CHAPTER III

CONSTRUCTION OF PARAQUAT RESISTANT MUTANTS

3.1 Growth of Chlamydomonas reinhardtii 137c

Cells were inoculated at the middle of light period on day zero with an inoculum size of 10^5 cells/ml. Initiation time of illumination was designated as the first hour of light-dark cycle.

It was observed by light microscope that C. reinhardtii 137c (wild type cell) began to form zoospores with maximally 4 daughter cells at hour 18 (6 hours in the dark) (Fig. 3.1a). Complete cell division occurred almost immediately after the light was turned on. The light micrograph of cells at hour 1 of illumination was shown in Fig. 3.1b .

Several parameters for growth of the alga could be demonstrated including cell density, turbidity of culture (OD_{540}), the content of chlorophyll a and protein (Fig. 3.2 a, b). Growth of C. reinhardtii 137c was immediately detected on the first day and reached the maximum on day third. However continuous increase in culture turbidity was observed until the end of day fourth.

Decrease in chlorophyll a content on day fifth culture was detected in correspondence with changing of the culture color from dark green to yellowish green. At this phase cells turned clumping and sedimentation occurred.

The amount of cellular proteins of the algal cells was highest

Figure 3.1 Growth of Chlamydomonas reinhardtii 137c.
Cells were grown photosynthetically and photograghs were taken by light microscopy (x 100).

(a) hour 18 (b) hour 1

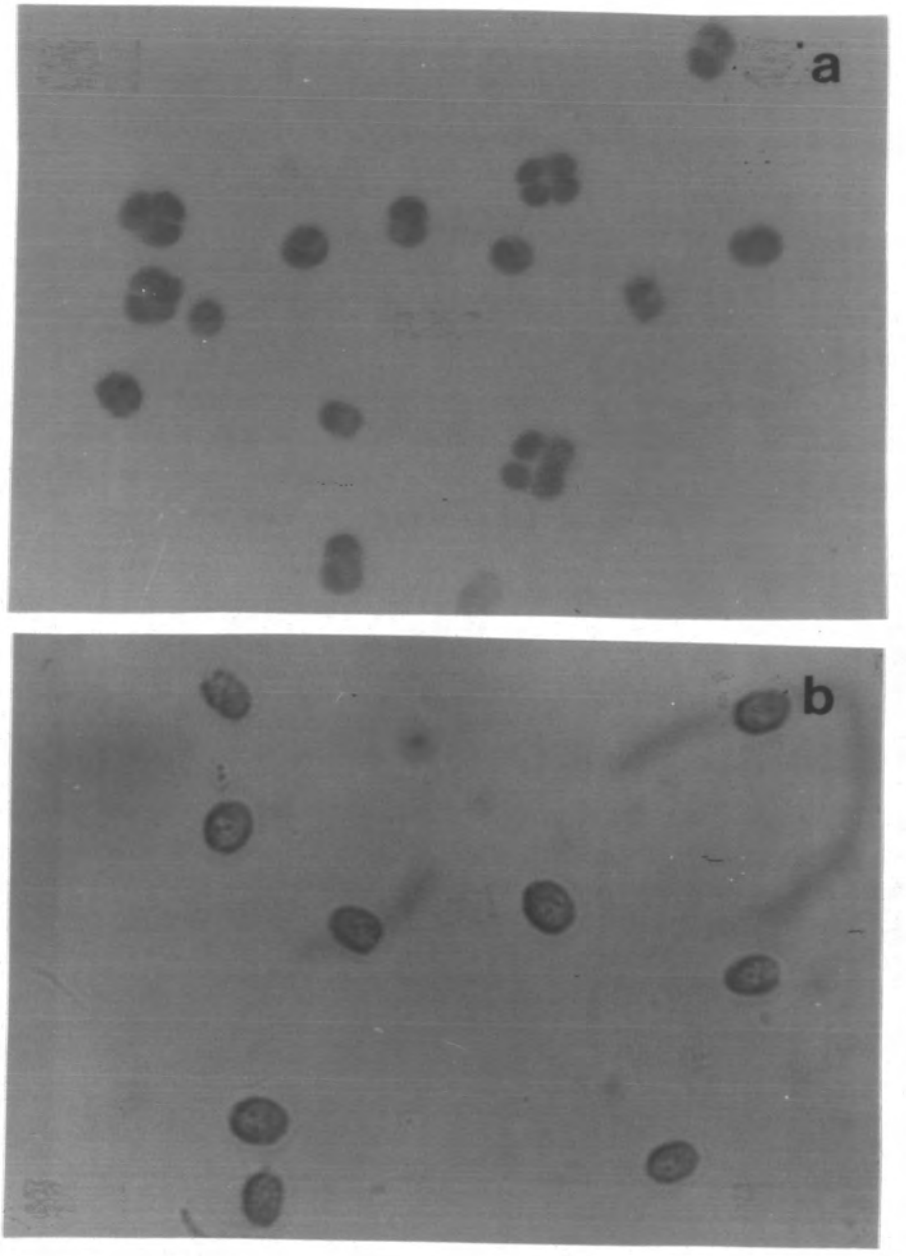
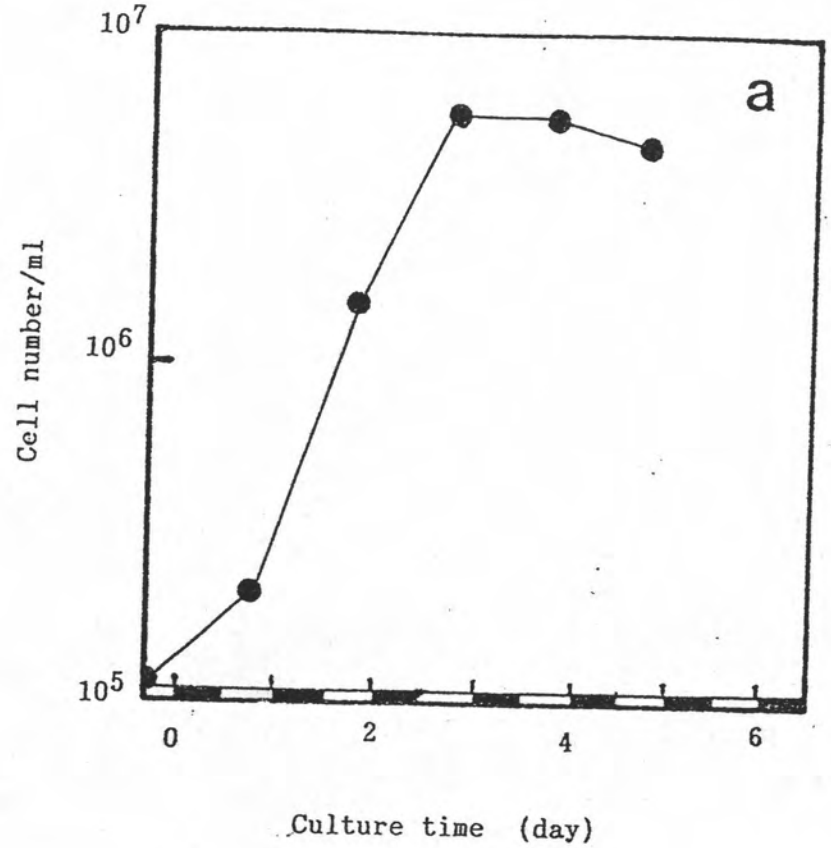


