CHLOROPHYLL A TRANSIENT FLUORESCENCE IN DETACHED LEAVES OF PEANUT, WHEAT AND SORGHUM UNDER DESICCATION; ACTIVITIES AND ISOZYMES OF SUPEROXIDE DISMUTASE AS AFFECTED BY CYANIDE AND PARAQUAT IN PEANUT AND WHEAT LEAVES

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GENERAL INTRODUCTION

Chlorophyll transient fluorescence, discovered in 1931 by Kautsky and Hirsch, is known to be correlated with photosynthesis. The kinetics of variable fluorescence reflect certain aspects of photosynthetic mechanisms. Therefore, it was suggested that transient fluorescence could be used as an intrinsic probe of photosynthesis (Papageorgiou, 1975). Based on this principle, a fluorescence technique has been developed to measure resistance of plants to various environmental stresses, such as extreme temperature, high light and UV irradiation, high salinity, water stress, and some chemical stresses, including herbicides and pollutants. The primary action site of water stress on photosynthesis can be approached conveniently by the fluorescence method (Govindjee et al., 1981; Downton, 1983). Others have also attempted to use this technique to measure the resistance of plants to drought (Wiltens et al., 1978; Hetherington and Smillie, 1982a). Leaf water deficit causes changes in transient fluorescence. However, it still remains unclear whether leaf water loss tends to increase the fluorescence yield or reduce it, because thus far the experimental results are inconsistent. In addition, the pattern alteration of fluorescence curves by leaf dehydration from the normal water content to low

water content has not yet been well monitored. The first part of this study was conducted by using several economic plants to determine how the leaf water loss affects the variable fluorescence yield. Several approaches have been used to try to find a better index for measuring the drought-resistance of plants.

The second part of this work is the continuation of previous study on superoxide dismutase (SOD) (Wu and Todd, 1985). SOD enzyme functions as a scavenger of oxygen radicals, was found to correlate with drought tolerance in certain plants (Dhindsa and Matowe, 1980; Wu and Todd, 1985). In this study, the SOD isozymes of leaves from different peanut species and from wheat have been partially characterized. The localizations of these isozymes in leaf cells and the quality of metalloproteins were also determined. Paraquat a common herbicide, injures plants by inducing free radicals including superoxides. It has been reported that a higher paraquat resistance was correlated with a higher level of SOD enzyme. I found that paraquat significantly reduced the SOD level in peanut leaves. Furthermore, the SOD enzyme in wild peanut species seemed to have a higher tolerance to paraquat than the cultivated peanuts. Starting from these facts, comparative studies between the wild species and cultivars of peanut were conducted, including enzymatic activities, pattern of isozymes as well as the localizations of distinct isozymes.

PART I

CHLOROPHYLL A TRANSIENT FLUORESCENCE IN DETACHED LEAVES OF PEANUT, WHEAT AND SORGHUM UNDER DESICCATION

CHAPTER I

LITERATURE REVIEW

Chlorophyll a Transitory Fluorescence As an Intrinsic Probe of Photosynthesis

The light absorbed by the photosynthetic pigments of plants creates a supply of singlet electronic excitation energy. The major part of this energy is utilized in photosynthesis. The remainder is lost as heat or radiated as fluorescence. Fluorescence emanates mostly from chlorophyll a of photosystem II (PSII).

Among the measurable fluorescence parameters, the relative fluorescence yield, which is proportional to the measured intensity, is the most convenient one for kinetic studies (Papageorgiou, 1975). Because the excitation energy can be dissipated either by light-induced electron transport or by the emission of fluorescence, the level of fluorescence yield is closely related to photosynthetic processes. A higher fluorescence yield usually represents lower photosynthetic efficiency. Kautsky and Hirsch (1931) first discovered that the intensity of chlorophyll fluorescence in vivo changed as a function of time when dark-adapted photosynthetic cells were illuminated. This is called the induction phenomenon of chlorophyll a fluorescence or the

"Kautsky effect". The transients of this fluorescence induction have been correlated with the underlying photosynthetic reactions (Lavorel and Etienne, 1971). The partial reactions of photosynthesis are reflected in parts of the complex fluorescence induction curves displayed upon a dark-light transition (Schreiber, 1978). The discovery of this phenomenon provided the possibility of using chlorophyll a fluorescence as a valuable intrinsic probe of photosynthesis (Papageorgiou, 1975). It also can be used to monitor plant responses to environmental factors, such as light, temperature, water, and salts which can affect photosynthetic metabolism directly or indirectly.

When plants are illuminated after being in the dark, the photosynthetic apparatus passes through several stages of accompaning transitory changes in chlorophyll a fluorescence before attaining steady-state photosynthesis. Both fast and slow changes can be distinguished. The fast change lasts only a few seconds which results in the first peak of variable fluorescence. However, the slow change can be maintained for several minutes. During this period the second peak of fluorescence may appear. These two parts of fluorescence changes had been shown with chloroplasts and red and green algae (Papageorgiou, 1975). Similar changes were found in higher plants. The pattern of fluorescence curves can be influenced by many factors. Different species of plant usually show some differences in the pattern of their fluorescence curves. The green,

red and brown marine algae were reported to have different patterns of fluorescence curves (Wu et al., 1966). Variable fluorescence is mainly contributed by chlorophyll a of PSII. Figure 1 shows the Z-scheme for electron flow in photosynthesis (Govindjee and R. Govindjee, 1975). The right-hand side of PSII is associated with the water splitting system. It is the photooxidizing side of PSII. On the contrary, the left-hand side of PSII is the photoreducing side. Q is the primary electron acceptor. If the reoxidation of reduced Q by the secondary electron acceptor of the electron transfer chain between the two photosystems becomes blocked entirely, the maximum yield of steady-state fluorescence will be reached since Q remains reduced in continuous exciting light. If an inhibition or damage occurs on the water splitting side of PSII, an effective quencher of variable fluorescence ($P680^{+}Q^{-}$) will accumulate and this will lead to a decrease in variable fluorescence yield. In the extreme situation the total inhibition on the water splitting side of PSII may cause a total loss of the variable fluorescence (Sundbom et al., 1982). So it can be concluded that an inhibition on the photooxidizing side of PSII will lower the yield of fluorescence, while a block on the photoreducing side of PSII will enhance the fluorescence yield. This principle has been widely used in works identifying the action sites of distinct environmental factors on photosynthetic processes.

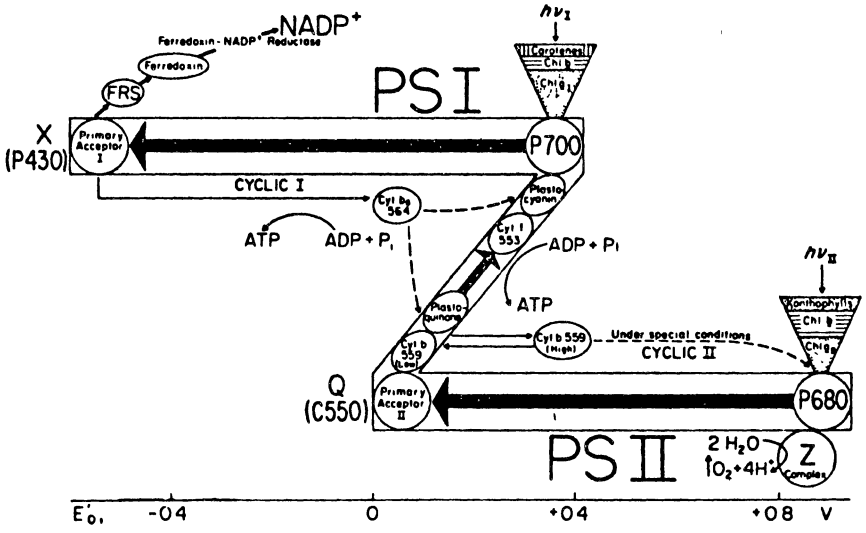

Figure 1. The Z-scheme for Electron Flow in Photosynthesis. The two bold horizontal arrows represent the light reactions; all other, dark reactions. (Govindjee, 1975)

Chlorophyll molecules are embedded in the thylakoid membrane and the yield of fluorescence is a sensitive indicator for the state of this membrane. Reactions depending on the intactness of thylakoid membrane are particularly water splitting and photophosphorylation (Schreiber and Berry, 1977). Although sufficient knowledge has not yet become available for giving a better explanation to the second peak of fluorescence curves, it was suggested that the slow fluorescence change SMT (see Figure 3) may reflect the processes in which the ultra-structure of thylakoid membrane plays a major role. These processes are related to the transmembrane ionic fluxes, and thus related to photophosphorylation (Papageorgiou, 1975).

Applications of Chlorophyll a Transitory Fluorescence to Evaluation of Plant Responses to Varied Environmetal Stresses

Many environmental factors affect photosynthesis. Among these, light intensity, leaf temperature and water supply are of major importance (Berry and Bjorkman, 1980). Leaf fluorescence should also be influenced by these factors. In recent years, the fluorescence technique has been developed as a useful tool to monitor cellular injuries caused by distinct environmental stresses. It has been reported that the effects of extreme temperature, high light

and UV irradiation, salinity stress, toxic pollutants and water stress on plants all can be evaluated by the chlorophyll fluorescence method. This kind of research is important not only for the studies of plant stress physiology but also for agricultural applications. For example, there is an increasing need for new stress-tolerant varieties. Physiological responses of plants to stresses can be used as indicators of genetic selection. However, reliable screening methods need to be established to enhance the selection process. Similarly, suitable methods to examine plant injury by environmental stress are also needed. The technique of measuring leaf fluorescence is relatively simple, rapid, sensitive and nondestructive to the tissue. So it appears promising for development as an effective tool to be used in both physiological studies and plant breeding.

Chilling injury has been of great concern for many years. Some plant species are sensitive to low temperature when they are exposed to nonfreezing temperatures. Such plants exhibit a marked physiological dysfunction (Mazur, 1969; Lyons, 1973). So far this phenomenon has not been well understood. Smillie and Hetherington (1983) measured chilling tolerance in bean, cucumber and cabbage plants by the chlorophyll fluorescence method. They succeeded in using the maximum rate of the fast rise IP (see Figure 3) as an indicator of chilling tolerance. It was found that this rate decreased as the length of cold treatment

increased. The time taken for the rise to decrease by 50% in leaves at 0^{o} C was used as a measure of chilling tolerance. They concluded that an inhibition developed on the photooxidizing side of PSII which resulted in a decrease of the variable fluorescence yield. The same technique was used to measure chilling tolerance within maize populations (Hetherington et al., 1983). The chilling tolerance of maize at 0^{o} C appeared to be negatively correlated with the loss of variable fluorescence in the intact leaf tissue. This result was consistent with the experimental results obtained from field investigations.

The frost tolerance of plants has been investigated. (Sundbom and Oquist, 1982). As temperature decreased from 20^{o} C to -20^{o} C, the steady state of variable fluorescence increased with a transient at the freezing point. They claimed that temperature lowering slowed down electron transport after Q, hence the maximum fluorescence yield was reached when Q was fully reduced. However, after repeated freeze-thaw cycles a total quenching of variable fluorescence was observed which indicated a damage developing in the water splitting system. In this case, variable fluorescence was quenched by the oxidized reaction center of PSII or reduced Q could not accumulate since no electrons were supplied from the water splitting side to the reaction center of PSII. In potato leaves, it was found that fluorescence increased at above-freezing temperature

for the frost-resistant clone, but decreased in frost sensitive clones. However, even in the frost resistant clone, variable fluorescence was lost after a single freeze-thaw cycle. With prolonged freezing stress, both variable fluorescence and fluorescence maximum declined. It was concluded that the primary effect of freezing injury occurred on the photooxidizing side of PSII.

