# CHAPTER I

# INTRODUCTION

## 1.1 Background of Herbicide Resistance

One approach to improve crop productivity is to overcome weeds in the planting areas. Application of herbicides provide the most cost-effective method in controlling weeds, far better than any classical mechanical means. Among the 200 major world-wide weed species, 60 species are troublesome to Thai farmers. A large number of structurally divergent chemicals are at present in use as herbicides which, regarding to their actions on weeds, can be categorized as selective and non-selective herbicides (Ramulu,1985).

In almost all instances, the selective herbicides have been developed based upon the selectivity of their effects on crops and weeds. An ideal herbicide is a compound which is toxic to a wide-range or all weeds encountered in an agricultural production system, but which is tolerated by one or several crop species. On another hand, various kinds of weeds are found in a cultivation area depending on geographic parameters, land management and culture practices, then more than one selective herbicide should be applied to potentially eliminate weeds. Non-selective herbicides, such as atrazine, glyphosate, and paraquat, which have broad spectrum to weeds species and consequently to crops as well, are therefore utilized with satisfaction.

It has been recently reviewed that after long-term the application, as well as in the case of insecticides or fungicides , a varieties of weeds have become resistant to the available herbicides (Bandeen et at, 1982 ; Watanebe et al, 1982 ; Kato et al , 1982 ; Stalker et al, 1985). Then larger dosage of herbicide should be applied which possibly causes crop damage. The herbicides in use at the present time have all been selected by empirical screening procedures involving analysis of very large number of chemicals. These processes give continuing decrease in the probability of identifying a new and successful market product. Moreover, it needs a lot of time and money to develope a new herbicide (Shaner, 1986). There has been therefore increased interest in extending the usage range of the existing ones. This can be done if herbicide resistant cultivars could be constructed especially those resistant to broad spectrum herbicides, such as atrazine resistance, glyphosate resistance, and paraquat resistance (Matsunaka, 1985).

Since 1980, breeding of crop plants which are resistant to a herbicide has remarkably been one of the popular research subject in weed science. To achieve a herbicide resistant crop , it is in need to understand the following information from many sources as basic criteria ;

1) The interesting herbicide would cost-effectively control weeds in a given crop.

2) Mode of the herbicide action should be clarified.

3) The herbicide resistance will not cause yield reduction of crops.

4) Different types of resistance should be considered to obtain the most appropriate resistant genotype.

Nevertheless, major problems in construction of herbicide resistant crops are the lack of resistance gene pools owing to the very rare number of resistant cultivar varieties which naturally occurred (Weed Research Organization, 1973). Another problem is concerned with the metabolic basis for herbicide resistance. There are many probable mechanisms by which cells can avoid the herbicide toxicity including uptake mechanism for the herbicide in whole plant system and detoxification of the herbicide. Very few studies have been devoted to determine mechanisms of herbicide resistance (Hughes ,1983). If a specific biochemical basis for herbicide resistance can be determined in conjunction with specific genetic basis, it might be possible to isolate and transfer the resistance gene via recombinant DNA technology.

Herbicide reistances have been investigated in many circumstances, yet molecular basis of the resistance is established in only a few cases, i.e. atrazine resistance (Erickson et al, 1985 1985; Hirschberg and McIntosh,1983), glyphosate resistance (Stalker et al,1985 ; Comai et al,1985), and sulfometuron methyl resistance (Larossa and Falco,1984).

## 1.2 Paraquat Toxicity

Paraquat (1,1-dimethyl-4,4-bipyridinium dichloride) is a

herbicide of much importance since data from Weed Science Society of Thailand indicate that it has been imported, from the year 1979, at the first rank (about 40% of the total herbicides) (ทนง พงษ์พานิช, 2527) and that the production of paraquat (the product of I.C.I. Company, England ; personal communication) is now recognized as the highest comparing to any other herbicide in the world.