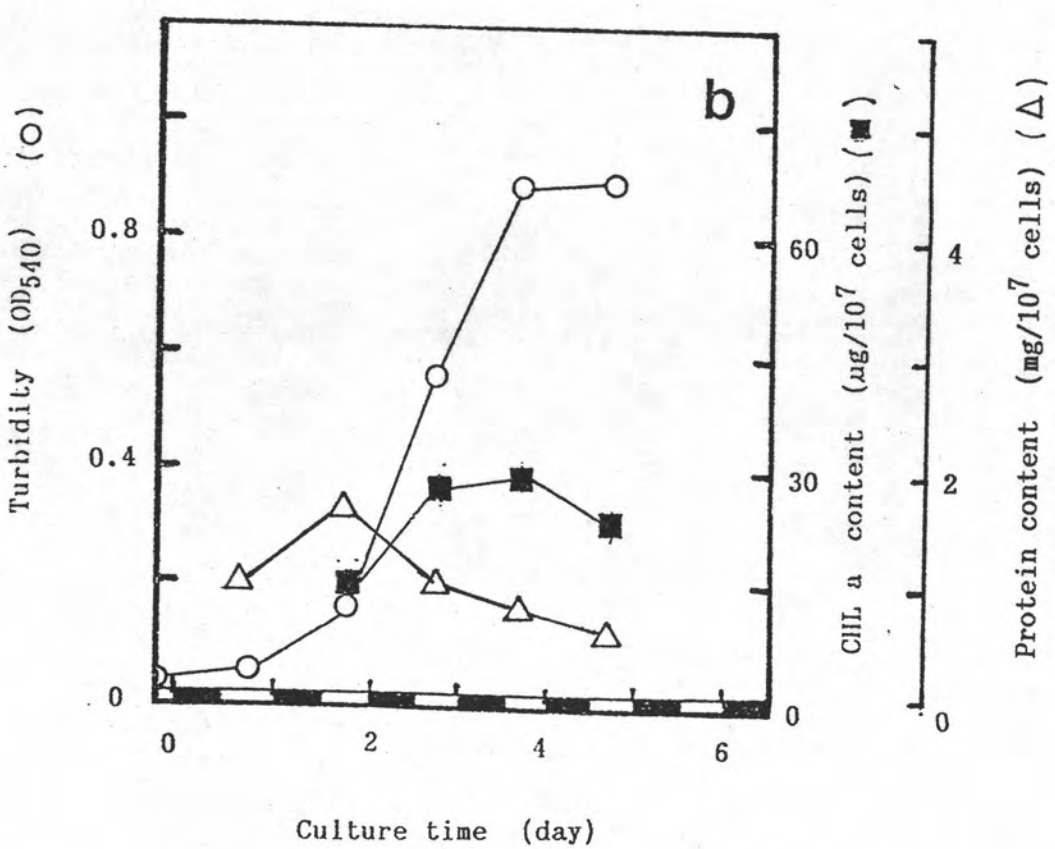
Figure 3.2 Growth curve of Chlamydomonas reinhardtii 137c.

Photosynthetic culture was initiated at hour 6 with an inoculum size of 10^5 cells/ml. Day 0 in the figure represents inoculation day. Growth was determined as

(a.) number of cell in the culture medium

(b.) turbidity at 540 nm, chlorophyll a (CHL a) content, and protein content.


a
10^7
10^6
10^5
Cell number/ml
0
2
4
6
Culture time (day)


b
Turbidity (OD_{540}) (○)
0.8
0.4
0
Chl a content (μg/10^7 cells) (■)
60
30
0
Protein content (mg/10^7 cells) (△)
4
2
0
0
2
4
6
Culture time (day)

at the mid-log period and declined thereafter.

3.2 Effect of Paraquat on C. reinhardtii 137c

3.2.1 Preliminary Test for Paraquat Susceptibility Toxicity of paraquat on the wild type cells was preliminarily screened by spot test. When algal suspension was spotted on solid growth medium containing 0-0.5 μM paraquat, green area could be clearly observed within 3 days after the inoculation (Fig. 3.3). However no cell growth could be illustrated when the concentration of paraquat was increased up to 1.0 μM at which the algal spots were completely bleached within 7 days after spotting.

3.2.2 Lethal Dose-50 Acute effect of paraquat on growth of C. reinhardtii 137c was easily evaluated by a method developed in this laboratory (section 2.5.2). The wild type cells were shortly exposed to paraquat under liquid culture conditions. After that the treated cells were washed and chemotrophically grown in the dark on paraquat free medium containing acetate as sole carbon source to stop the herbicide influx and its action on photosynthesis. Toxicity of paraquat was demonstrated as shown in Fig. 3.4. The concentration of the herbicide above 0.2 μM could partially inhibit the growth of wild type 137c, whereby complete growth inhibition was shown at 1.0 μM paraquat.

From this result, LD_{50} of paraquat to the wild type cell was evaluated to 0.36 μM.

Figure 3.3 Spot test of paraquat effect on Chlamydomonas reinhardtii 137c.

Cells were spotted on medium containing paraquat and exposed to light (12 hours) and dark (12 hours) alternately. Bleaching phenomena were observed on day 0, 3, 5, and 7.

(lane 1) paraquat free medium

(lane 2) 0.25 μM paraquat

(lane 3) 0.50 μM paraquat

(lane 4) 1.0 μM paraquat

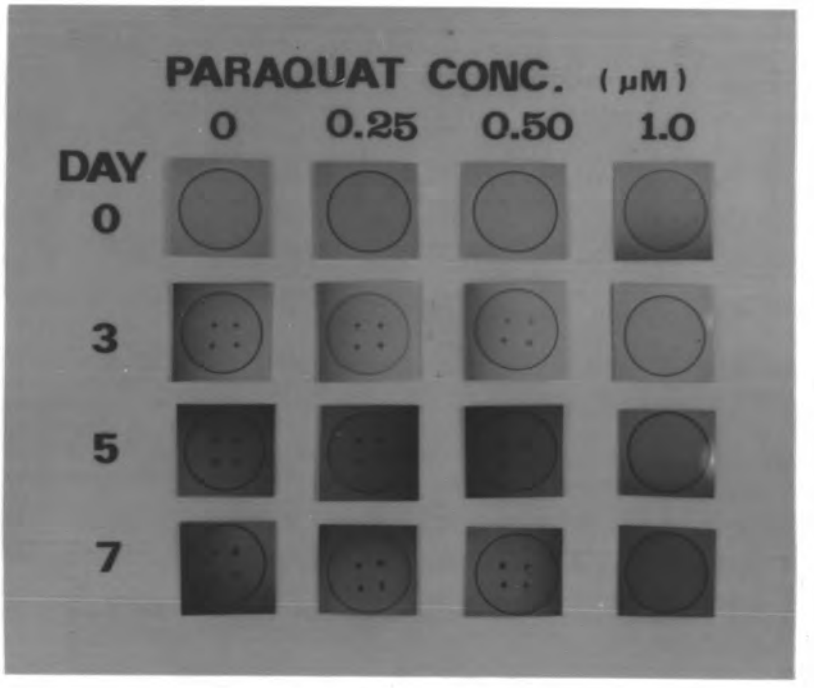
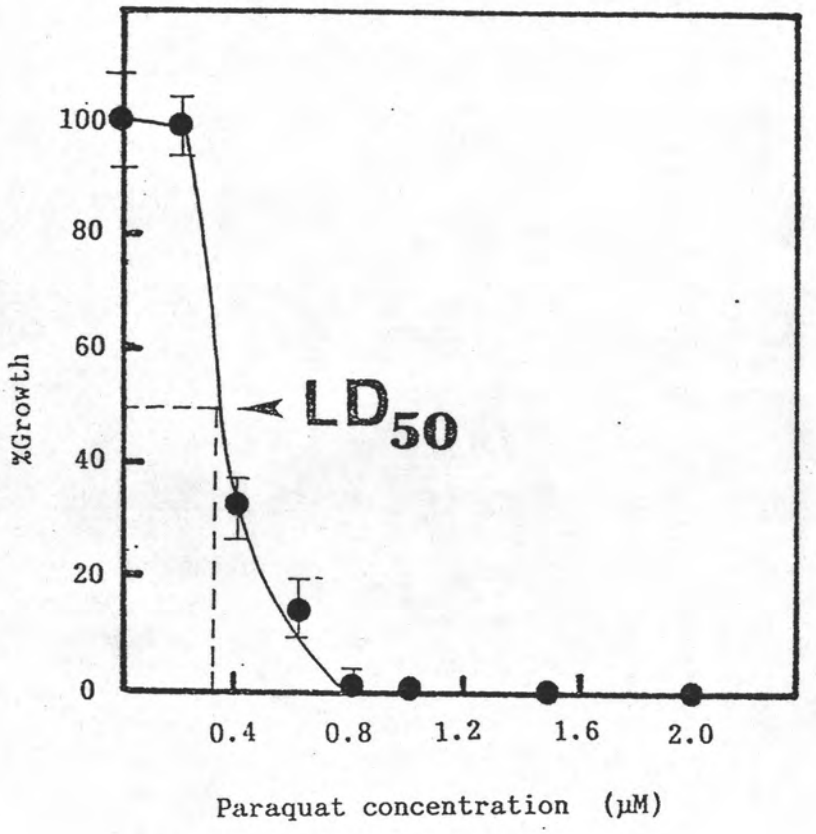


Figure 3.4 Effect of paraquat on growth of Chlamydomonas reinhardtii 137c.

Cells from exponential culture were exposed to paraquat for two cycles of 6 hours illumination. After treatment cells were grown on acetate medium in the dark.