In three species of Atriplex, the fluorescence yield of intact leaves increased by 2 to 3-fold with an increase of temperature into the range where heat damage occurred. The thylakoid membrane was thought to be the most heat-sensitive (Schreiber and Berry, 1977). On the contrary, fluorescence was found to decrease after heat treatment in clones of potato plant. The decrease in the rate of the fast rise of fluorescence after heating was used to determine relative heat tolerance (Hetherington et al., 1983).

Progressive salt accumulation in soil has been a serious problem in numerous areas of the world. Excess salinity already affects one-third of the agricultural land under irrigation (Epstein and Norlyn, 1980). Development of salt-tolerant plants would make possible improved utilization of saline soils and water. New methods are required for both selection of salt-tolerant strains and monitoring of salt stress in physiological studies. The measurement of chlorophyll a fluorescence in vivo to detect cellular responses to salt stress was performed in some of

crop plants. It was found that distinct species of plant were affected by salinity in quite different ways. In a salt tolerant sugar beet, salinity caused an increase in the rate of the fast rise of fluorescence, but a decrease in the rate of subsequent fluorescence decay. In a moderate salt tolerant plant, sunflower, the fast rise of fluorescence was depressed while the rate of fluorescent decay became faster when the leaf was subjected to salt stress. In a salt-sensitive bean plant, the young leaves showed an increase in the fast rise but a decrease in the decay of variable fluorescence (Smillie and Nott, 1982). These results indicated that the responses of plants to the same environmental stress may depend on the species.

Cucumber plants grown in low intensity light then irradiated with high intensity light exhibited a lessened variable fluorescence. Longer irradiation caused irreversible photoinhibition. The sensitive site for photoinhibition in vivo was on the photooxidizing side of PSII (Critchley and Smillie, 1981). Variable fluorescence was found to be lost progressively in pea leaves irradiated with UV light (Smillie, 1982). Thus the damage caused by UV light was first seen on the photooxidizing side of PSII.

It was reported that ozone injury in bean leaves can be predicted by fluorescent measurement at least 20 hours before any visible sign of leaf damage appeared. The suppression of the fast rise indicated a gradual loss of

the activity of the water splitting system (Schreiber, 1978).

Chlorophyll a Transitory Fluorescence as a Valuable Probe to Investigate the Drought Tolerance of Plants

The injury of plants caused by drought and plant tolerance to water loss is a subject of substantial interest since the agricultural economic losses due to drought are a serious problem year after year in many countries of the world. Drought tolerance of plants varies widely from species to species. For some species even mild desiccation causes irreversible changes, while other species can tolerate extreme water loss (Gaff, 1977; Gwóźdź and Bewley, 1975). Desiccation tolerance usually depends on the type of environment to which a plant species is adapted. Water stress leads to several changes in the photosynthetic apparatus of green plants (Mohanty and Boyer, 1976). Lower water potential causes a decrease in the quantum yield of oxygen evolution in chloroplasts and leaves (Mohanty and Boyer, 1976). It also lowers the binding ability of the coupling factor (Younis et al., 1979). Only recently have scientists tried to use chlorophyll a variable fluorescence to measure plant tolerance to drought. Water stress affects photosynthesis and at the same time, it alters the kinetics of fluorescence. The ratio of the maximum (P level) to the minimum

(O level) of fluorescence (see Figure 3) was regarded as a good indicator of the activity of PSII (Govindjee et al., 1981). The effect of leaf desiccation on the pattern of fluorescence curves has been investigated independently in several laboratories. However, the experimental results have not been consistent.

The thin thalli of marine algae have been used as an ideal experimental material for fluorescent measurement since less irradiation is scattered or reabsorbed by cell layers. The algae of higher intertidal regions tend to tolerate extended periods of desiccation and are more resistant to desiccation than those living in lower intertidal regions. The chlorophyll a fluorescence induction of the red algae Porphyra sanjuanensis undergoing desiccation was investigated by Wiltens et al (1978). It was found that a gradual loss of the characteristic fluorescence transient occurred with a progressive decrease of water content in the thallus. It appeared that desiccation only changed the pattern of fluorescence curve but had no effect on the maximum yield of variable fluorescence (OP). The OP value remained unchanged until the water content dropped to 20% (-50 bars). Below -50 bars the OP value diminished. The range of water content within which the pattern of fluorescence curve changed was species dependent. For P. sanjuanensis the range was as low as 25-20% (-37 bars to -50 bars). The data obtained from fluorescence measurement agree well with those obtained

from the measurement of photosynthetic rates. In both a desiccation-tolerant red algae Porphyra tenera and a desiccation-tolerant fern, photosynthesis ceased at 25-20% water content. For sunflower this value was about -20 bars. In soybean it was -25 bars. Thus soybean and sunflower, like the lower tidal algae, were desiccation sensitive plants. The most significant differences between desiccation-tolerant and desiccation-sensitive species in marine algae was the extent of reversibility upon rehydration. In tolerant species rehydration resulted in rapid recovery of variable fluorescence, whereas no such recovery can be found in desiccation-sensitive species (Enteromorpha linza; Ulva scagelii) when the water content decreased below a critical range. By analyzing the changes of fluorescence curves upon rehydration, the authors suggested that the water splitting system recovered more slowly than intersystem electron transport. The reappearance of variable fluorescence during rehydration was accompanied by oxygen evolution (Wiltens et al., 1978).

In the leaves of three species of higher plants (Nerium oleander; Atriplex triangularis; Tolmiea menziesii), Govindjee et al (1981) reported that the OP value diminished as leaf water potential decreased. On watering, recovery of OP occurred in N. oleander and A. triangularis but no recovery was found with T. menziesii. They suggested that water stress blocked electron flow on the water splitting side of PSII in the

three species examined. In the grapevine plant, Downton (1983) also found a rapid loss of variable fluorescence when leaves were subjected to desiccation. Grapevine is capable of turgor maintenance through osmotic adjustment to avoid the loss of variable fluorescence until a much lower water potential was reached.

Hetherington and Smillie (1982a) first reported that there was an increase in the variable fluorescence during leaf desiccation in the desiccation-tolerant plant Borya nidita. After leaf drying of 28 hours the OP value decreased. Dehydration caused degreening while rehydration induced regreening. The early stage of degreening up to 28 hours showed a pronounced increase of variable fluorescence. The authors suggested that these changes could result from increased PSII activity linked to oxygen evolution or from a partial block in electron transport after PSII. The slower decline (PS) also shown during desiccation indicated a gradual reduction in photosynthetic electron flow subsequent to PSII, a reduced rate of reoxidation of PSII acceptors. They believed that the recovery of PSI activity is faster than that of PSII.

Therefore, so far three different kinds of experimental results have been reported in the literature:

1. A decrease in the chlorophyll variable fluorescence yield was found in the early stages of desiccation (Govindjee et al., 1981; Downton, 1983).

2. An increase in the chlorophyll variable

fluorescence yield was found in the early stages of desiccation (Hetherington and Smillie, 1982).

3. No substantial changes in variable fluorescence yield were found in the early stages of desiccation (Wiltens et al., 1978). No discussion concerned with the differences in these results is to be found in the literature. Meanwhile, similar research on crop plants is lacking.

In my thesis, data will be presented on the relationship between the variable fluorescence and leaf water content in peanut, wheat as well as sorghum plants. The results suggest that leaf water deficit caused a substantial increase in chlorophyll variable fluorescence yield in the early stages of leaf desiccation and slowed the fluorescence decline PS in all cases examined. Variable fluorescence yield eventually decreases as water content drops below a critical point.

CHAPTER II

MATERIALS AND METHODS

Plant Materials

Peanut (Arachis hypogaea cv. EC-5 and Tamnut), wheat (Triticum aestivum cv. KanKing and Ponca) and sorghum (Sorghum bicolor cv. BOKIII; IN-2 and ROKY62) seeds obtained from the Oklahoma Agricultural Experiment Station were grown in a controlled environmental chamber at 25°C ± 1°C. Vermiculite, contained in plastic pots (DOW. 0.5L) was used as a replacement for soil. Light intensity was maintained at 230 $\mu E\ m^{-2} s^{-1}$ with a 12 hour light and 12 hour dark daily cycle. After seed germination, the vermiculite was moistened regularly with water and half strength Hoagland's nutrient solution. Leaf samples selected for fluorescence measurements were from the most recently mature leaves. The ages of most leaves used in experiments were less than one month. Pots were placed in a layer of water to protect from any possible water deficiency. Leaf samples of wild species of peanut plants (Arachis helodes and Arachis villosulicarpa) were obtained from a greenhouse grown USDA plant collection maintained by R. Pittman and H. A. Melouk.

Leaf Dehydration and Rehydration

Detached leaves were placed in a desiccator over anhydrous $CaCl_2$ for a certain length of time. The desiccator was maintained at room temperature (24-27° C) under low light intensity (10-20 $\mu E\ m^{-2} s^{-1}$). Dehydrated leaves were rehydrated by floating them in water in a covered petri dish. Leaf samples were weighed before and after dehydration or rehydration. Finally, leaf samples were dried in an oven at 80° C for 48 hours to obtain the dry weight (W_d). The water content was calculated by the following formula:

$$\% \text{ Water content} = \frac{W_f - W_d}{W_f} \times 100$$

W_f ----- Leaf fresh weight.
W_d ----- Leaf dry weight.

Measurement of Chlorophyll a Transitory Fluorescence

Chlorophyll a fluorescence induction kinetics were measured with a fluorometer, Model SF-10 (Richard Brancker Research Ltd. Ottawa) connected to a recorder (Linear 1200). The SF-10 sensor contains both light source (red light, 17 $\mu E\ m^{-2} s^{-1}$) and photodiode. The light source is used to exite chlorophyll and the fluoresced light is measured by the photodiode. The sensor was positioned on

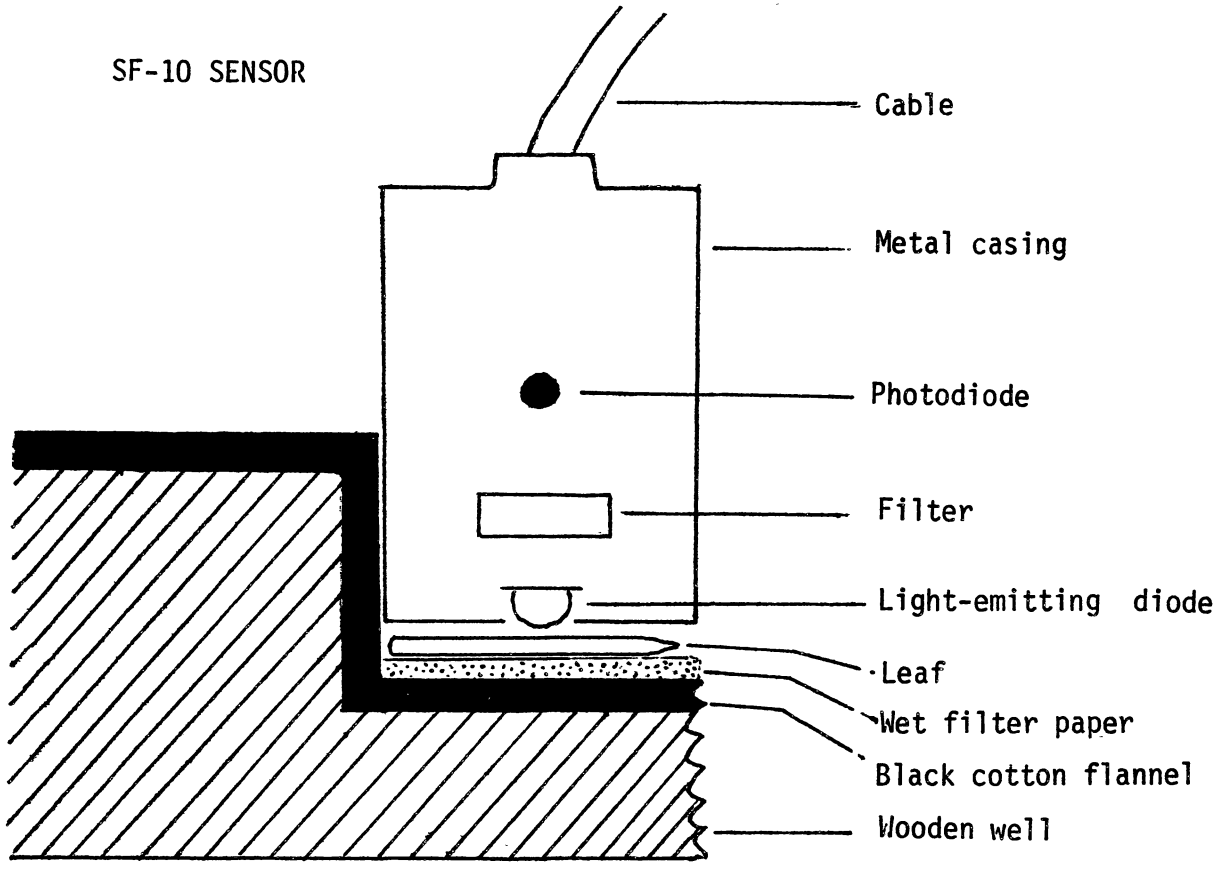

Figure 2. Arrangement for measurements of Fluorescence Induction Curves of Leaves by a Fluorometer Model SF-10.

the center of an intact leaf within a dark cell (shown in Figure 2) and the fluorescence curves were measured at different water content of detached leaves. Two different chart speeds (3 cm/min and 30 cm/min) were used to record the slow change and the fast change of chlorophyll fluorescence. Most experiments reported here were repeated at least once while many were repeated several times.