Paraquat is a broad spectrum contact herbicide with efficient kill of annual plants and severe defoliation of perennial species. It is used as a non-selective herbicide prior to planting of a crop and also as a dessicant. It is more effective to grasses and a wide range of weed species including pre-emergence weed control. The mode of action involves photosynthesis, light, and, molecular oxygen (Merkle et al, 1965 ; Brian, 1967 ; Dodge 1982). It has a strong negative redox potential ($E_0$ = -o.446 V) which is very similar to that of ferredoxin ($E_0$ = -0.430 V), an electron acceptor in photosystem I (PS I) (Fig. 1.1). Hence paraquat is proposed to compete electron transfer in photosystem I at the site of ferredoxin, then it is directly photoreduced to a monovalent cation radical. Paraquat radical is highly reactive with triplet oxygen ($^3O_2$) (Fig. 1.2) to produce superoxide radical ($O_2^-$) at a rate of $7.7 \times 10^8$ $M^{-1}.sec^{-1}$ (Farrington et al,1973). This hypothesis is strongly evident by the findings that in the presence of the herbicide, illuminated chloroplasts generated higher level of superoxide ( Epel and Neuman, 1973 ; Miller and McDowell, 1975 ; Harbor and Bolton, 1975). Excess superoxide results in the

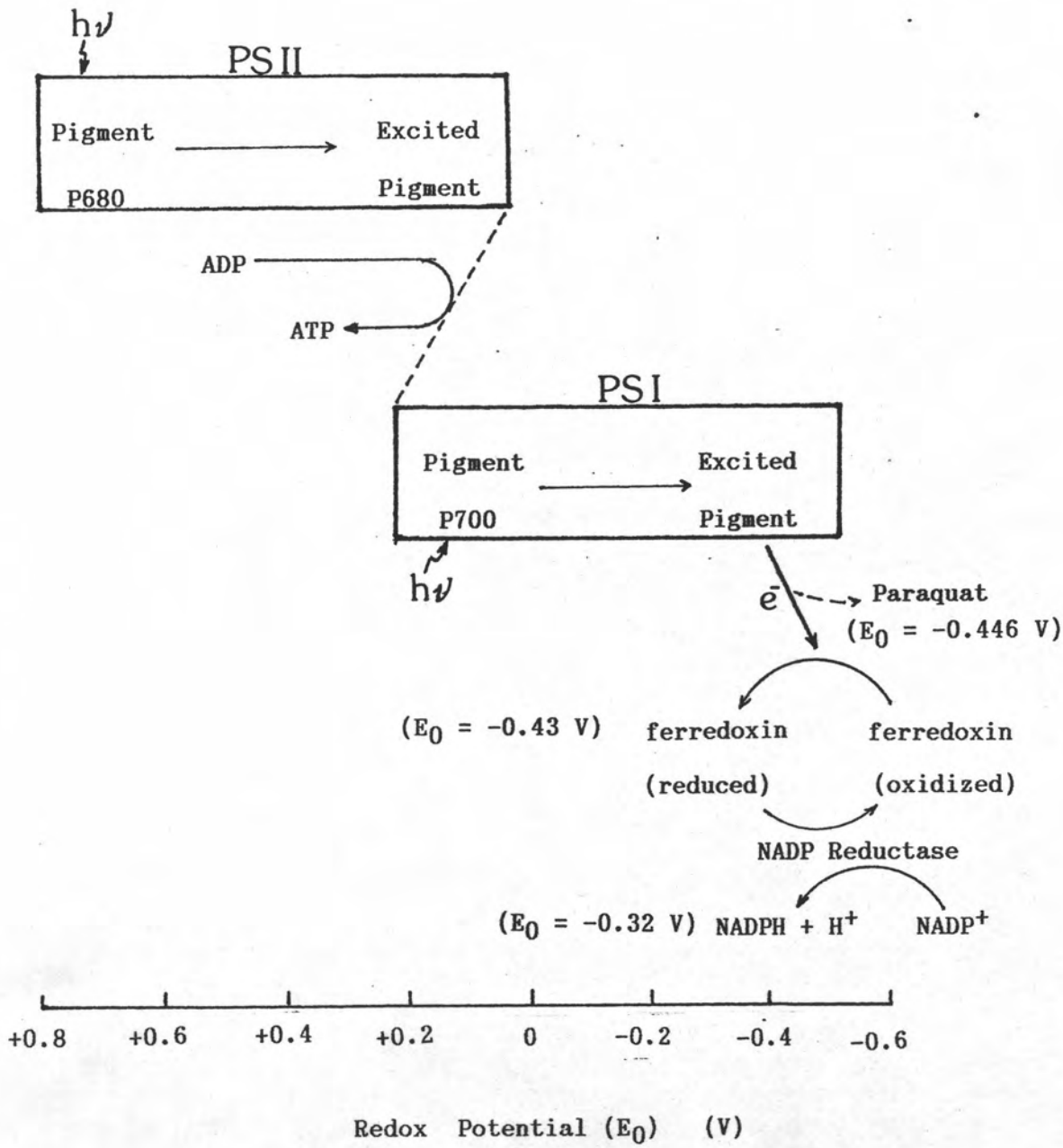


Figure 1.1 Proposed mechanism of paraquat action at the site of photosynthesis in plant (Calderbank and Slade, 1976)

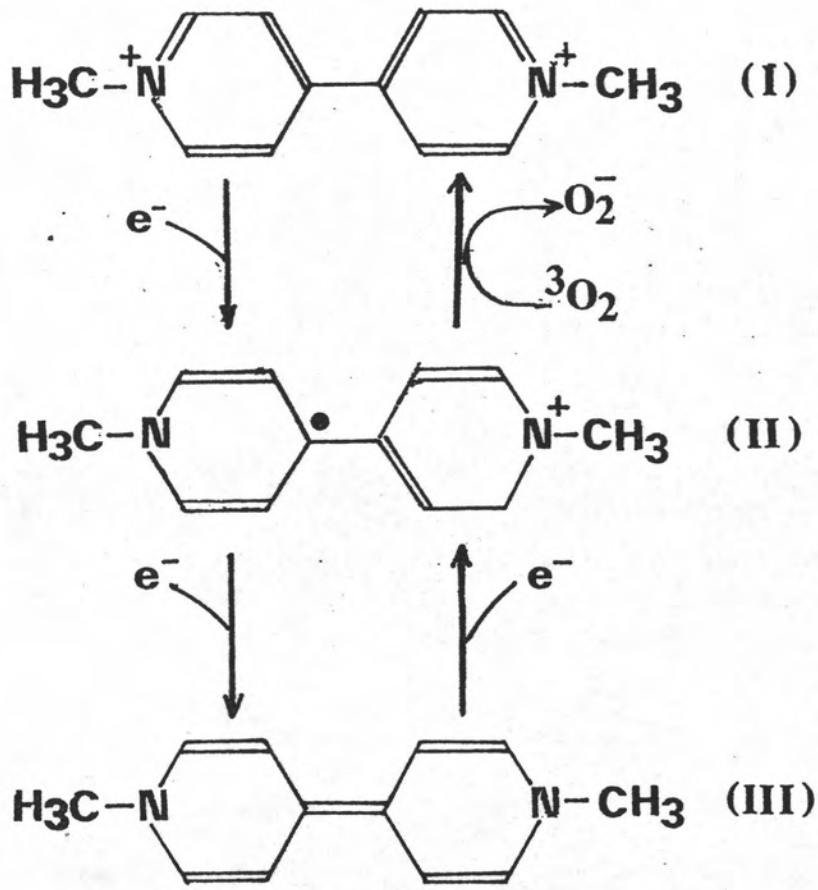


Figure 1.2 Formation of paraquat radical and oxygen radical formula (I): oxidized paraquat, formula (II): one-electron reduced cation paraquat radical, and formula (III): two-electron reduced paraquat (Asada and Takahashi ,1987).

production of hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^{\cdot}$) (Winterbourn,1981 ; Winterbourn and Sutton, 1984). These highly reactive species generally cause peroxidation of unsaturated fatty acids with damage to cellular and subcellular membrane (Chia et al ,1981 ; Vaughn and Duke, 1983). From electron microscopic analysis of plant cells affected by paraquat toxicity (Calderbank and Slade, 1976), it revealed destruction of lipid bilayer conformation at chloroplast membrane.