The percentage of growth was calculated by counting the number of colonies as a fraction of control growth without the herbicide treatment.



3.2.3 Effect on Viability Wild type cells of C. reinhardtii were photosynthetically grown in liquid medium containing various levels of paraquat and growth was determined by cell counting together with mortality staining. The result was demonstrated in Fig. 3.5 that the increment of the herbicide upto 0.1 μM did not cause abnormal cell division as well as cell mortality during exponential growth, however slight effect of paraquat on cell viability could be seen on day fourth which was one day after stationary phase. When paraquat concentration was increased to 0.2 μM, growth inhibition of C. rienhardtii 137c was clearly observed and the dead cells were earlier detected. By the inhibitory effect at this paraquat level, over 90% of cell mortality was observed at the stationary growth phase.

3.4 Construction of Paraquat Resistant Mutants by Selection under the Herbicide Pressure

3.4.1 First Step Selection When the preliminary toxicity of paraquat was determined by spot test the concentration of 1 μM completely killed C. reinhardtii 137c wild type cell in one week. However the herbicide concentration at 2 μM was chosen as selective pressure of the first selection. Ten series of 10^6 wild type cells were directly plated on the medium containing 2 μM paraquat. Mutants were recovered at the frequency of $1x10^{-6}$. One mutant, namely PPQ-1 , was selected for further study due to its relatively fast growing with bright green colony on paraquat medium.

Figure 3.5 Effect of paraquat on growth and viability of Chlamydomonas reinhardtii 137c.

Cells (10^5cells/ml) were grown photosynthetically under various concentrations of paraquat.

(a.) Microscopic observations of 137c cells from day 4 culture in the presence of 0.2 µM paraquat after staining with erythrosine B (x200). Dead cells were red stains as indicated by arrows

(b.) Growth which was measured by cell number in culture medium at various growth periods.

- ■ —— paraquat free medium
- ▨ ---- 0.05 µM paraquat
- ⁘ ····· 0.10 µM paraquat
- ▩ —·—· 0.20 µM paraquat

The number of dead cells was also indicated by blank space.

Fig 3.5a

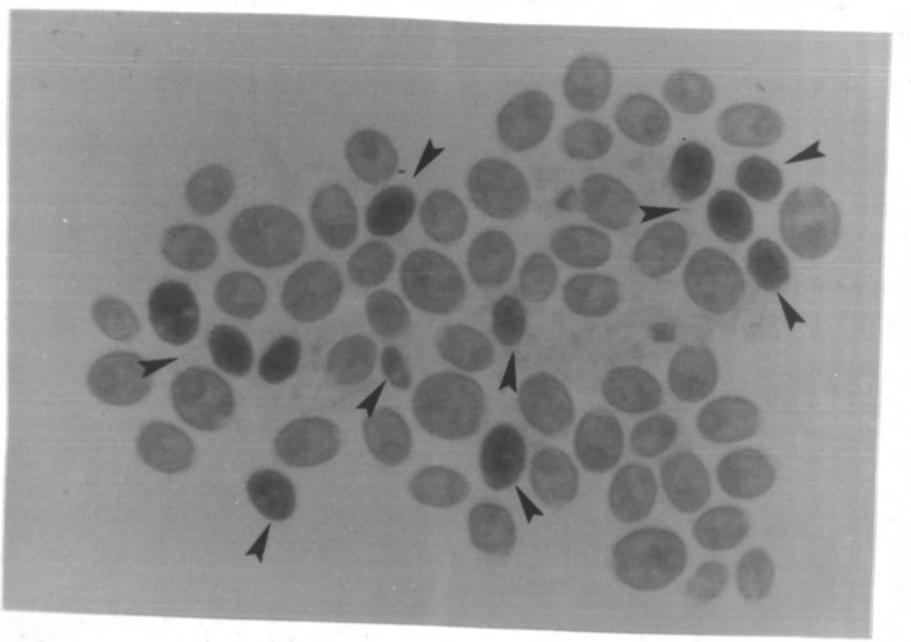
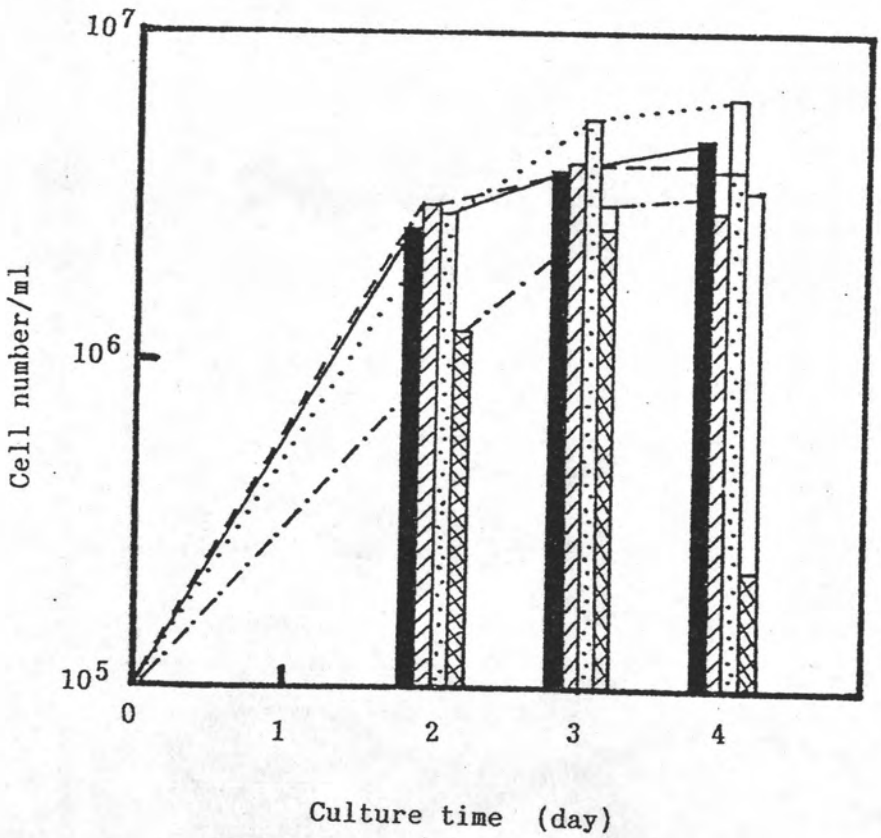


Fig. 3.5b



It was found that PPQ-1 hardly grew in liquid medium supplemented with 2 μM paraquat. However results which could be seen in Fig. 3.6 illustrated that the growth pattern of PPQ-1 in paraquat free medium or the medium plus 0.5 μM paraquat was not significantly different from the growth pattern of the original wild type. Supplementation of paraquat at 1 μM into the growth medium affected PPQ-1 growth. It should be noted that under liquid culture conditions wild type was completely killed by 0.5 μM paraquat.

After PPQ-1 was successively subcultured in 1 μM paraquat medium for a period of time, adaptation of cell to the medium was noticed (Fig. 3.7). The maximum growth of PPQ-1 after the fourth subculture (PPQ-1/sub 4) increased about 5 folds in comparison with the original culture (PPQ-1/sub 0). This growth pattern of PPQ-1/sub 4 remained constant through a long term subculture to ten times.

Measurement for the LD_{50} of PPQ-1 to paraquat was performed with cells at various time intervals of subculturing (Fig. 3.8). It was found that successive subcultures of PPQ-1 brought about significant decrease in the LD_{50} value, that was 3.25 μM for PPQ-1/sub 4 and 2.55 μM for PPQ-1 /sub 9.

3.4.2 Second Step Selection In order to achieve the higher paraquat resistant strains of C. rienhardtii, the tenth subculture of PPQ-1 was further exposed to the herbicide on selective medium. Dark green colonies of eleven mutants of C. reinhardtii were obtained , at selection frequency of 1.4×10^{-6}, under the selective pressure

Figure 3.6 Growth pattern of Chlamydomonas reinhardtii PPQ-1 comparing to the 137c strain.

Photosynthetic culture was initiated on day 0 with an inoculum size of 10^5 cells/ml. Growth was determined each day at hour 6 by measuring the turbidity at 540 nm.