Applications of Photosynthetic Inhibitors

Atrazine (10^{-4}M, in 0.2% Triton X-100), 2,4- Dichlorophenol (DCP, 10^{-4}M) and N,N-Dicyclohexylcarbodiimide (DCCD, 5×10^{-5}M) were used to treat leaf samples. Before the treatment, the leaf sample was maintained at room temperature and under a light intensity of 230 μE $m^{-2}s^{-1}$ for thirty minutes, then the fluorescence curve was measured as the control. After that, the same leaf was floated in a solution of inhibitor under the same conditions for a given time length. Then the fluorescence curve was recorded again. The result was compared with the pre-treatment control.

CHAPTER III

RESULTS AND DISCUSSION

Analysis of Chlorophyll a Fluorescence Induction Curves

The fluorescence induction curves of a detached leaf of peanut cultivar EC-5 are shown in Figure 3. The fast change of fluorescence is shown in Figure 3A. At the onset of illumination, chlorophyll a fluorescence reached a low level (O) very rapidly. Fluorescence yield was low because the first electron acceptor (Q) of PSII was fully oxidized. The level (O) was regarded as not being responsive to photochemical processes occurring in the chloroplast thylakoid membrane. The ensuing increase (OI) and (IP) represented the reduction of Q and the further reduction linked to the water splitting system. The fast rise (IP) was the major portion of the variable fluorescence and it accounted for about seven seconds required to increase fluorescence from (O) to (P) in EC-5 leaves. The level (S) represented the steady state of fluorescence. The decay (PS) corresponded to the reoxidation of reduced Q by a second electron acceptor which was interpreted as an increasing photochemical utilization of electron excitation energy. The fast change

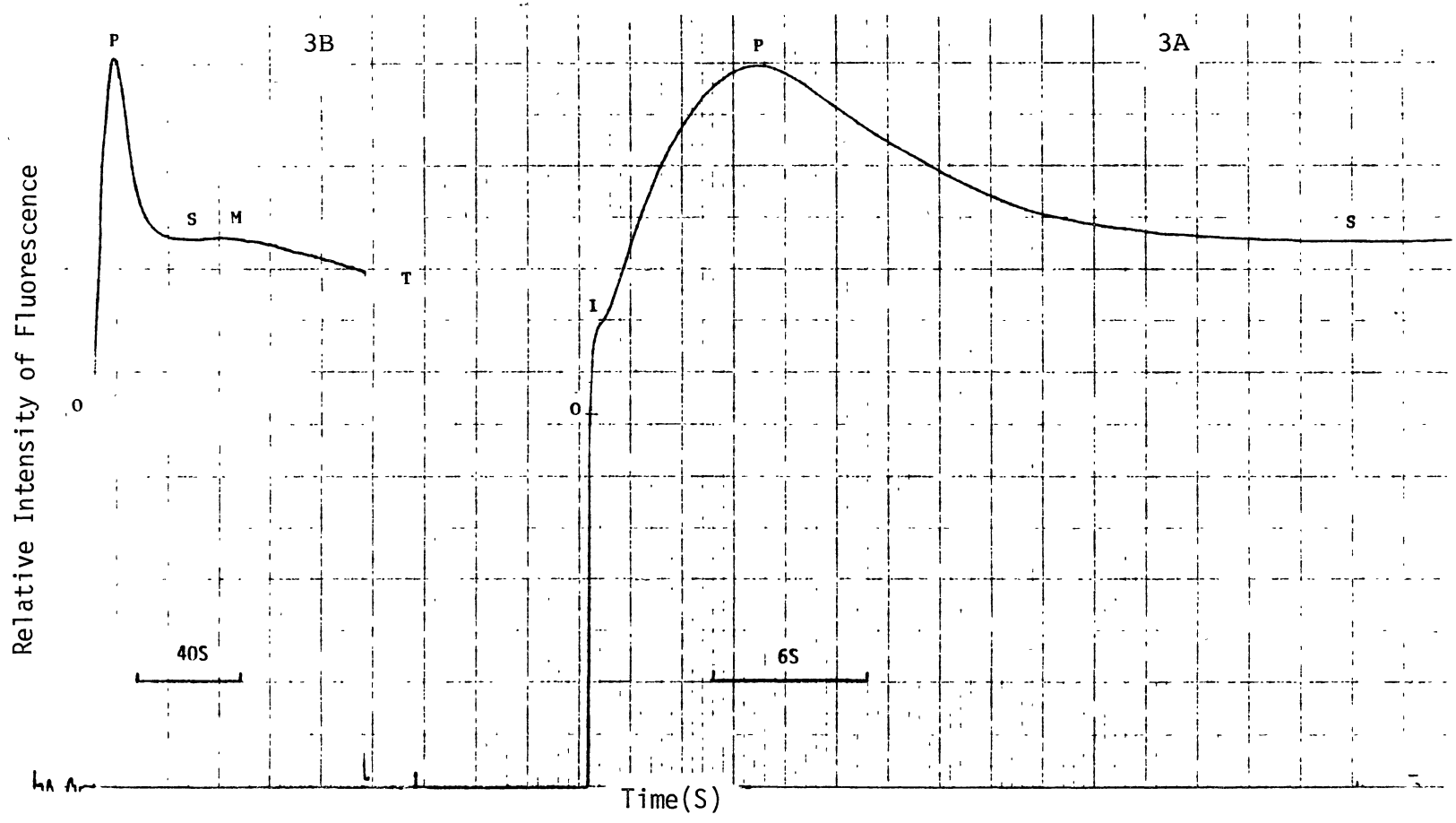

Figure 3. Chlorophyll a Fluorescence Induction Curves of Leaves of Cultivated Peanut A. hypogaea cv. EC-5. 3A: the fast change of transitory fluorescence. 3B: both the fast and the slow changes.

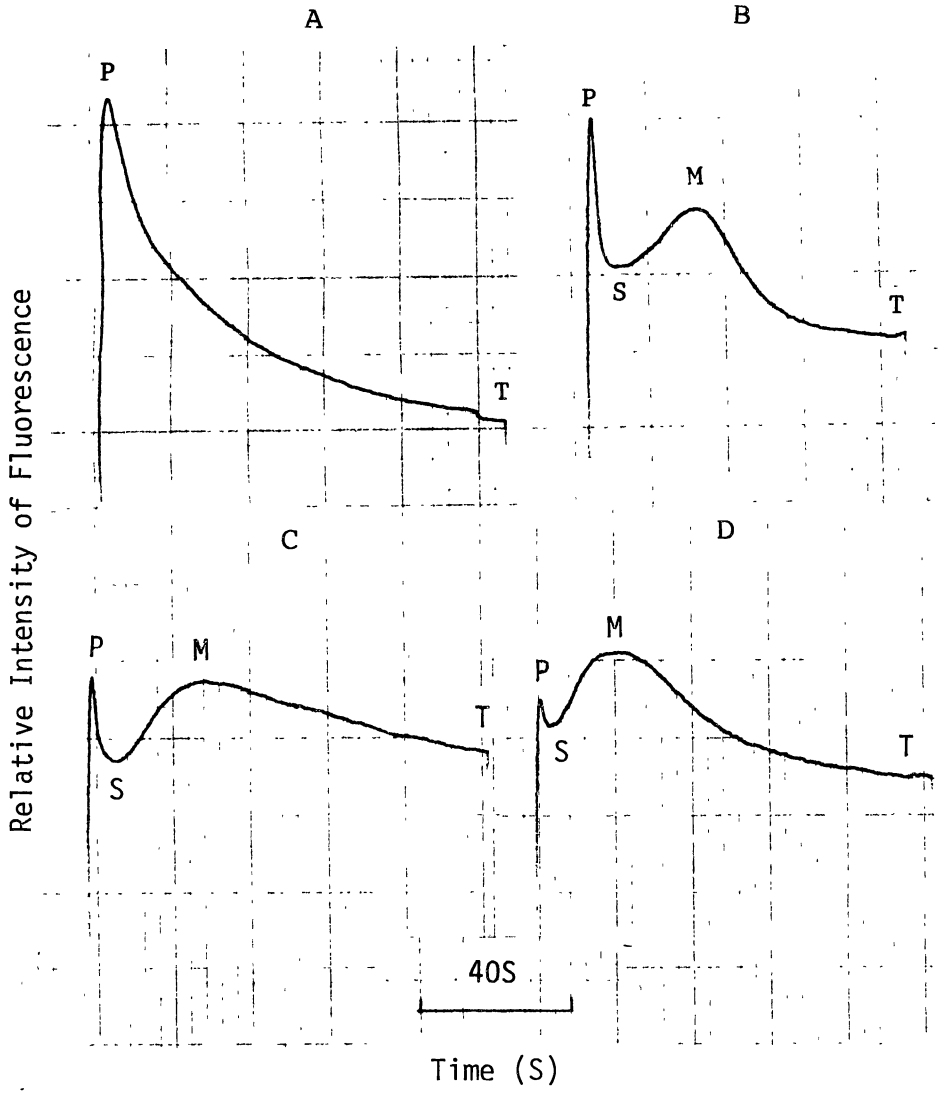

Figure 4. Patterns of Chlorophyll a Fluorescence Induction Curves Recorded from Leaves of Cultivated Sorghum. A: ROKY62 (sparse), no second peak of fluorescence. B: ROKY62(bloomless), second peak appeared but it was lower than the first peak. C: ROKY62(sparse), second peak equaled to the first peak. D: ROKY62(bloomless), second peak was higher than the first peak.

of variable fluorescence was represented by the first peak (OPS) which accounted for about 30 seconds. Figure 3B shows the fluorescence curve of the same leaf but recorded at a chart speed of 3 cm /min. In these cases, both the fast change (OPS) and the slow change (SMT) are included. During the slow change the fluorescence yield increased again from (S) to M giving a second fluorescence peak, then it declined to a lower terinal level (T). Sorghum, a C_4 plant, sometimes showed a much stronger second peak than the other plants tested. The relative height of the second peak of fluorescence as compared with the first fluorescence peak varied in different leaves (Figure 4). The mechanism for causing the second fluorescence wave is not quite clear.