Hydrogen peroxide is not only produced by the enzymatic disproportionation of superoxide dismutase, but it is also generated by spontaneous reduction of superoxide by ascorbate, thiols, ferredoxin, and manganese ions. The importance of hydrogen peroxide is that it displays a common molecule in production of hydroxyl radical, the most reactive molecule known up to date, both by interaction with superoxide and paraquat radicals which are non-enzymatically catalyzed by transition metals, especially iron and copper ions. Many workers have reported the toxicity of hydroxyl radical in various biological systems (Brown and Fridovich,1980 ; Gutteridge and Halliwell, 1982), it should be however considered that because of the high reactivity of hydroxyl radical with almost all cell components at nearly diffusion controlled rates, this reactive radical interacts with cell components only at the site where it is produced (Asada, 1987).

## 1.3 Enzyme Defense System Against Paraquat Toxicity

Superoxide dismutases (SOD) (EC 1.15.1.1) are a class of metalloproteins that catalyze the disproportionation of superoxide radical to oxygen and hydrogen peroxide. Their presence in all aerobic organisms has led to suggestions that they play a vital role in protecting cells against oxidative stress. Three metalloenzyme types of superoxide dismutases have been found in different subcellular organelles and among diverse species (Table 1.1).

Superoxide dismutases catalyze the disproportionation of superoxide through the oxidation-reduction cycle of the prosthetic copper, manganese, and iron as shown in the subsequent equations below, where $M^n$ stands for Cu (II), Mn (III), and Fe (III).

$$SOD\text{-}M^n + O_2^- \longrightarrow SOD\text{-}M^{(n-1)} + O_2$$

$$SOD\text{-}M^{(n-1)} + 2H^+ + O_2^- \longrightarrow SOD\text{-}M^n + H_2O_2$$

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$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

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Hydrogen peroxide is proposed to be scavenged by two alternative enzymes, i.e. catalase and ascorbate peroxidase owing to the common substrate. Both enzymes are distinguishable in the affinity to hydrogen peroxide and their cellular compartmentation. Catalase has a relatively high apparent $K_m$ for hydrogen peroxide and is found concentrated in a crystalline state in leaf peroxisomes

Table 1.1 Distribution of metalloenzymes of superoxide dismutase (Asada ,1987)

| Enzyme source | Cu/Zn-SOD | Fe-SOD | Mn-SOD |
|---|---|---|---|
| Prokaryote | | | |
| cytoplasm | - | + | + |
| Eukaryotic algae | - | + | + |
| Eukaryote | | | |
| cytoplasm | + | - | - |
| mitochondria | + | - | + |
| Higher plants | | | |
| cytoplasm | + | - | - |
| mitochondria | - | - | + |
| chloroplast | + | + | + |

(Asada, 1987), whereas ascorbate peroxidase is found with high catalytic activity in chloroplast and it has very low $K_m$ for the substrate hydrogen peroxide. No specific scavenger of hydroxyl radicals has been found in chloroplasts or in other organelles and other organisms (Asada and Takahashi, 1987).

Those three enzymes are proposed to co-operatively function in detoxification of oxygen reactive molecules generated by paraqaut action. Carlioz and Touati (1986) has recently described a crucial role of superoxide dismutase in protecting Escherichia coli from paraquat toxicity. Growth of the E. coli mutant having disfunction of gene coding for superoxide dismutase was inhibited by paraquat at lower concentration than the normal cell strain.

## 1.4 Paraquat Resistance in Living Organisms

Investigation of paraquat resistance was first demonstrated in Escherichia coli (Gregory et al,1973). When E. coli was grown under sublethal dose of paraquat pressure, where the production rate of superoxide was high, the biosynthesis of superoxide dismutases in this organism was induced by 4 folds within one hour after the herbicide treatment. Such increase in superoxide dismutase content was associated with an augmentation of the activity of the manganese type isozyme.