- ● wild type in paraquat free medium
- ○ wild type in 0.5 μM paraquat
- ▲ PPQ-1 in paraquat free medium
- ■ PPQ-1 in 0.5 μM paraquat
- □ PPQ-1 in 1.0 μM paraquat

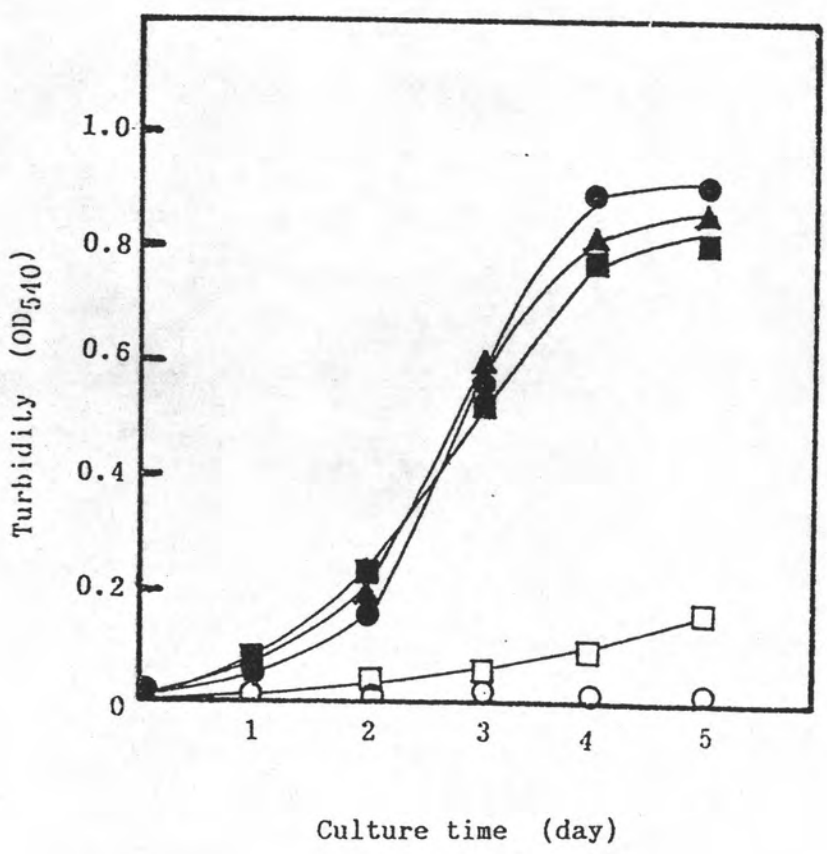


Figure 3.7 Adaptation of Chlamydomonas reinhardtii PPQ-1 in paraquat supplemented medium.

● wild type in paraquat free medium

□ PPQ-1/sub 0 in 1.0 μM paraquat

⊠ PPQ-1/sub 4 in 1.0 μM paraquat

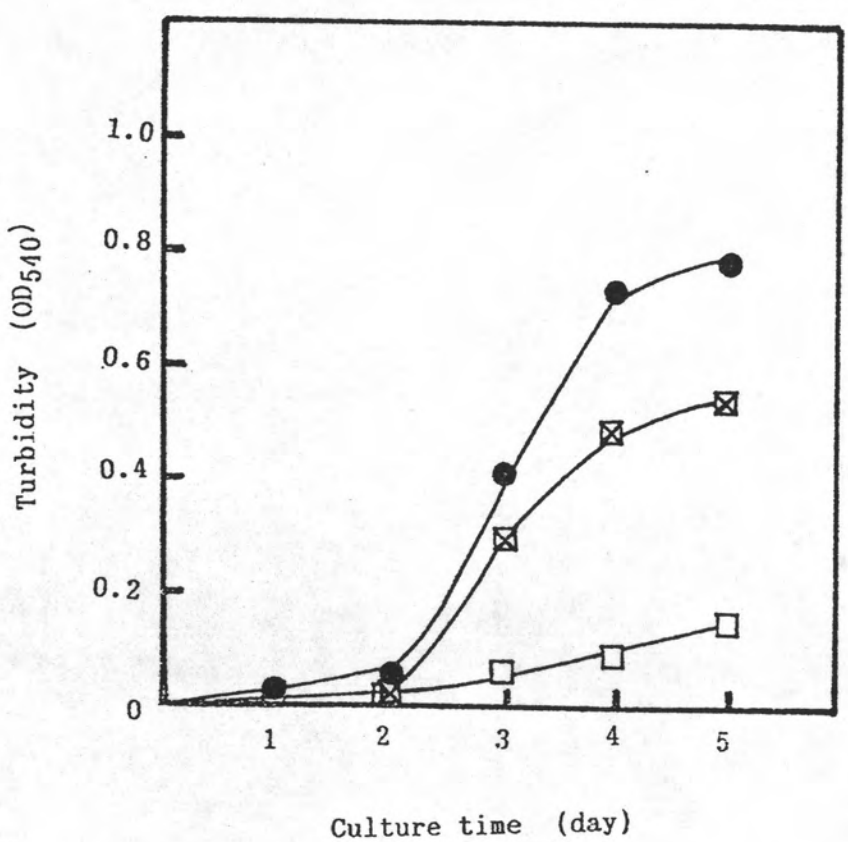


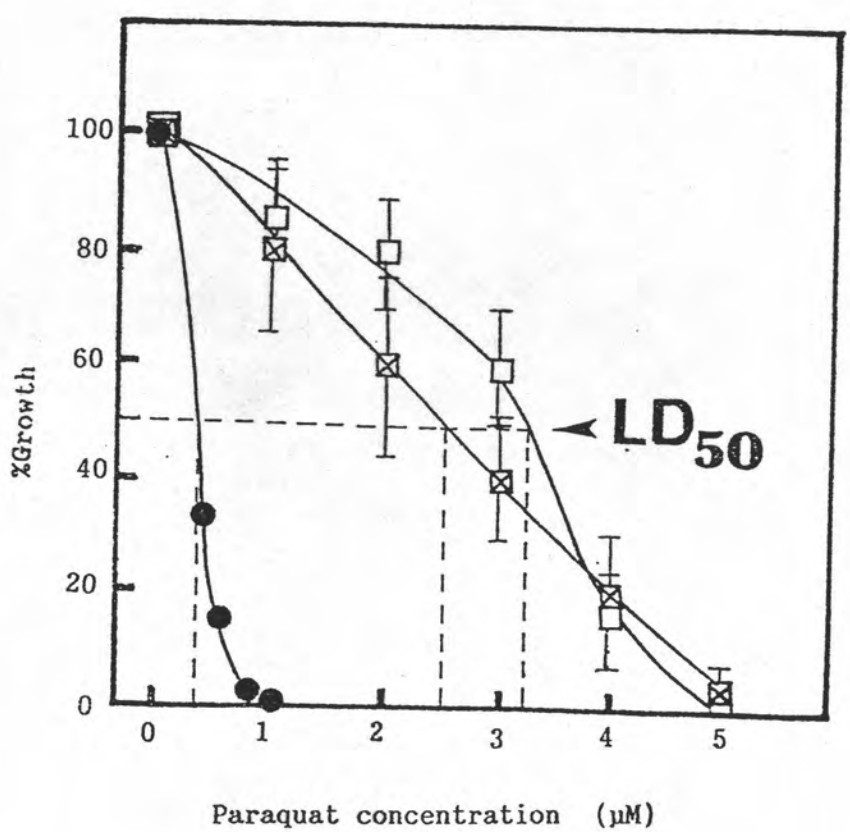
Figure 3.8 Effect of paraquat on growth of Chlamydomonas reinhardtii PPQ-1.

Cells from exponential culture were exposed to paraquat for two cycles of 6 hours illumination. After treatment cells were grown on acetate medium in the dark. The percentage of growth was calculated by counting the number of colonies as a fraction of control growth without the herbicide treatment.