Other Factors Affecting Transitory Fluorescence besides Desiccation

Dark Period

A minimum length of time of the dark treatment on leaves prior to illumination is required for inducing the transitory fluorescence. In the dark period, a sufficient amount of oxidized Q and other oxidized electron acceptors is accumulated in chloroplasts that is necessary for the development of the chlorophyll transient fluorescence. The chlorophyll fluorescence induction curves of detached peanut leaves following different dark periods (0, 5 and 10 minutes) are shown in Figure 5. With an increase in length

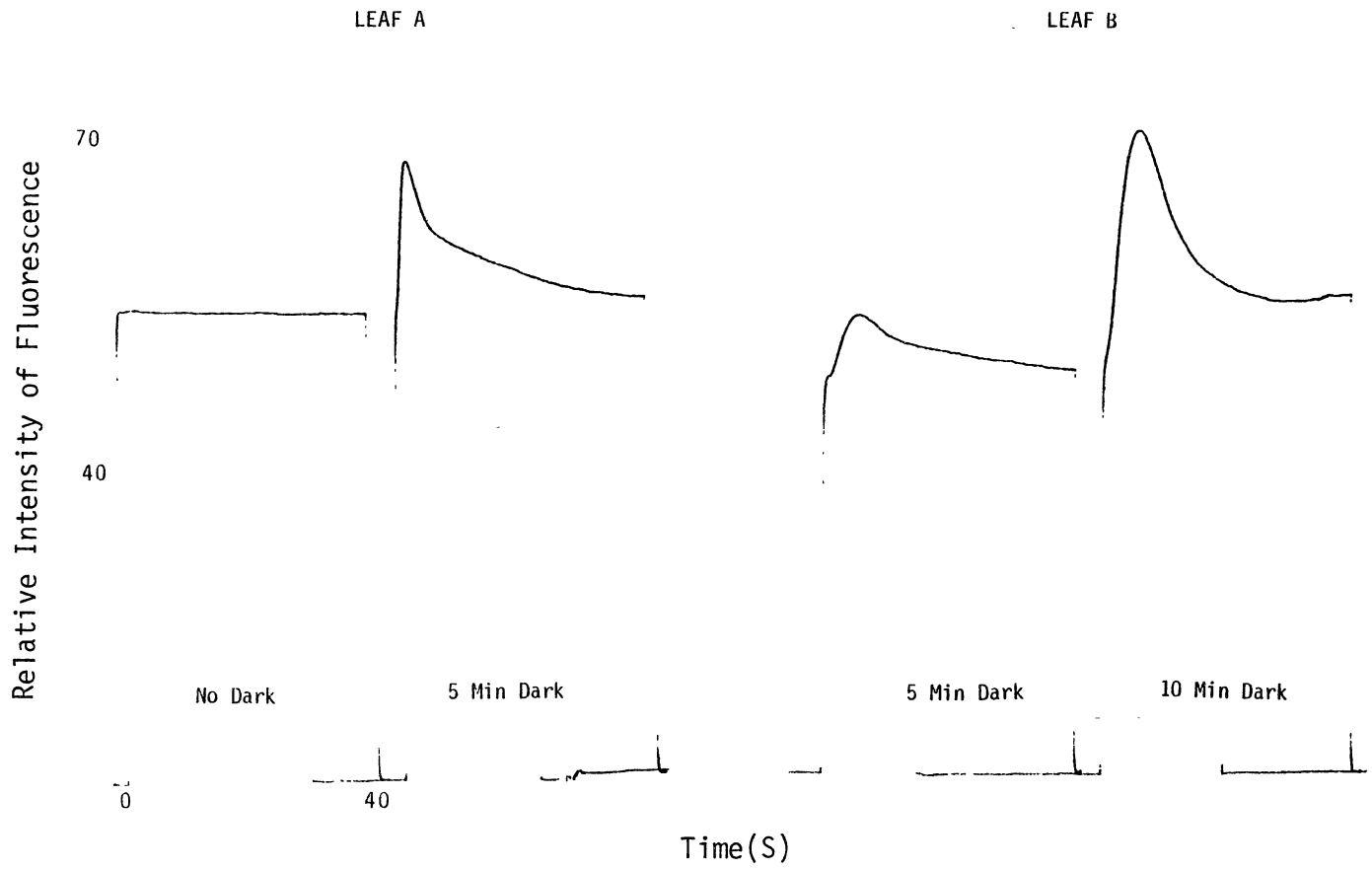

Figure 5. The Effect of the Dark Adaptation Period on Fluorescence Induction Curves Recorded from Wild Peanut A. helodes.

of the dark period from zero to ten minutes, the variable fluorescence rose and the transient of chlorophyll fluorescence became stronger and clearer. Therefore, ten minutes was chosen as the time length for dark pre-treatment throughout this study.

Leaf Removal Time

The time of leaf removal during the day was usually not mentioned in the literature. However, leaves removed from the plant at different times during the day may give different fluorescence curves. Schreiber (1978) recorded fluorescence curves in 1-min light/15-min dark cycles. After four cycles a constant control curve was reached. However, this light-dark cycle pre-treatment may not succeed because chlorophyll fluorescence can also be influenced by factors other than light. I found in this study that the time of detachment should be considered, especially for certain species of plants. For example, the leaflets of peanut A. helodes detached at night gave fluorescence curves that differed from those displayed by the leaflets picked off the next morning. In a repeated experiment, two pairs of leaflets (A,B and C,D) of A. helodes leaves were used in order to minimize the differences between individual leaves (Figure 6). One leaflet (A or C) was picked off at midnight when the plant was still under weak light. The others (B or D) were detached the next morning. After leaflet detachment

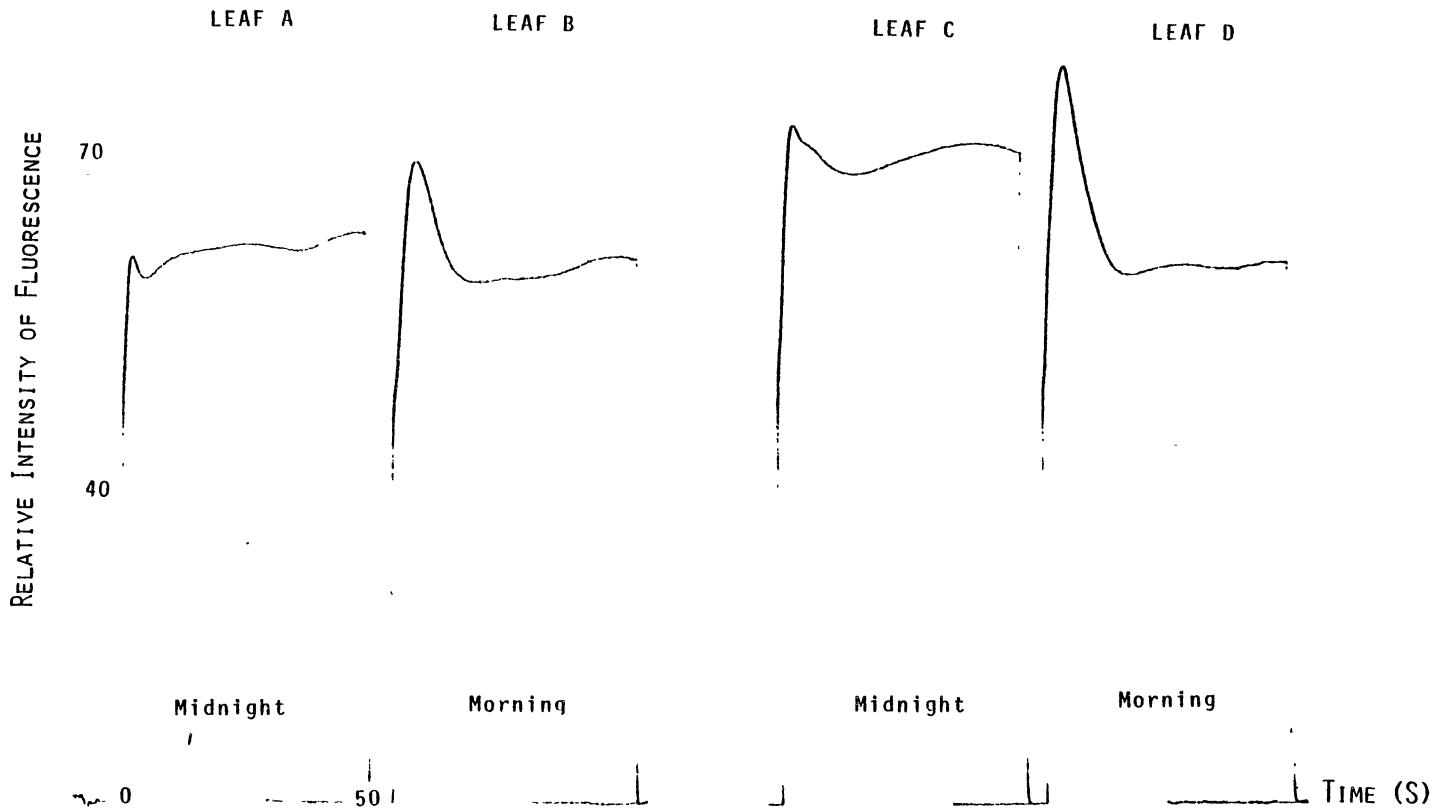

Figure 6. The Effect of the Time of Leaf Removing from the Plant on Fluorescence Curves Recorded from Wild Peanut A. helodes.

fluorescence induction curves were measured immediately. Two different patterns of fluorescence curves were obtained. Morning detachment (leaflet B and D) showed a higher variable fluorescence yield. On the contrary, midnight detachment (leaflet A and C) displayed a lower variable fluorescence yield. It may indicated that the activities of photosystems changed with the alternation between day and night.

As mentioned before, sorghum leaves often displayed a stronger second peak of fluorescence. It was shown that this phenomenon was also related to the time of detachment. In three cultivars of sorghum (IN-2, BOKIII and ROKY62) the second peak of fluorescence usually appeared in leaves detached in the afternoon when the time of illumination exceeded 8 hours. However, there was no second peak found in leaves when the time of illumination was less than 4 hours (Figure 7). Considering the fact that photosynthesis depends on light and it is influenced by the accumulation of photosynthetic products, it is not suprising that chlorophyll variable fluorescence can be affected by time during the day. Therefore, for most of this study, leaf samples were detached in the morning, then the first fluorescence curve was recorded immediately as the experimental control.

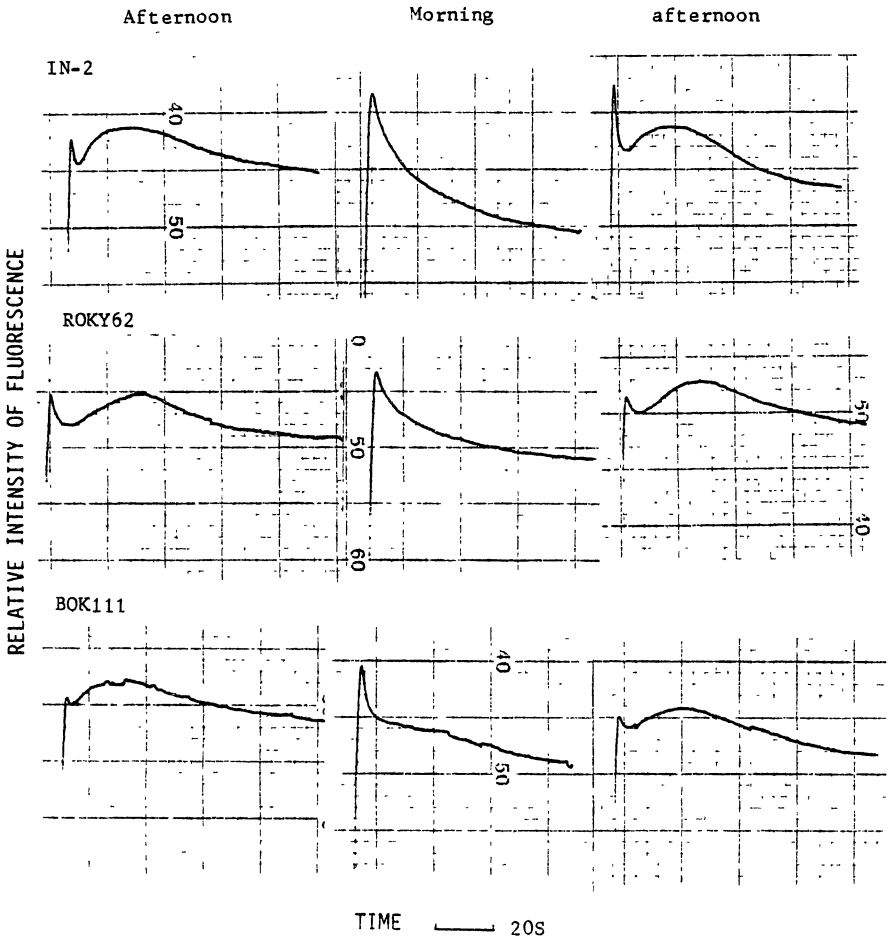

Figure 7. The Effect of the Time of Leaf Removing from Plant on Fluorescence Induction Curves Recorded from Cultivated Sorghum.