Hassan and Fridovich (1977) reported the co-operative function of superoxide dismutase with either catalase or peroxidase in detoxification of paraquat in E. coli. Addition of paraquat at

very low concentration that did not affect active aerobic growing cells caused 10 folds induction of superoxide dismutase biosynthesis in comparison to the normal growing cells. Slight increase in catalase content (2.5 folds higher) was also detected. While there was no significant change in the total activity of peroxidase.

Paraquat was shown to increase the superoxide dismutase content in a green alga Chlorella sorokiniana, and also to cause the appearance of a new electrophoretically distinct isozyme (Rabinowitch et al ,1983). Cells grown in the absence of paraquat contained one manganese-superoxide dismutase and two iron-isozymes , while the herbicide-grown cells produced an additional manganese-superoxide dismutase. C. sorokiniana which was cultivated in the presence of 25 μM paraquat possessed elevated level of the enzyme up to 3 folds comparing to the control cells. The increment of enzyme content was observed only when the paraquat was included in the growth medium under photosynthetic conditions. Thus it appeared that the herbicide enhanced the production of superoxide radical in C. sorokiniana and that the augmentation of the cellular content of superoxide dismutase was an adaptation response which provided protection against this herbicide.

Paraquat resistant calluses of tobacco (Nicotiana tabacum) was produced by three successive screening of protoplast-derived calluses in a paraquat containing medium (Furusawa et al ,1984). Growth of normal calluses was inhibited by the herbicide at the concentration less than 2 μM, whereas the resistant cell lines could

propagate on medium with the herbicide up to 250 μM. Specific activity of superoxide dismutase varied among the callus population, however, in paraquat resistant calluses, the enzyme specific activity was reported to be 14- to 159-folds higher than that of the intact leaf cells. The catalase and ascorbate peroxidase activity of the resistant calluses were similar to those of the sensitive ones. Hence, in this case the resistance to paraquat was supposed to be confered by a high superoxide dismutase content.

A paraquat resistant biotype of Conyza bonariensis was taken from vine and citrus plantations in Egypt. This resistant plant was studied for the resistance mechanisms with an emphasis on in vivo uptake metabolism (Fuerst, 1985). When whole leaves were thoroughly sprayed with [$^{14}$C]paraquat, the herbicide translocation was traced by autoradiography and fluorescence spectroscopy. The results led to a conclusion that paraquat was excluded from the chloroplast in the resistant biotype. Basis of resistance was also shown not concerning with the alteration at the electron acceptor level of photosystem I .

The mechanism of resistance to paraquat was also investigated in a resistant biotype of weed Hordeum glaucum (Powles and Cornic, 1987). Examination at cellular level of this biotype could not establish any difference between the resistant and normal plants. However, it was concurrently found in the subsequent work with the same weed biotype (Bishop et al , 1987) that the mechanism of resistance to paraquat might be exclusion of this herbicide from the

cytoplasm by sequestration in the apoplast.

Gametophytes of a fern Ceratopteris richardii were developed resistance to paraquat. Biochemical studies with respect to the enzyme defense system including superoxide dismutase, catalase, ascorbate peroxidase, and two more enzymes, i.e. glutathione reductase and dehydroascorbate reductase revealed no differences from those found in the susceptible gametophyte. Uptake of paraquat by the whole gametophyte was also equivalent in both phenotypes. The fact that gametophyte is a one-cell-thick thallus, the paraquat resistance in this organism was apparently not due to the sequestration mechanism of the herbicide far away from the symplasm. The resistance mechanism was supposed to somewhat be based on the alteration of the photosynthetic system (Carroll et al ,1988).

In Thailand paraquat has been widely applied in many plantings , and the case of resistance biotype was demonstrated in Paspalum conjugatum, a weed species in rubber planting in the southern part (Boonsrirat et al, 1982). No characterization of the resistance basis has ever been described yet.