● wild type

□ PPQ-1/sub 4 in 1.0 µM paraquat

⊠ PPQ-1/sub 9 in 1.0 µM paraquat



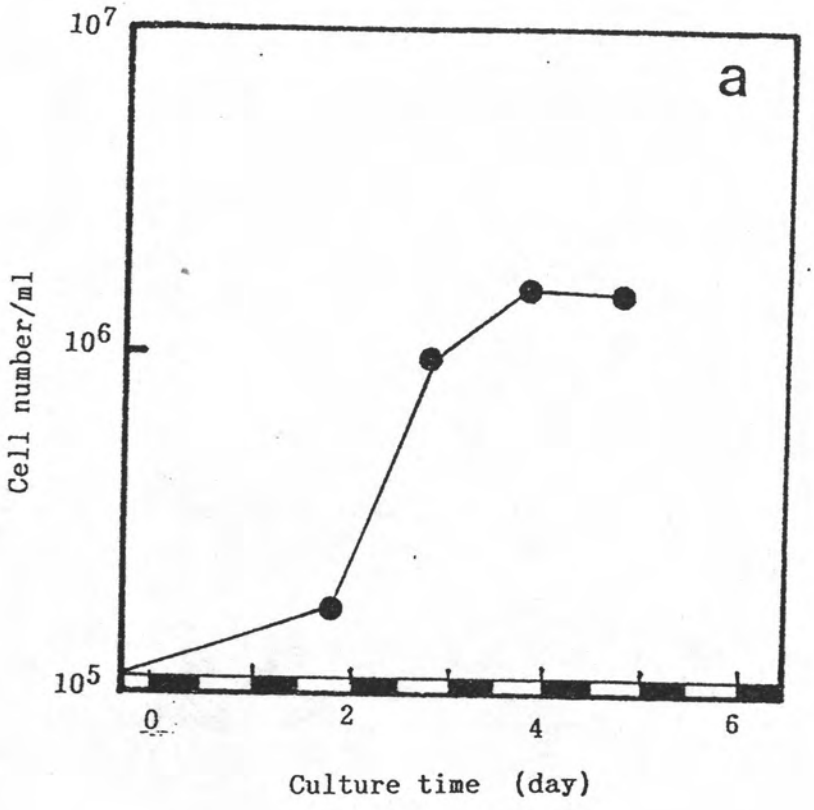
of 10 µM paraquat. Among these, three mutants, namely PPQ-10/1, PPQ-10/2, and PPQ-10/3, were picked up on the basis of their relative fast growing character. Unfortunately the first two isolates lost their resistance to paraquat during maintenance under 6 µM paraquat pressure.

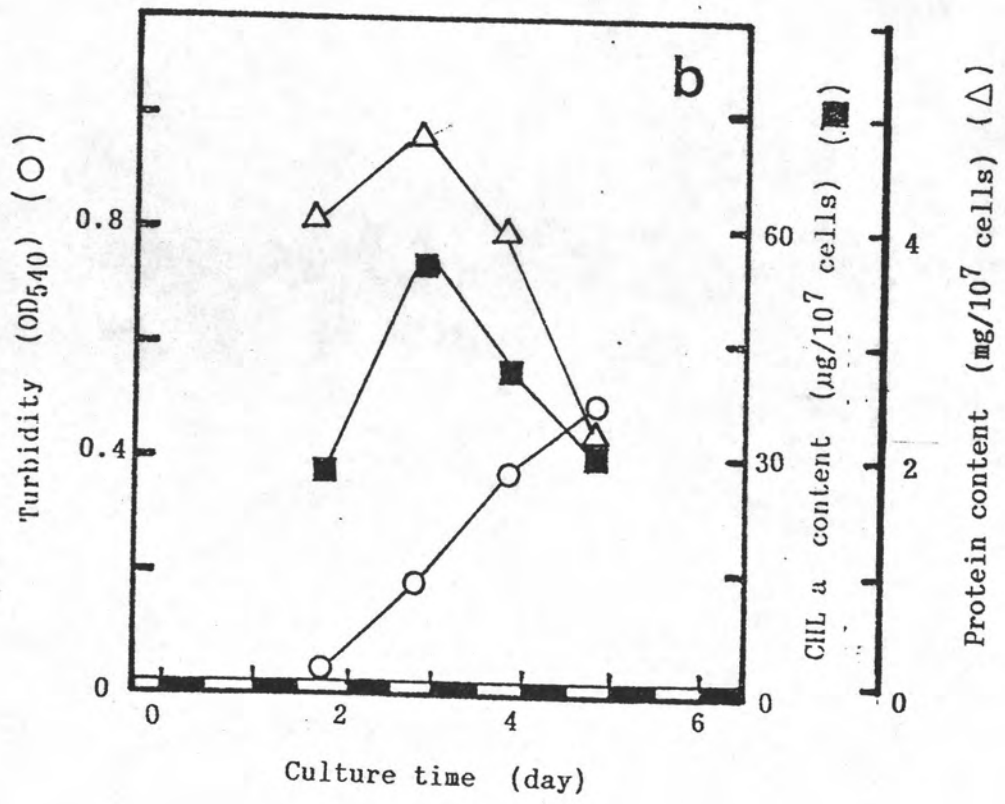
3.4.3 Growth Characteristic of C. reinhardtii PPQ-10/3 Growth pattern of PPQ-10/3 cell line in the paraquat free medium was different from that of the wild type (Fig.3.9). When started with an equal inoculum (10^5 cells/ml), PPQ-10/3 grew maximally to $1.0x10^6$-$2.0x10^6$ cells/ml in four days (Fig. 3.9a) which was about 5-7 times lower than that found in the wild type. Cell turbidity was also corresponded to the increment of cell density throughout the growth period (Fig. 3.9b).

The amount of chlorophyll a in PPQ-10/3 (Fig. 3.9b) was 2-3 folds higher than that of the wild type. The level of chlorophyll a varied from approximately 28 µg/10^7 cells at early log phase to a peak in active growing cells during mid log phase (54 µg/10^7 cells) and then declined to a normal level thereafter.

The profile of protein content along the growth of PPQ-10/3 was the same as that illustrated for 137c with the maximum of 4.72 mg /10^7 cells at the mid log period which was about 2.5 folds higher than that of 137c.

Figure 3.9 Growth curve of Chlamydomonas reinhardtii PPQ-10/3. Photosynthetic culture was initiated at hour 6 with an inoculum size of 10^5 cells/ml. Day 0 in the figure represents inoculation day. Growth was determined as (a.) number of cell in the culture medium (b.) turbidity at 540 nm, chlorophyll a (CHL a) content, and protein content.


a
10^7
10^6
10^5
Cell number/ml
0
2
4
6
Culture time (day)


b
Turbidity (OD_{540}) (○)
0.8
0.4
0
60
30
0
Chl a content (μg/10^7 cells) (■)
4
2
0
Protein content (mg/10^7 cells) (△)
0
2
4
6
Culture time (day)

3.4.4 Effect of Paraquat on C. reinhardtii PPQ-10/3

3.4.4.1 Preliminary Test for Paraquat Resistance

Spot test was first used for testing of PPQ-10/3 response to the herbicide. As shown in Fig. 3.10, despite that this isolate was obtained by selection under 10 µM paraquat, partial inhibition of its growth could be observed at this herbicide level which was illustrated by the retardation at the early period of darkening of the green areas. However C. reinhardtii PPQ-10/3 strain was shown, by the spot test, to resist to paraquat as high as 20 µM since it could somewhat grow at this herbicide concentration.

3.4.4.2 Lethal Dose-50 Using the same procedures as described for wild type, it was found that exposure of PPQ-10/3 to paraquat at 6.0 µM did not have any effect on the algal growth. From the inhibitory curve of paraquat (Fig. 3.11), LD_{50} of the herbicide to PPQ-10/3 could be evaluated at 6.48 µM which was approximately 20 times higher than the value obtained with the wild type (0.36 µM). It was also obviously noticed that substantial increment of paraquat up to 7.2 µM had slight additional effect on PPQ-10/3.

3.4.4.3 Effect on Viability The growth pattern of PPQ-10/3 cells in the medium containing 1 µM paraquat as determined by the total cell count was similar to that of the normal growth in paraquat free medium in which dead cells were considerably

Figure 3.10 Spot test of paraquat effect on Chlamydomonas reinhardtii PPQ-10/3.

Cells were spotted on medium containing paraquat and exposed to light (12 hours) and dark (12 hours) alternately. Bleaching phenomena were observed on day 0, 3, 5, and 7.