Chlorophyll Fluorescence Induction Curves of Detached Leaves without Desiccation

There are usually differences in fluorescence curves of different leaves even though they are outwardly highly similar. Therefore the fluorescence curves of a pre-treatment single leaf (or leaflet) were used as a basis of comparing treatment effects. In this case, if fluorescence curves of a single leaf changed with time after detachment from the plant without desiccation, it would make the comparison more complicated. Fortunately, this was not the case. When the detached leaf was placed on a water-saturated filter paper in a closed petri dish at room temperature and under weak light, the pattern of the fluorescence curve could be maintained for over 24 hours without obvious alteration (Figure 8). A slight fluctuation in fluorescence might be observed in some leaves, but it was insignificant comparing with the marked changes of the fluorescence curve caused by desiccation.

The Chlorophyll a Fluorescence Induction Curve of Attached Leaves

The fluorescence curve could possibly change due to effects of leaf detachment, and this would lead to artifactual results. To examine this possibility, tests on leaves still attached to the plant were made. Figure 9

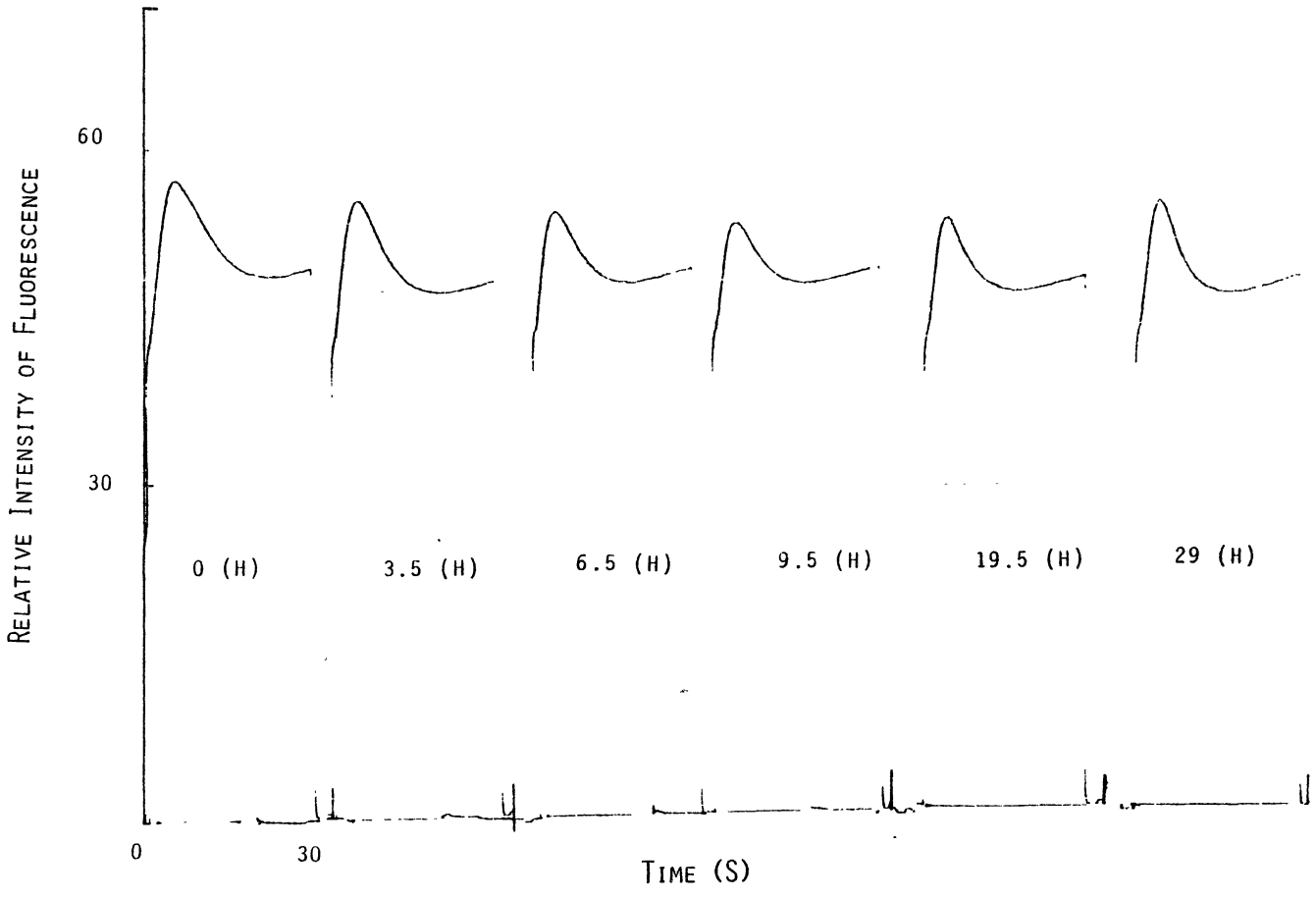

Figure 8. The Fluorescence Induction Curves of Detached leaf of Peanut A. helodes Maintained in a Saturated Atmosphere.

shows the fluorescence curve recorded from attached leaves of sorghum (In-2; ROKY62) in the morning and in the afternoon. Results were similar to those obtained from detached sorghum leaves. The second peak of fluorescence always appeared on the curves recorded in the afternoon. It seems that there is no essential difference in fluorescence curves between attached and detached leaves.

Alterations of Chlorophyll a Fluorescence Induction Curves Caused by Leaf Dehydration

In order to investigate the effects of leaf water deficit on patterns of fluorescence curves, a meaningful comparison can be made by making measurements starting with the natural state of the leaf (higher water content) and allowing dehydration to occur to reach a lower leaf water content. Thus any changes in the pattern of fluorescence curve can be monitored through the entire process of desiccation. The advantages of this approach are:

1. The fluorescence is labile. It is influenced by many factors, some of which may be unknown under the experimental conditions. A series of measurements displays the entire process instead of a single point, so the result should be more reliable than those derived from a single measurement.

2. The changes of a fluorescence curve may be complicated. They may include several different stages which may

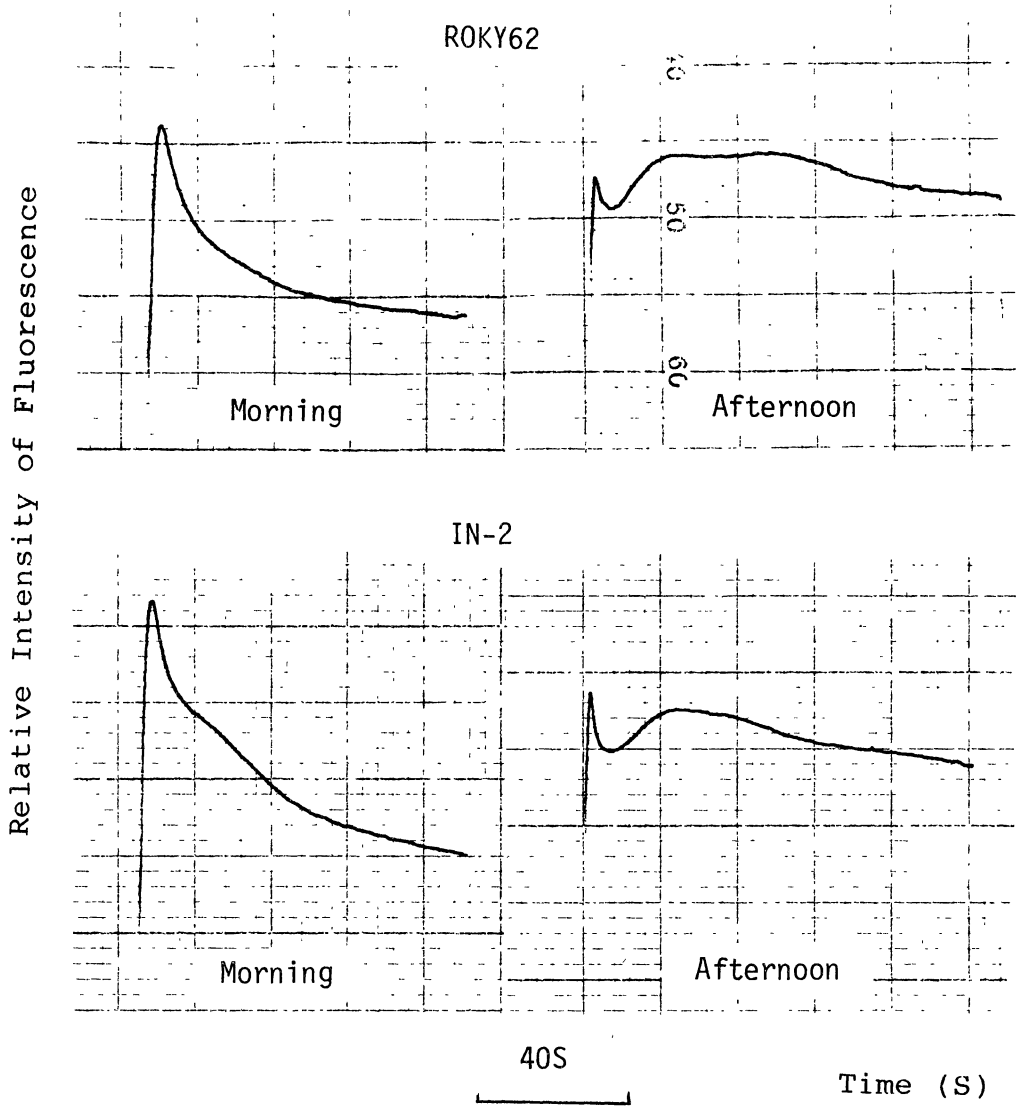

Figure 9. The Fluorescence Induction Curves of Attached Leaves of Cultivated Sorghum Recorded at Different Times of the Day.

not be apparent by a limited number of measurements.

3. There are few reports in the literature of this kind of measurement.

In this study, fluorescence curves were measured at leaf water content ranged from the natural state of the leaf (as the control) to a low leaf water content (<20%). The typical model of pattern alteration of fluorescence curves caused by desiccation in peanut, wheat and sorghum are shown by Figures (10-15).