In conclusion at this moment the research works which have been reviewed in attempting to understand the mechanisms of paraquat resistance in higher plants come to a point that the resistant biotypes have been restricted. The construction of paraquat resistant plant and cell lines in the laboratory cannot be carried out consistently to serve as suitable sources for molecular studies. Moreover, the greatest obscure is that, in almost all works which

have been established up to now, the specimens in test can explain the paraquat resistance mechanisms only at physiological level. Investigation of paraquat resistance mechanisms in a bacterum E. coli could not be introduced to the explanation of the herbicide resistance in higher plants. Then an alternative approach is to look for other appropriate model organisms

Several hypotheses have been addressed to explain the mechanisms of paraquat resistance. These are (1) alteration in the membrane integrity or in the specific route for paraquat entry such that the herbicide penetration into cells is diminished (2) alteration in compartmentation of paraquat resulting in reduced localization of the herbicide at the target site (3) detoxification of the superoxide radical or other reactive oxygen molecules produced in the presence of paraquat and (4) alteration in the redox potential of the photosystem I electron acceptors, which has been proposed as the primary site of action of paraquat, such that the potential herbicide would be less efficient in competing electron fluxes from the photosystem I.

## 1.5 General Properties of Chlamydomonas reinhardtii

Chlamydomonas reinhardtii is a haploid unicellular green algae, generally ovoid in shape, which is classified to be a kind of lower plant in class Chlorophyceae. It contains a single and large cup-shaped chloroplast occupying about 40% of the cell volume. The life cycle of this organism is showm in Fig. 1.3. Under favor-