(lane 1) paraquat free medium

(lane 2) 10 μM paraquat

(lane 3) 15 μM paraquat

(lane 4) 20 μM paraquat

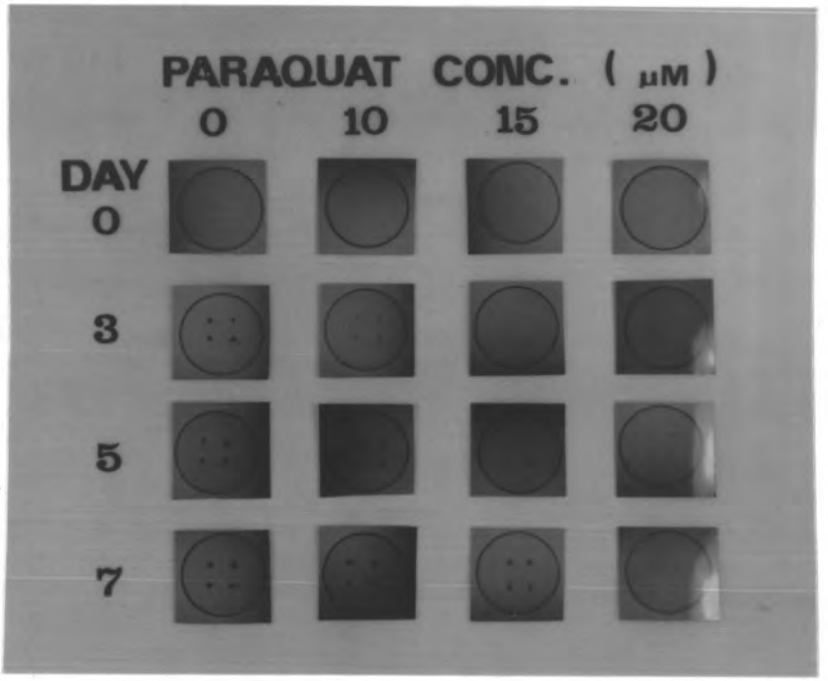


Figure3.11 Effect of paraquat on growth of Chlamydomonas reinhardtii PPQ-10/3.

Cells from exponential culture were exposed to paraquat for two cycles of 6 hours illumination. After treatment cells were grown on acetate medium in the dark. The percentage of growth was calculated by counting the number of colonies as a fraction of control growth without the herbicide treatment.

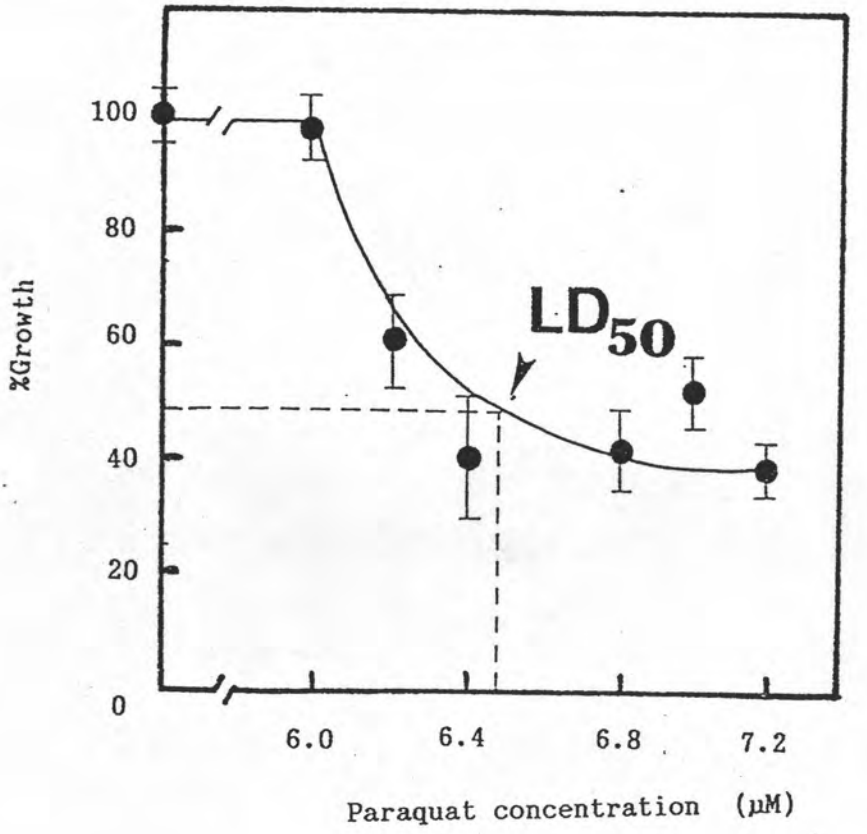
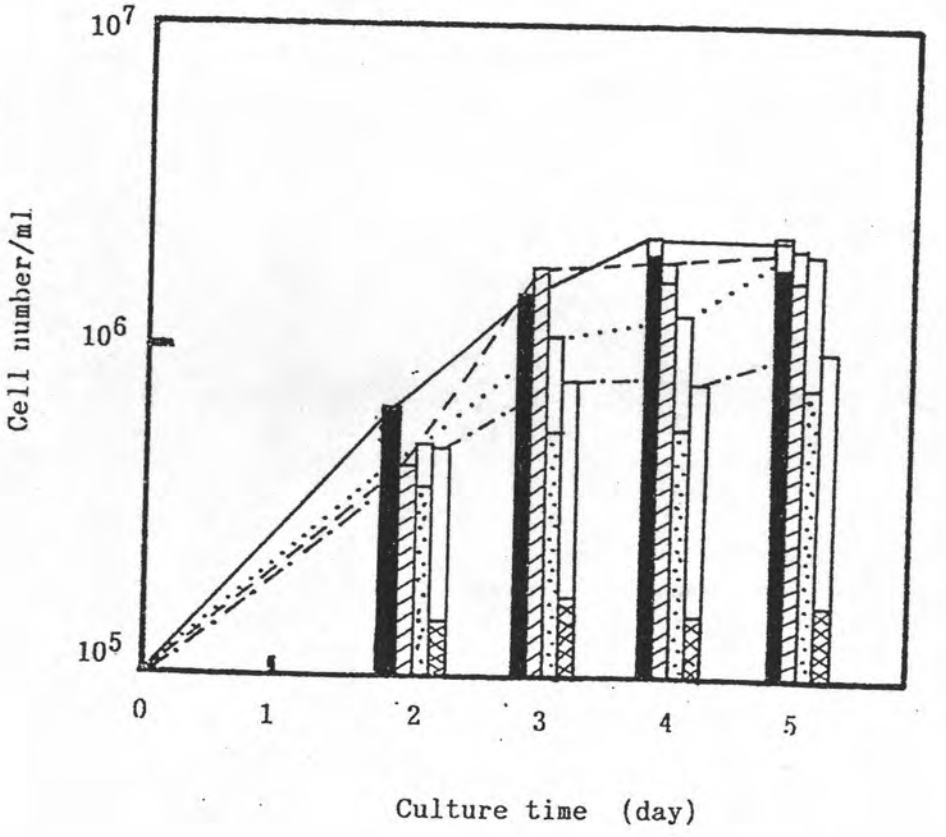


Figure 3.12 Effect of paraquat on growth and viability of Chlamydomonas reinhardtii PPQ-10/3.

Cells were grown photosynthetically at an inculum size of 10^5 cells/ml. Growth was measured by cell counting. Dead cells were determined by erythrosine B staining.

- ——— paraquat free medium
- – – – 1 μM paraquat
- · · · · · 2 μM paraquat
- —·—·— 4 μM paraquat

The number of dead cells was indicated by blank space.



detected at stationary phase (Fig. 3.12). The toxicity of the herbicide which could be seen at the higher concentration was indicated by growth retardation accompanying with an increase in number of dead cells throughout the culture time. During cultivation of PPQ-10/3, higher number of dead cells of 72-78% was observed in the presence of 4 μM paraquat while only 19-26% was found at 2 μM paraquat.

3.5 Construction of Paraquat Resistant Mutants by 5-Fluorodeoxyuridine (FdUd) Mutagenesis

3.5.1 First Mutagenesis Mutagenesis was achieved by 1 mM FdUd and most of the non-desired mutants along with non-mutagenized cells remaining were destroyed by treatment with 0.5 μM paraquat. In the last step, the paraquat resistant mutants were screened on medium containing 2 μM of paraquat. Under these conditions, no mutant was found from the FdUd non-treated C. reinhardtii 137c (wild type cells).

The resulting mutants were classified into 2 categories (a) mutants with normal green colonies growing either fast or slow , and (b) slow growing mutants with pigment deficiency , which appeared as yellowish green colonies. Among 260 isolates of the fast growing mutants, only 68 isolates remained after a few subcultures on the selective medium. The resistance of these mutants to paraquat was investigated. It was found that, by using spot test, five mutants namely UPQ-1, UPQ-2, UPQ-3, UPQ-10, and UPQ-20 exhibited resistance against paraquat up to 6 μM.

The UPQ-2 isolate was chosen as a representative for second mutation on the criteria that it was the only mutant which could be capable to be cultivated in liquid medium containing the herbicide as high as 2 µM.