For the wild species of peanut A. helodes the variable fluorescence yield (OP) was markedly enhanced with the loss of leaf water. Meantime, the level of steady state fluorescence (S) was also raised by desiccation (Figure 10) Sometimes, the enhanced fluorescence caused by desiccation can be as high as two or three times the original level. This indicates a block which was initiated on the photo-reducing side of PSII immediately after loss of water. The block developed with the progressive loss of leaf water until the pattern of the fluorescence curve changed entirely. The peak of fluorescence was transferred into a fluorescence plateau at which point the maximum fluore-scence yield was usually reached. The leaf water content at this point was found to be species dependent. For A. helodes it was around 30%. Below this water content the variable fluorescence declined indicating damage occurred to the water splitting system. Similar results were found in leaves of peanut cultivars EC-5 (Figure 11)

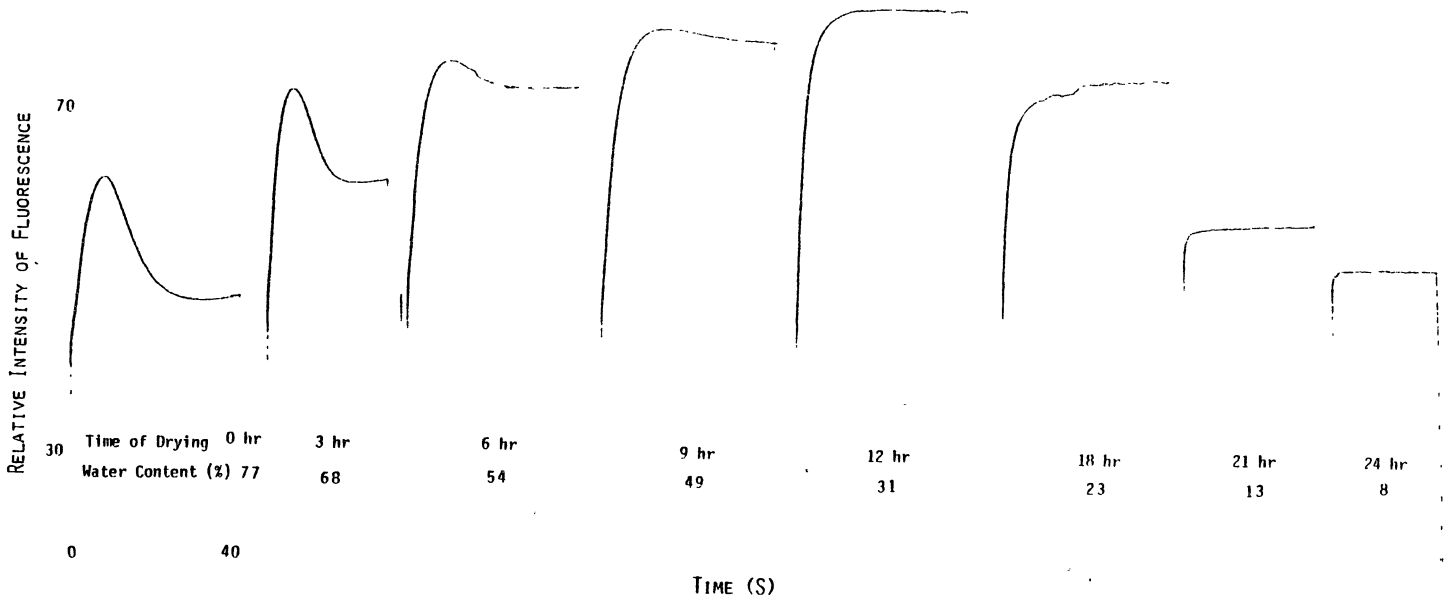

Figure 10. The Pattern Alteration of Fluorescence Induction Curves of a Detached Leaf of A. helodes by Dehydration.

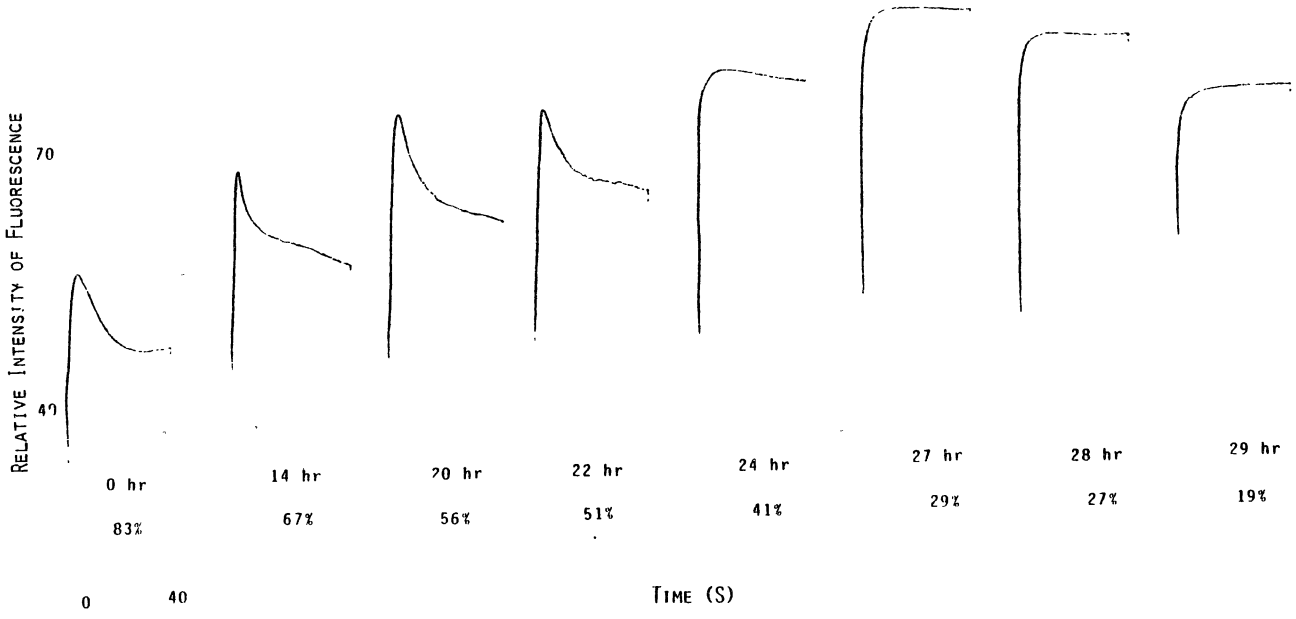

Figure 11. The Pattern Alteration of Fluorescence Induction Curves of a Detached Leaf of Cultivated Peanut A. hypogaea L. cv. EC-5 by Dehydration.

and Tamnut (Figure 12). The increased fluorescence caused by leaf desiccation was followed by the appearance of a fluorescence plateau, then fluorescence yield declined as leaf water content dropped below a certain point. For EC-5 leaves this point was found to be also around 30% water content (Figure 11).

For measuring the fluorescence curves of wheat leaves, four 2-cm long leaves were arranged side by side in the dark cell. The SF-10 sensor was placed in the center of the sample. The changes in patterns of fluorescence curves for two wheat cultivars, KanKing (Figure 13) and Ponca (Figure 14) caused by leaf dehydration were similar to those observed in peanuts. However, the leaf water content at the point of plateau formation was about 40%, a higher water content than for peanuts.

Sorghum leaves also showed enhanced fluorescence by desiccation up to 60% water content. Then the variable fluorescence declined slightly and the pattern of fluorescence was transferred into a plateau. Thus the yield of fluorescence at the plateau was not the highest yield through out the process of desiccation (Figure 15).

Therefore, a model of pattern alteration of fluorescence curves by desiccation included three major stages: 1) A progressive increase of both the variable fluorescence and the steady state of fluorescence; 2) The formation of a fluorescence plateau at which time the maximum fluorescence yield was usually attained; 3) A decline in fluorescence

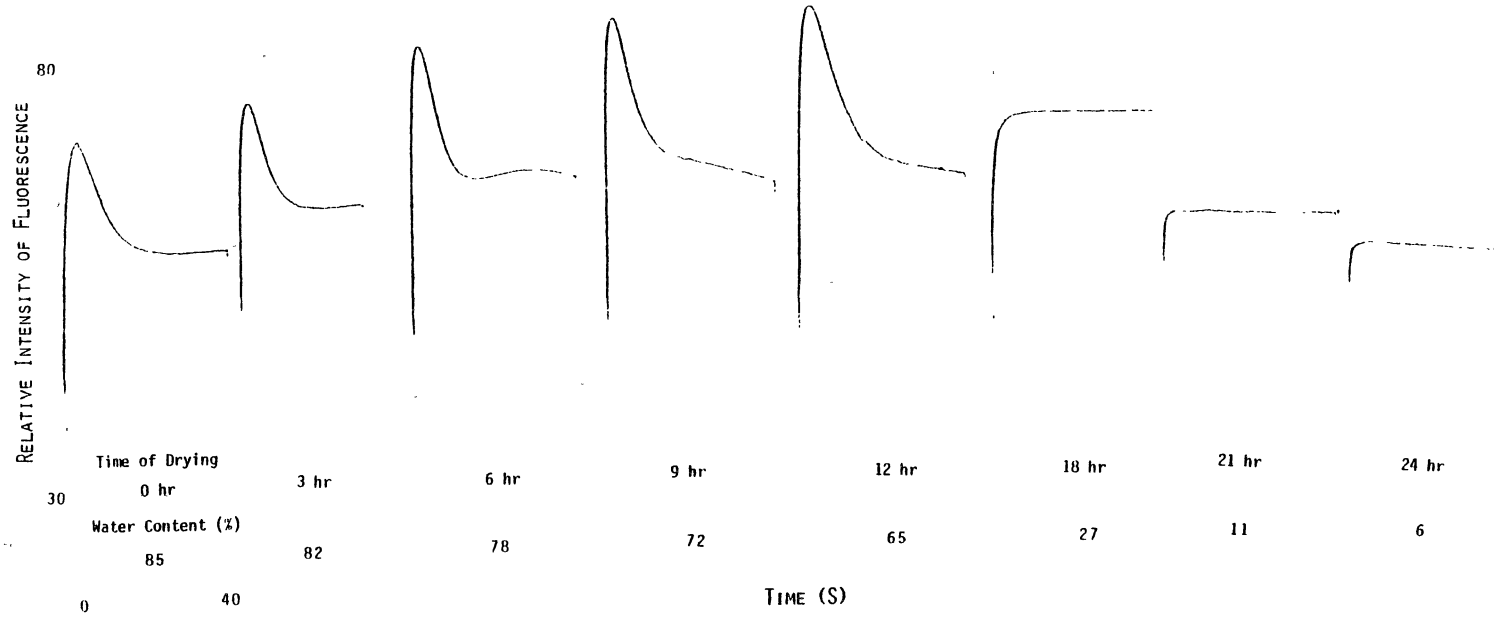

Figure 12. The Pattern Alteration of Fluorescence Induction Curves of a Detached Leaf of Cultivated Peanut A. hypogaea cv. Tamnut by Dehydration.