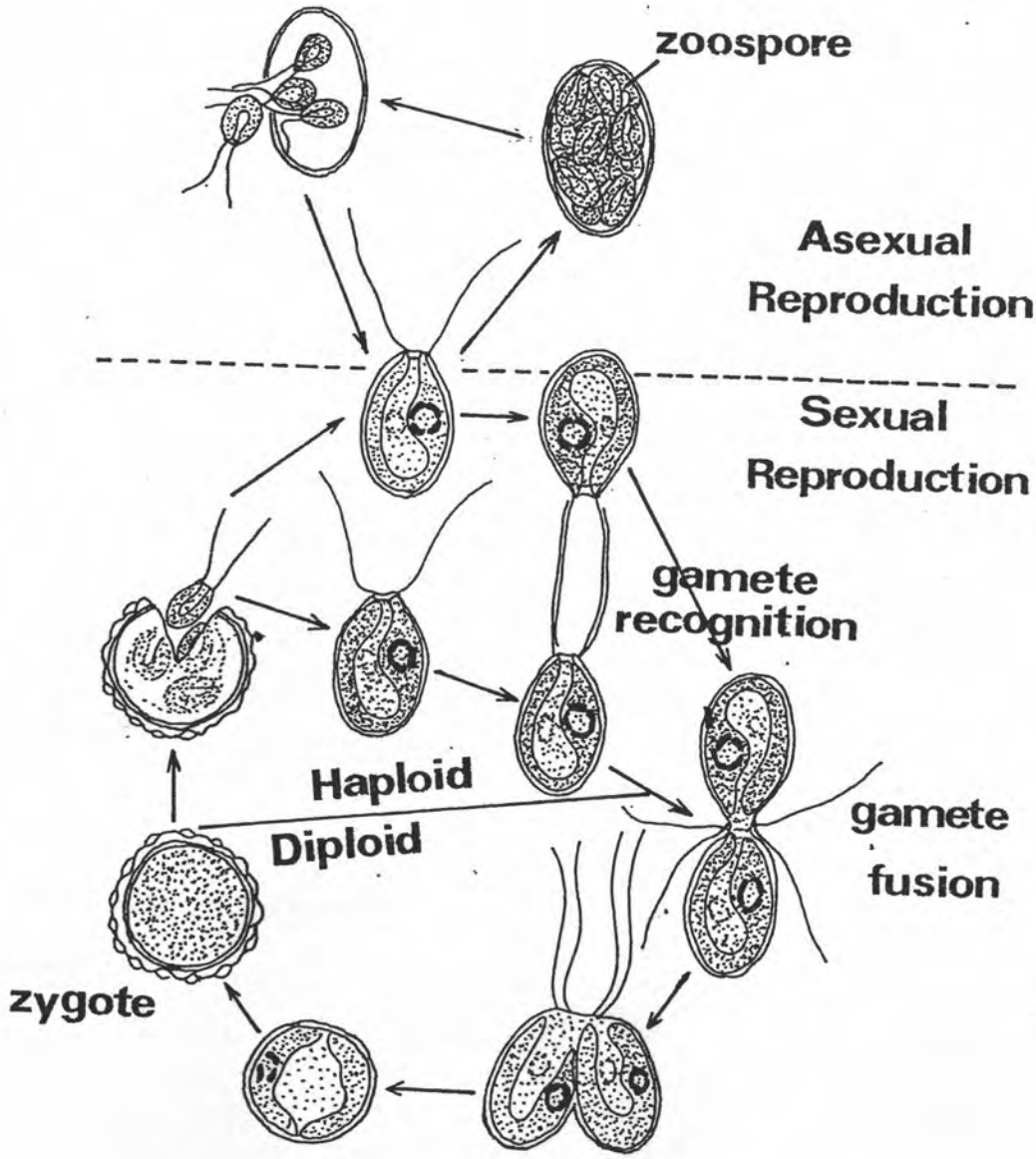


Figure 1.3 Life cycle of Chlamydomonas reinhardtii (Lee, 1980).

able conditions, such as photosynthetic conditions, mitotically asexual reproduction usually takes place with 2, 4, 8, or 16 daughter protoplasts. Each daughter protoplast secretes a wall of its own, while still in the parent cell. When the flagella developed, each daughter cell was liberated by the gelatinization of the cell wall and gradually develops into adult Chlamydomonas cell.

Under unfavorable conditions, especially when nitrogen is deficient, vegetative cells differentiate into opposite gametes, i.e. mating type plus (mt+) gamete and mating type minus (mt-) gametes (resemble egg and pollen of higher plants). These phenotypes are inherited by nuclear genomic DNA. The pairing gametes recognize each other at their anterior and fusion of their protoplasm and nuclei occurs. This is the only diploid stage of this alga. The resultant zygote is quadriflagellate structure which soon loses the flagella and undergoes maturation. At the time of germination, the zygote meiotically divides into 4, 8, or 16 uninucleate protoplasts and these contents are extruded after splitting off the zygote wall. They soon develop flagellas and swim away.

It is important to notice that shortly after zygote formation, the two parental chloroplasts fuse forming one large chloroplast. Striking feature of the sexual cycle of C. reinhardtii is that chloroplast genes are inherited uniparentally whereby in most circumstances (over 95%) the chloroplast genome of the mt+ parent is

transmitted to the offsprings (Sager, 1954). Only exceptional zygote exhibits biparental inheritance. It is at present postulated from the discoveries of Ogawa and Kuroiwa (1985a and b) that fertilization brings about destruction of male (mt-) chloroplast DNA by $Ca^{2+}$ nuclease c, whereas female (mt+) chlroplast DNA is resistant to the enzyme activity because the DNA is protected by methylation during the differentiation period.

C. reinhardtii possesses three genetic systems located in the nucleus, chloroplast, and mitochondria, respectively. Chloroplast genome and mitochondrial genome are present in multiple copies. Electron microscopic studies (Behn and Herrmann, 1977) and restriction nuclease analysis (Rochaix, 1978) have revealed that the chloroplast DNA of C. reinhardtii consists of 190 kb in circle.

## 1.6 Herbicide Resistance in Chlamydomonas reinhardtii

The green alga C. reinhardtii represents particularly a well suited model organism for the linked understanding of herbicide resistance to the higher plants (Erickson et al, 1984), especially for a joint cellular and molecular analysis of herbicide resistance that concerns photosynthetic system. Its photosynthesis resembles the higher plants that photosynthetic electron transport takes place in the thylakoid membrane mediated by the membrane protein complexes of photosystem I (PS I) and photosystem II (PS II) (Levine, 1960 ; Levine and Smillie, 1963 ; Levine and Tokasaki, 1965 ; Levine, 1968 ; Levine, 1969 ; Levine and Goodenough,1970 ; Levine and Paszewski, 1970).