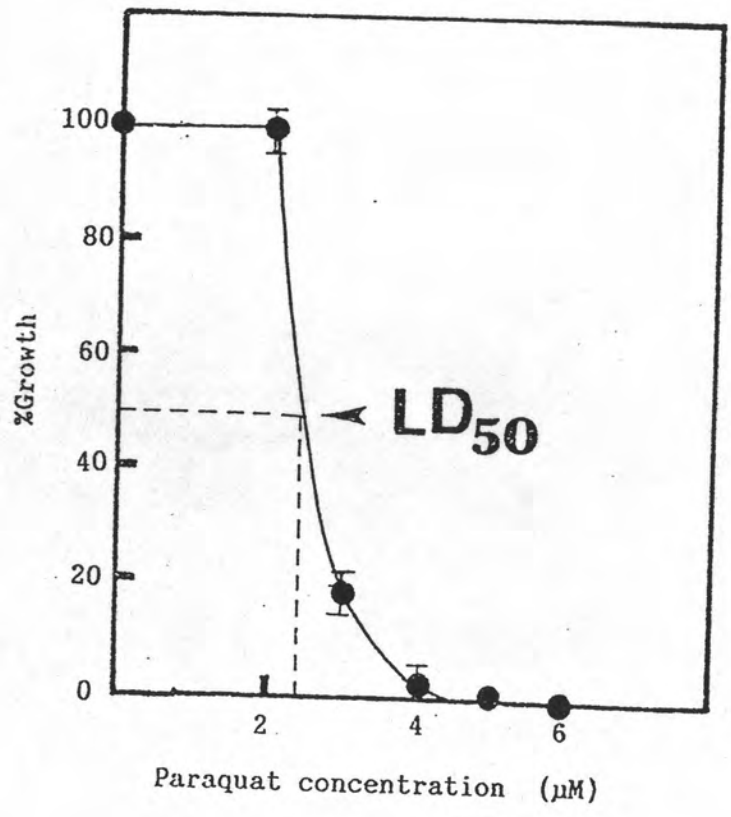
UPQ-2 was subcultured in TMP medium for 5 weeks to establish the cell suspension appearance before measuring the paraquat LD_{50}. It was evaluated (Fig. 3.13) that UPQ-2 was 50% killed by paraquat at the level of 2.4 µM.

3.5.2 Second Mutagenesis Following the procedures of the first mutagenesis step, cell suspension of UPQ-2 after 5 subcultures in TMP medium was subjected to second FdUd mutagenesis by carrying out the same procedure as described in the first mutation. After enrichment by exposing the treated cells to paraquat at 4 µM with a period of illumination as designed for LD_{50} determination, paraquat resistant mutants were recovered on selective plates containing 10 µM paraquat. The frequency of mutation was 2.86×10^{-6}.

From 96 colonies selected from 10 µM paraquat containing media, 81 mutants were lost in the first subculture on the selective medium. When the remaining mutants were maintained for a few passages, most of them gradually faded by the toxicity of paraquat even though the concentration of the herbicide was reduced to 6 µM. Only two mutants, namely UPQ-S1 and UPQ-S6, were recovered. However UPQ-S1 was chosen for further studies of paraquat resistance mechanisms comparing to the PPQ-10/3 owing to its relatively fast growing.

Figure 3.13 Effect of paraquat on growth of Chlamydomonas reinhardtii UPQ-2.

Cells from exponential culture were exposed to paraquat for two cycles of 6 hours illumination. After treatment cells were grown on acetate medium in the dark. The percentage of growth was calculated by counting the number of colonies as a fraction of control growth without the herbicide treatment.



3.5.3 Growth Characteristic of UPQ-S1

The C. reinhardtii UPQ-S1 cells were grown under the same conditions as described for the wild type and PPQ-10/3 in order to compare the properties and characteristics of paraquat resistant mutants. It was illustrated in Fig. 3.14a that the UPQ-S1 culture in paraquat free medium reached stationary phase in four days with a maximal cell density of $3x10^6$ cells/ml. This was merely about one-half of the wild type growth. The amount of chlorophyll a in UPQ-S1 was fairly constant in the range of 25-30 $\mu g/10^7$ cells throughout exponential growth which was not significantly different from that of the wild type cell. However the chlorophyll a content of UPQ-S1 tended to decrease during the stationary growth period (Fig. 3.14b).

The cellular protein content of UPQ-S1 cells was also higher than that of the wild type 137c with the maximum value at 4.64 mg/ 10^7 cells.

3.5.4 Effect of Paraquat on UPQ-S1

3.5.4.1 Preliminary test for Paraquat Resistance

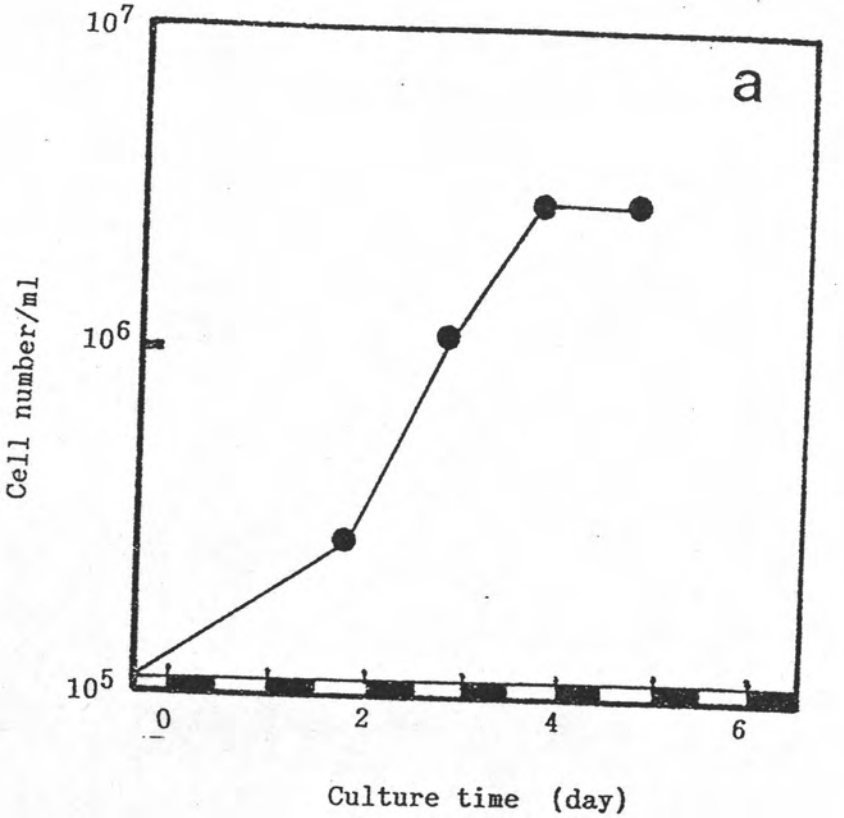
This could be done as reported in the wild type and PPQ-10/3, the paraquat toxicity to UPQ-S1 was also evaluated by spot test. Apparently this strain was lower resistant to the paraquat than PPQ-10/3 because greening of the algal area was retarded by 8 μM of the herbicide (Fig. 3.15). The increment of paraquat concentration up to 10 μM clearly inhibited growth of UPQ-S1.

Figure 3.14 Growth curve of Chlamydomonas reinhardtii UPQ-S1.