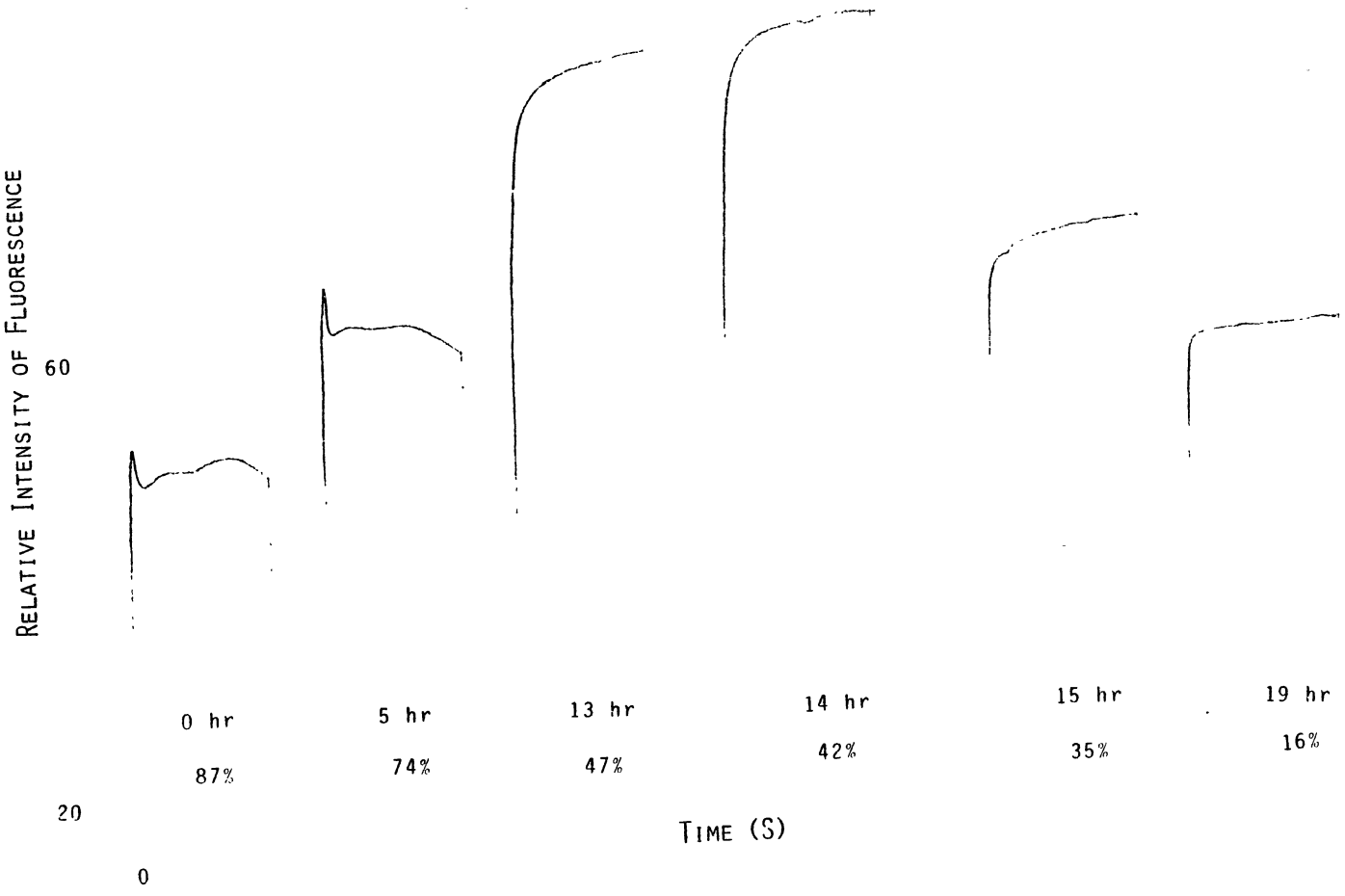

Figure 13. The Pattern Alteration of Fluorescence Induction Curves of a Detached Leaf of Cultivated Wheat Triticum aestivum L. cv. KanKing by Dehydration.

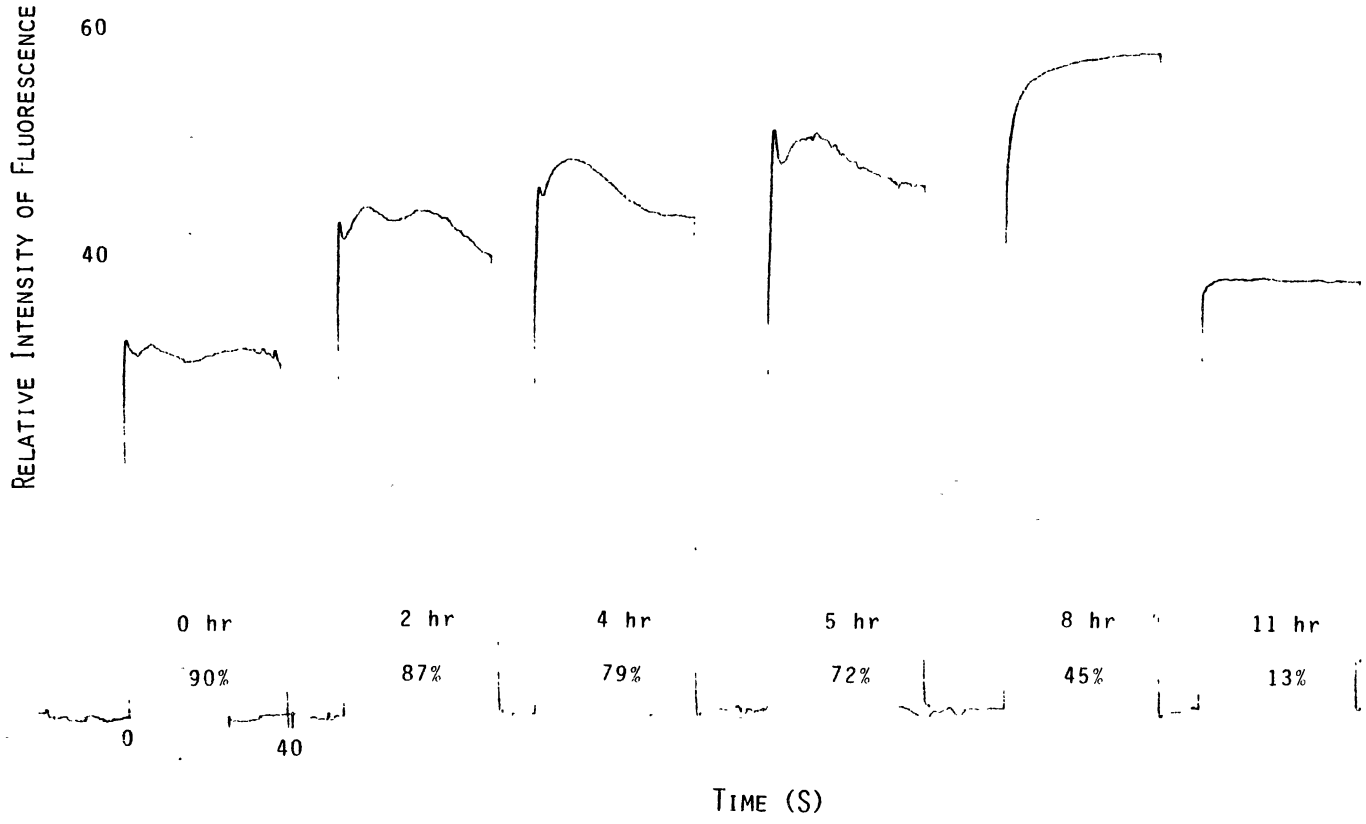

Figure 14. The Pattern Alteration of Fluorescence Induction Curves of a Detached Leaf of Cultivated Wheat Triticum aestivum L. cv Ponca by Dehydration.

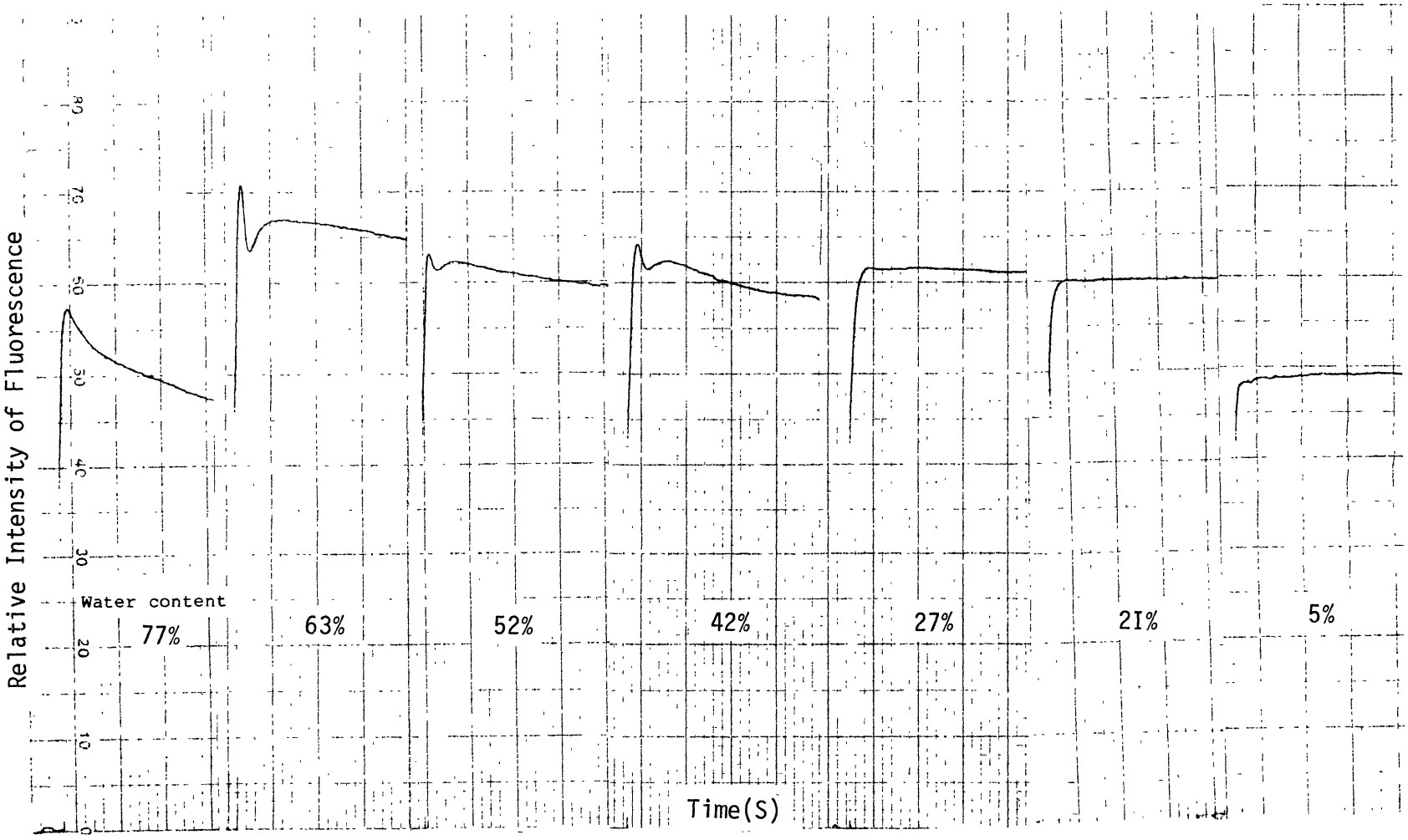

Figure 15. The Pattern Alteration of Fluorescence Induction Curves of a Detached Leaf of Cultivated Sorghum S. bicolor. cv. IN-2 by Dehydration.

yield after the plateau leading to the formation of fluorescence platform. However, this type of pattern was not always followed. A more complicated situation was observed in peanut leaves. As shown in Figure 16, one A. helodes leaf did not show fluorescence enhancement immediately when leaf water content dropped from 84% (natural state) to 66%, but after further water loss the pattern was similar to the model described above. In another leaf of A. helodes, a marked fluorescence enhancement was displayed after 12 hours of leaf dehydration, then the fluorescence yield decreased with further leaf water loss. The plateau was attained at the 24th hour after desiccation. Meanwhile, the maximum yield of fluorescence was attained (Figure 17). In many EC-5 leaves, it was confirmed that leaf fluorescence increased up to about 75% water content, then it decreased and further loss of leaf water led to the formation of a fluorescence plateau at which point the fluorescence yield nearly equaled to the maximum yield (Figure 18).

