the leaf; that surface of the leaf that is closer to the internodes above it and which usually faces upwards is referred to as the adaxial surface, and the other surface as the abaxial surface. In this study anatomies of plant were observed under microscope before and after exposure to NO_2. It was found that stomata of dumb cane found in upper epidermis and scatter all of leaves but found orderly stomata in lower epidermis for little prayer plant. After exposed to NO_2, stomata of dumb cane and little prayer plant were seared; size of stoma and epidermis cell after NO_2 exposure become smaller. The anatomical change of leaf and stem of plant after exposed to NO_2 are found in Tables 3.4 and 3.5, which are usually regular in shape and which pass through the entire leaf. In dumb cane and little prayer plant, NO_2 may penetrate deep into the tissue of the stem. This type of structure can be seen in the leaves of plants. An injury

leaves from necrosis lead to cell browning and died. Chlorophyll in leaf layer break down from cell and disrupted out of cell. Hypothesis that affect may from NO_2 passages and destroy cell membrane. Some of the above changes, such as the increase in the number of stomata, allow a higher rate of gas exchange under conditions of favorable water supply.

Table 3.9 Micrographs of NO_2 exposed and unexposed leaves of dumb cane

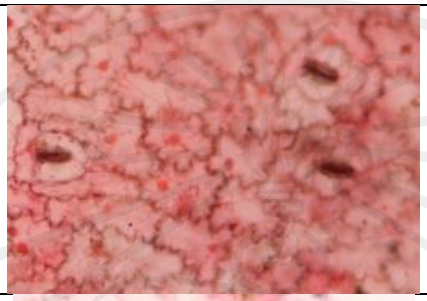
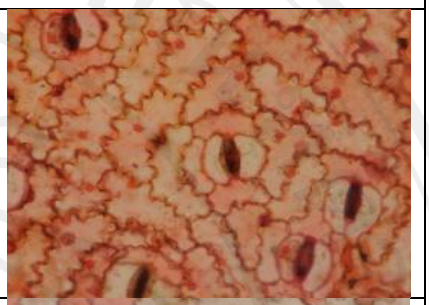
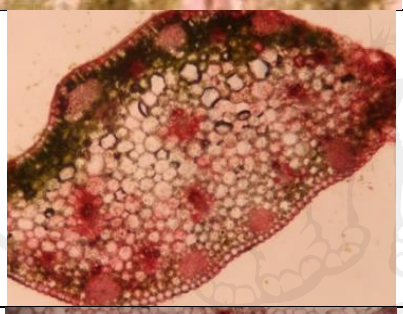
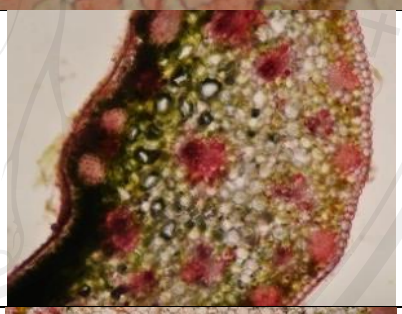
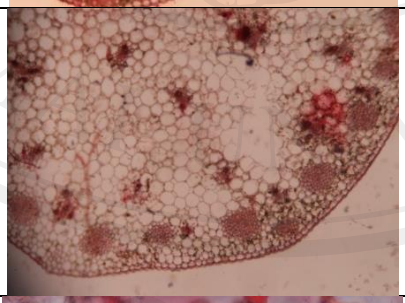
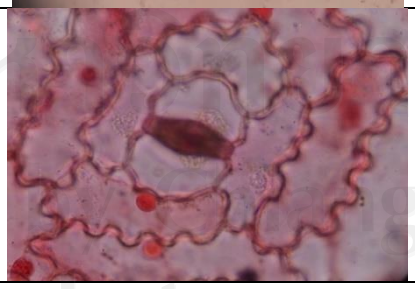
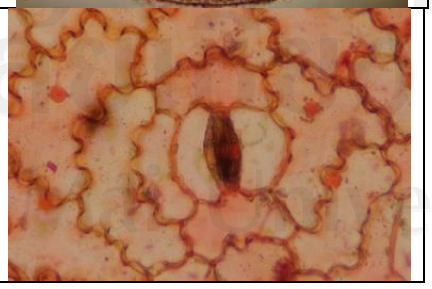
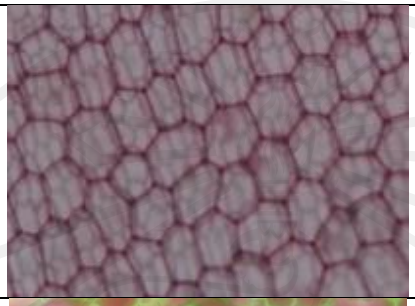
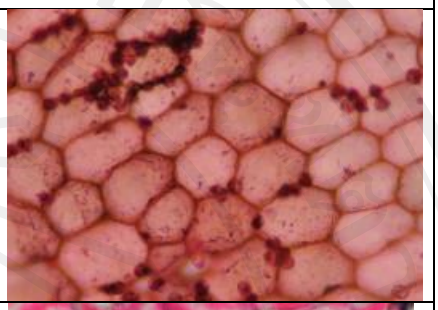
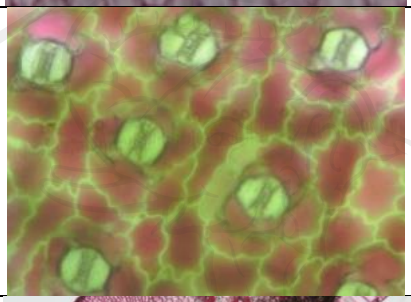
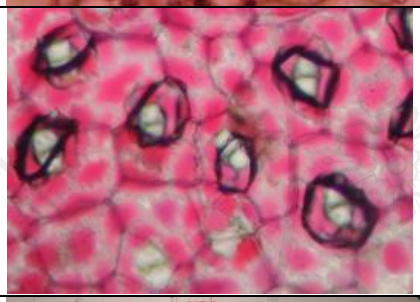
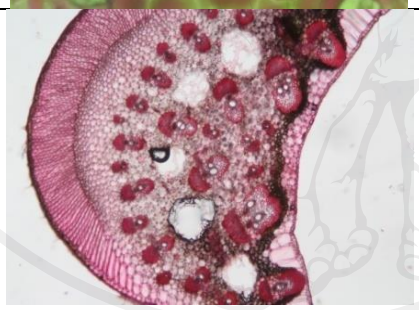
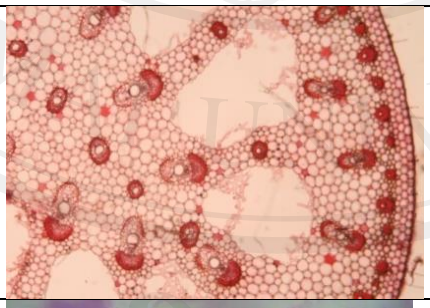
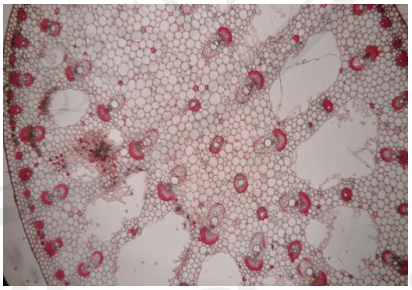
Elements	**Unexposed leaves**	**NO_2 exposed leaves**
Upper epidermis		
Lower epidermis		
Cross section axial of leaf		
Stem		
Stoma		

Table 3.10 Micrographs of NO_2 exposed and unexposed leaves of little prayer plant

Elements	**Unexposed leaves**	**NO_2 exposed leaves**
Upper epidermis		
Lower epidermis		
Cross section axial of leaf		
Stem		
Stoma