Photosynthetic culture was initiated at hour 6 with an inoculum size of 10^5 cells/ml. Day 0 in the figure represents inoculation day. Growth was determined as

(a.) number of cell in the culture medium

(b.) turbidity at 540 nm, chlorophyll a (CHL a) content, and protein content.


a
10^7
10^6
10^5
Cell number/ml
0
2
4
6
Culture time (day)

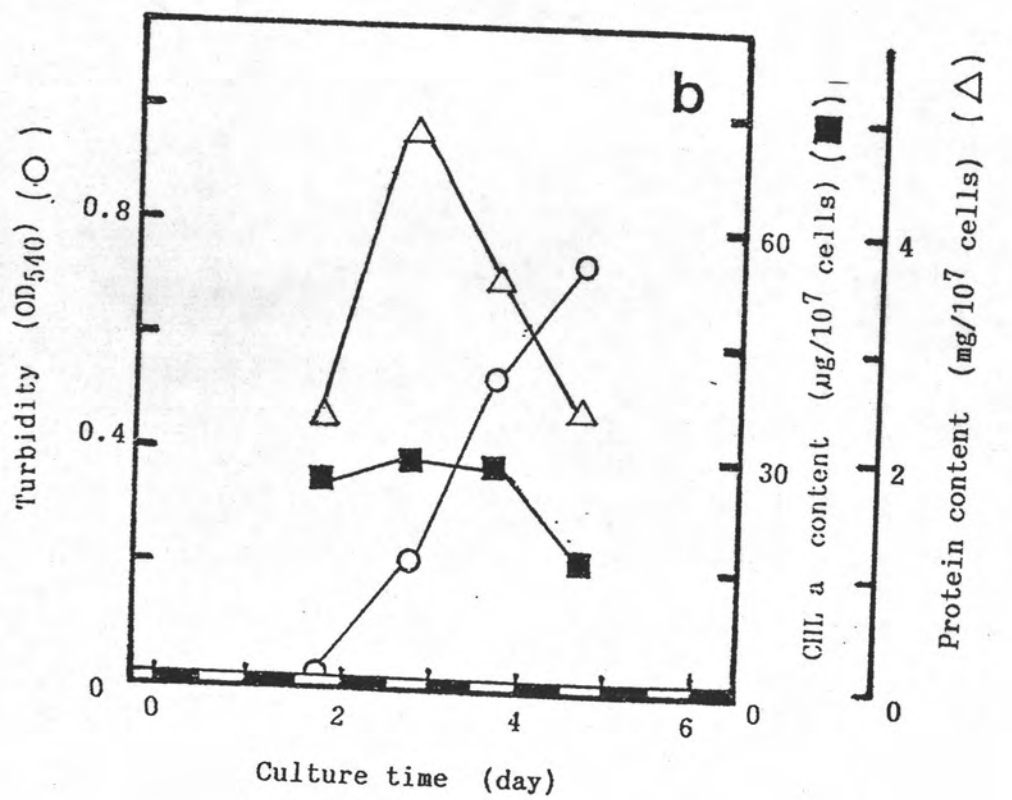

b
Turbidity (OD_{540}) (○)
Chl a content (μg/10^7 cells) (■)
Protein content (mg/10^7 cells) (△)
0
0.4
0.8
30
60
2
4
0
2
4
6
Culture time (day)

Figure 3.15 Spot test of paraquat effect on *Chlamydomonas reinhardtii* UPQ-S1.

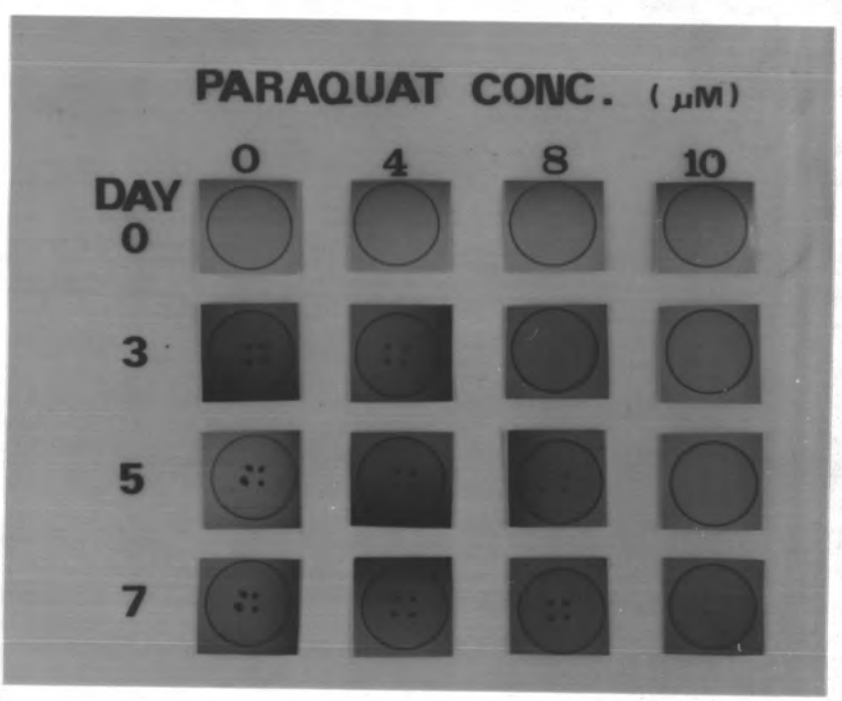
Cells were spotted on medium containing paraquat and exposed to light (12 hours) and dark (12 hours) alternately. Bleaching phenomena were observed on day 0, 3, 5, and 7.

(lane 1) paraquat free medium

(lane 2) 4 μM paraquat

(lane 3) 8 μM paraquat

(lane 4) 10 μM paraquat



3.5.4.2 Lethal Dose-50 The level of paraquat toxicity to UPQ-S1 was also determined in terms of LD_{50}. Cells were exposed to paraquat solution and illumination under the same conditions as of wild type and PPQ-10/3. From the response curve (Fig. 3.16), the paraquat LD_{50} of UPQ-S1 was estimated to be 3.07 µM.

3.5.4.2 Effect on Viability When 1 µM paraquat was included in liquid medium, photosynthetic culture cells of UPQ-S1 established slight decrease in growth yield and viability counting (7-14% death) during the stationary growth phase (Fig. 3.17). The increase in cell mortality (20%) was also accounted at higher herbicide concentration (2 µM) which was observed together with a slower growth rate. However it was also noticed that while most of the UPQ-S1 was killed by 4 µM paraquat, a portion of cells still survived, enable the cells to maintain their growth and division.

Figure 3.16 Effect of paraquat on growth of Chlamydomonas reinhardtii UPQ-S1.

Cells from exponential culture were exposed to paraquat for two cycles of 6 hours illumination. After treatment cells were grown on acetate medium in the dark. The percentage of growth was calculated by counting the number of colonies as a fraction of control growth without the herbicide treatment.

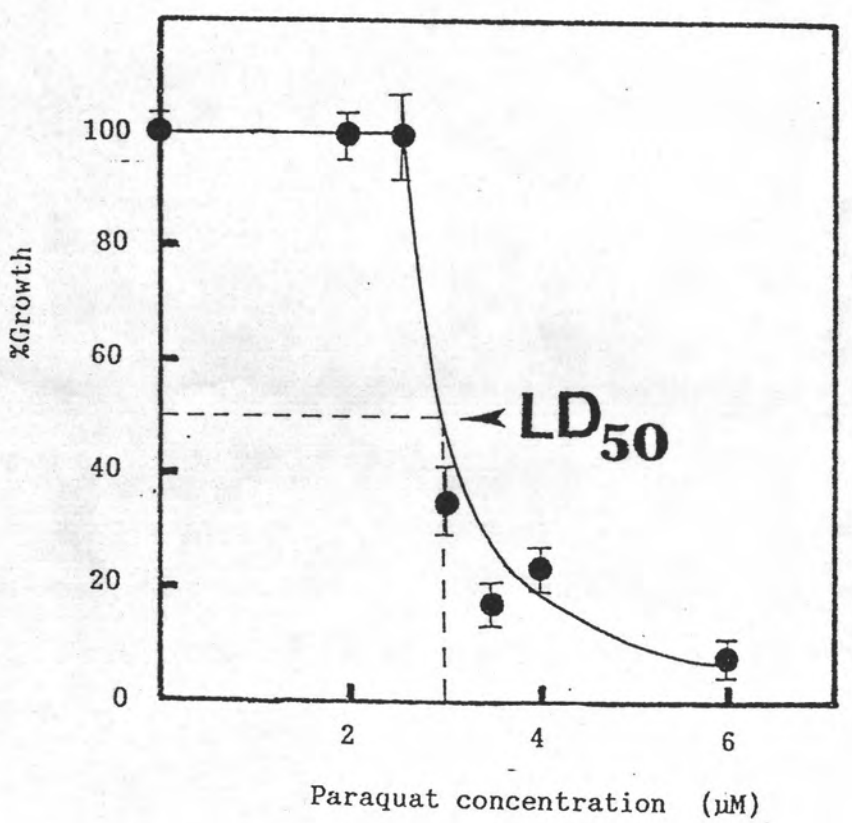


Figure 3.17 Effect of paraquat on growth and viability of Chlamydomonas reinhardtii UPQ-S1.

Cells were grown photosynthetically at an inculum size of 10^5 cells/ml. Growth was measured by cell counting. Dead cells were determined by erythrosin B staining.

- ■ —— paraquat free medium
- ▨ ----- 1 μM paraquat
- ⁘ ····· 2 μM paraquat
- ▩ —·—·— 4 μM paraquat

The number of dead cells was indicated by blank space.