The algae has developed resistance to many chemicals both by spontaneous selection, physical mutation, and chemical mutation direct at nuclear or chloroplast genome (Lawrence, 1967 ; Loppes, 1968 ; Mets and Bogorad, 1971 ; Rosen and Ebersold, 1972). Based on sexual reproduction of the alga, it is possible to analyze the genetic basis of the resistance in combination with the biochemical basis. Moreover, vegetative growth of C. reinhardtii yields large number of cells in a relatively short period of time.

Erickson et al (1984) reported the isolation and characterization of a uniparental mutant from 5-fluorodeoxyuridine mutagenesis of C. reinhardtii that resisted to DCMU and atrazine. The mutant showed an alteration in 32 kilodalton protein of photosystem II resulting in less affinity to the herbicides as described for the resistant biotype of higher plants (Hirschberg and McIntosh, 1983).

Another group of C. reinhardtii resistant to atrazine was achieved via mutagenesis by ethyl methane sulfonate along with 5-fluorodeoxyuridine (Galloway and Mets, 1984). The mutants so obtained were demonstrated to include some secondary mechanisms of resistance other than the alteration in the 32 kilodalton protein of photosystem II. However the resistance was shown to be inherited uniparentally by chloroplast DNA.

An increase in understanding of herbicide susceptibility and resistance not only has obviously practical applications for crop production but also reveals much about the basic photosynthetic apparatus in the chloroplast of green organisms. It might as well

provide interesting new insights into the function and regulation of chloroplast genes.

Despite the fact that paraquat resistance has long been investigated in the resistant biotypes of weeds with diverse results, no one has ever tried constructing such resistance in C. reinhardtii.

In the present research, paraquat resistant cell lines would be constructed from a standard strain of C. reinhardtii via two alternative methods. First, selection under the herbicide pressure is designed to obtain paraquat resistant mutants in the way that the resistant biotypes of weed occur naturally due to long term exposure to paraquat. Second, the paraquat resistant mutants would be constructed by 5-fluorodeoxyuridine mutagenesis to obtain mutants with chloroplast DNA mutation which is possibly attributed as gene pools for paraquat resistance.

## 1.7 Aims of the Research

1.7.1) Development of paraquat resistant cell lines of *Chlamydomonas reinhardtii*.

1.7.2) Comparative studies of the morphology and physiology of paraquat resistant and wild type strains which including fine structure analysis and photosynthetic rate of *C. reinhardtii*.

1.7.3) Comparative studies of biochemical aspects possibly concerning paraquat resistance in paraquat resistant and wild type strains of *C. reinhardtii* .

1.7.4) Studies of genetic aspects of paraquat resistance in *C. reinhardtii*.

## 1.8 Research approaches

1.8.1) Construction of *C. reinhardtii* paraquat resistant mutants by selection under paraquat pressure.

1.8.2) Construction of *C. reinhardtii* paraquat resistant mutants by 5-fluorodeoxyuridine mutagenesis

1.8.3) Scanning electron microscopic analysis of cell surface of the paraquat resistant strains of *C. reinhardtii* in comparison with the wild type.

1.8.3) Transmission electron microscopic analysis of cross-sectioned structure of the paraquat resistant strains of *C. reinhardtii* in comparison with the wild type.

1.8.5) Measurement of photosystem I activity of the paraquat resistant strains of *C. reinhardtii* in comparison with the wild type.

1.8.6) Investigation of paraquat uptake and paraquat distribution in the paraquat resistant strains of C. reinhardtii in comparison with the wild type.

1.8.7) Comparative investigation of superoxide dismutase, catalase, and ascorbate peroxidase in the paraquat resistant strains of C. reinhardtii in comparison with the wild type.

1.8.8) Comparative analysis of chloroplast DNA restriction patterns of the paraquat resistant strain of C. reinhardtii from 5-fluorodeoxyuridine mutagenesis in comparison with that of the wild type.