It was reported that variable fluorescence was lost in grapevine leaves subjected to rapid drying. If leaves were slowly dried the variable fluorescence was not lost until much lower water potential was reached (Downton, 1983). The author attributed this to leaf osmotic adjustment by which the leaf turgor presure can be maintained temporarily during desiccation. Thus possible damage to photosystems can be avoided or delayed. The deviant

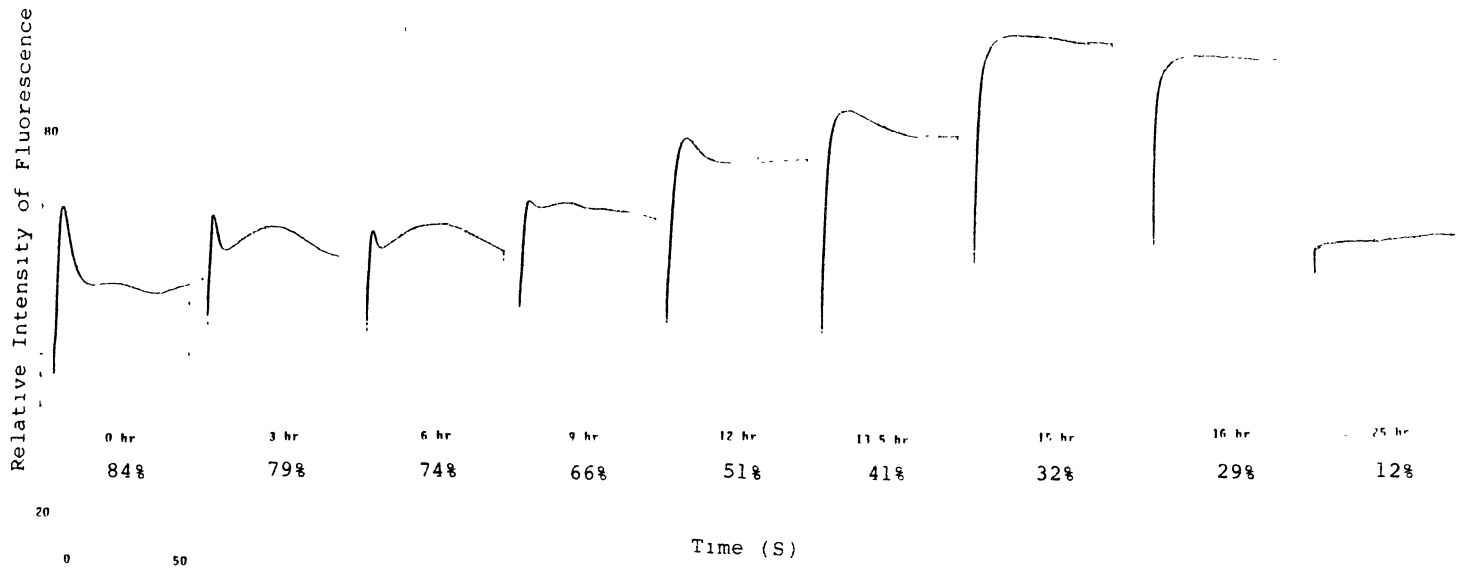

Figure 16. The Pattern Alteration of Fluorescence Induction Curves of a Detached Leaf of Peanut A. helodes (with adjustment in eary stage) by Dehydration.

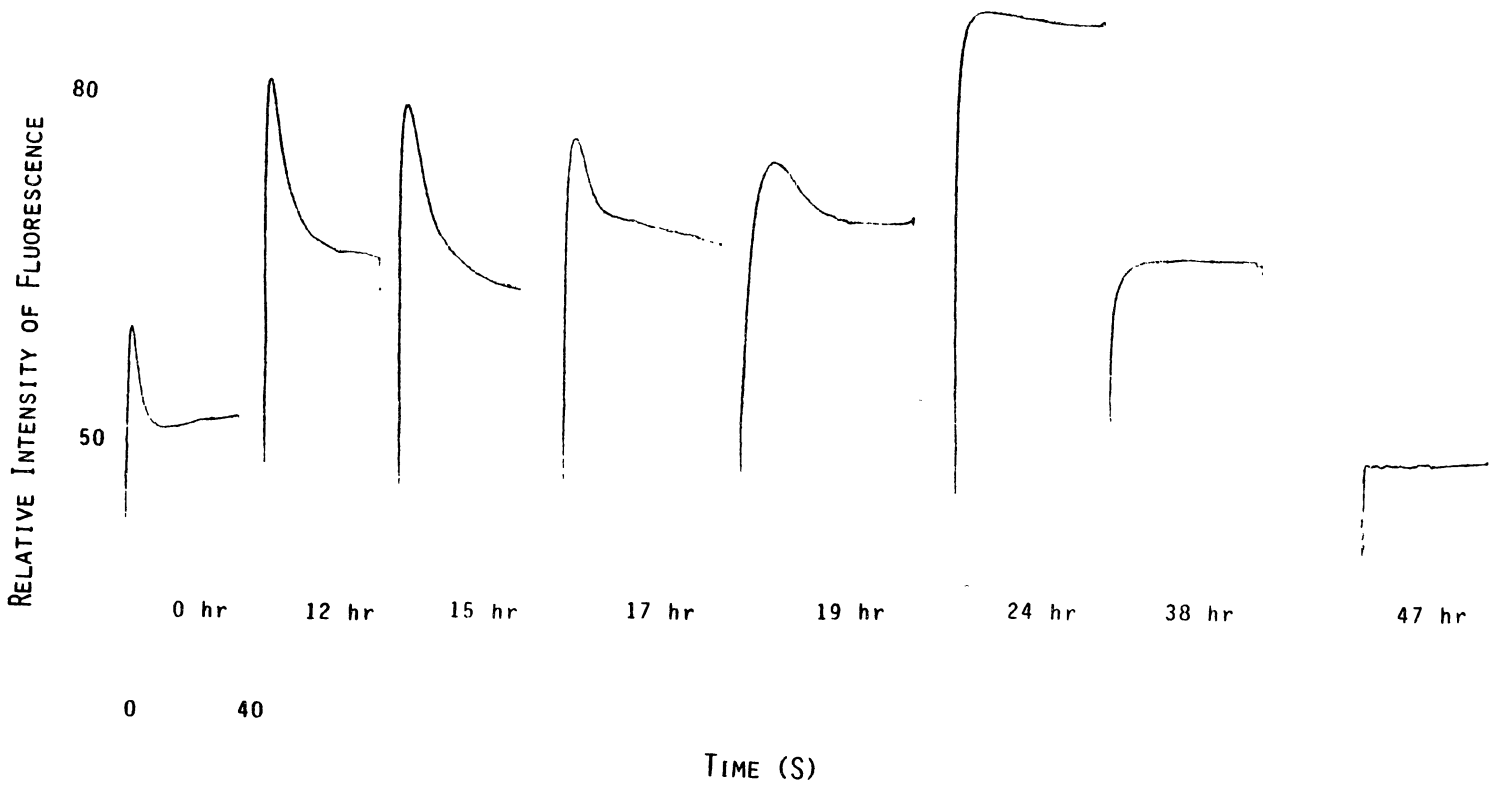

Figure 17. The Pattern Alteration of Fluorescence Induction Curves of a Detached Leaf of Peanut A. helodes (with adjustment in later stage) by Dehydration.

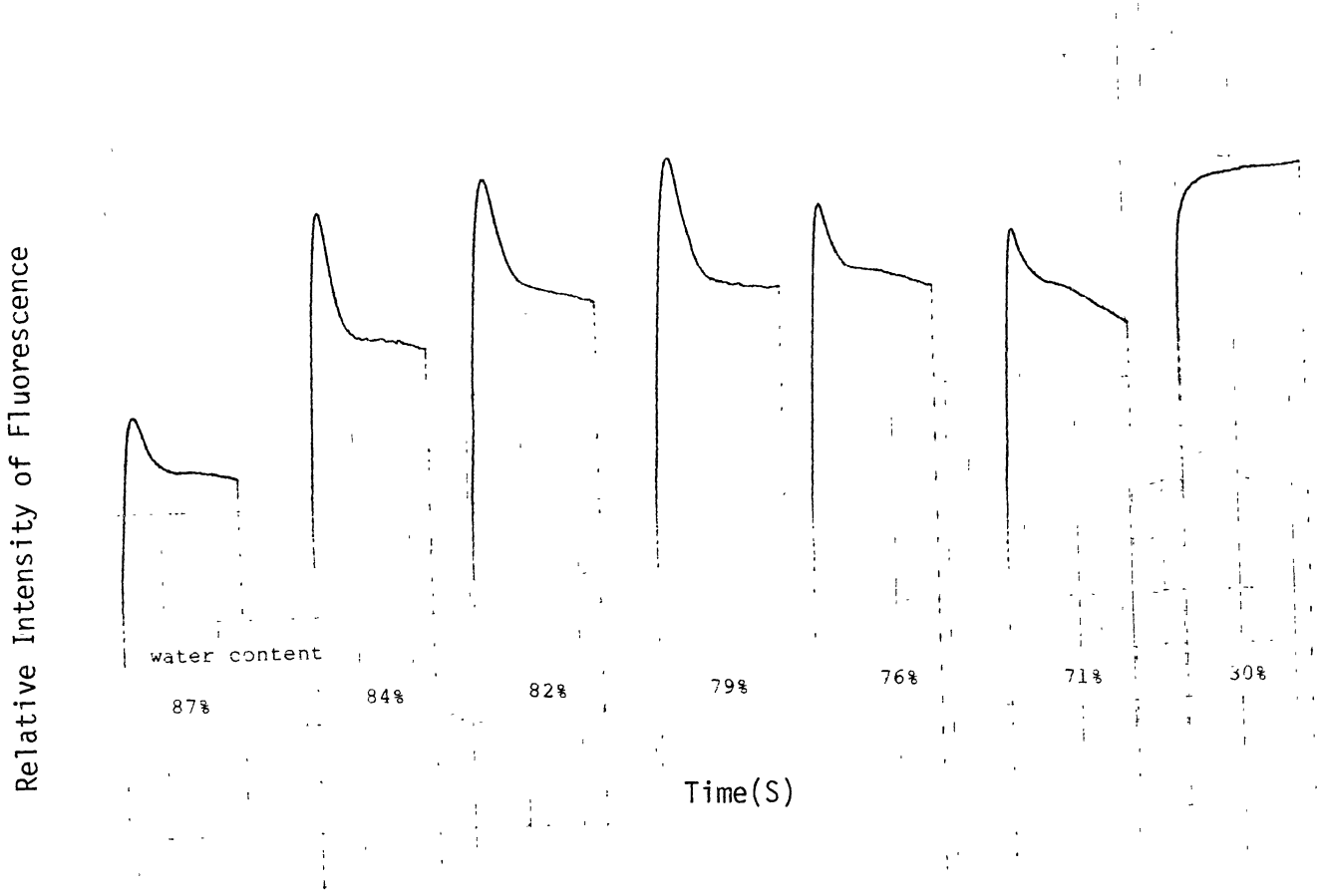

Figure 18. The Pattern Alteration of Fluorescence Induction Curves of a Detached Leaf of Cultivated Peanut EC-5 by Dehydration.

behavior found in this study may be caused by the same effect although an increase rather than a decrease in fluorescence yield was found here. It is likely that a block initiated on the photoreducing side of PSII can be relieved by leaf adjustment, hence the enhanced fluorescence declines.

A fluorescence curve of the attached leaf of peanut EC-5 was recorded while the plant underwent water stress. The water content of the intact plant was lowered by withholding water from the pot. Water loss from the plant was fairly slow. It took one month to lower the leaf water content from 82% to 50%. The fluorescence yield increased as leaf water declined in a pattern similar to detached leaves. The maximum fluorescence yield was reached around a leaf water content of 70%, then fluorescence declined (Figure 19). It indicates that there is no essential difference in the type of fluorescence curves obtained whether the leaves were attached or detached when subjected to water stress.

In other plant species (peanut A. villosulicarpa and cotton plant Gossypium hirsutum (See Appendixes) the immediate response of the variable fluorescence to leaf dehydration was found to be enhancement. Detached leaves may or may not exhibit an adjustment during the desiccation process. A leaf with this ability will produce an altered model of fluorescence under desiccation.

In leaves of three species of higher plants, it was

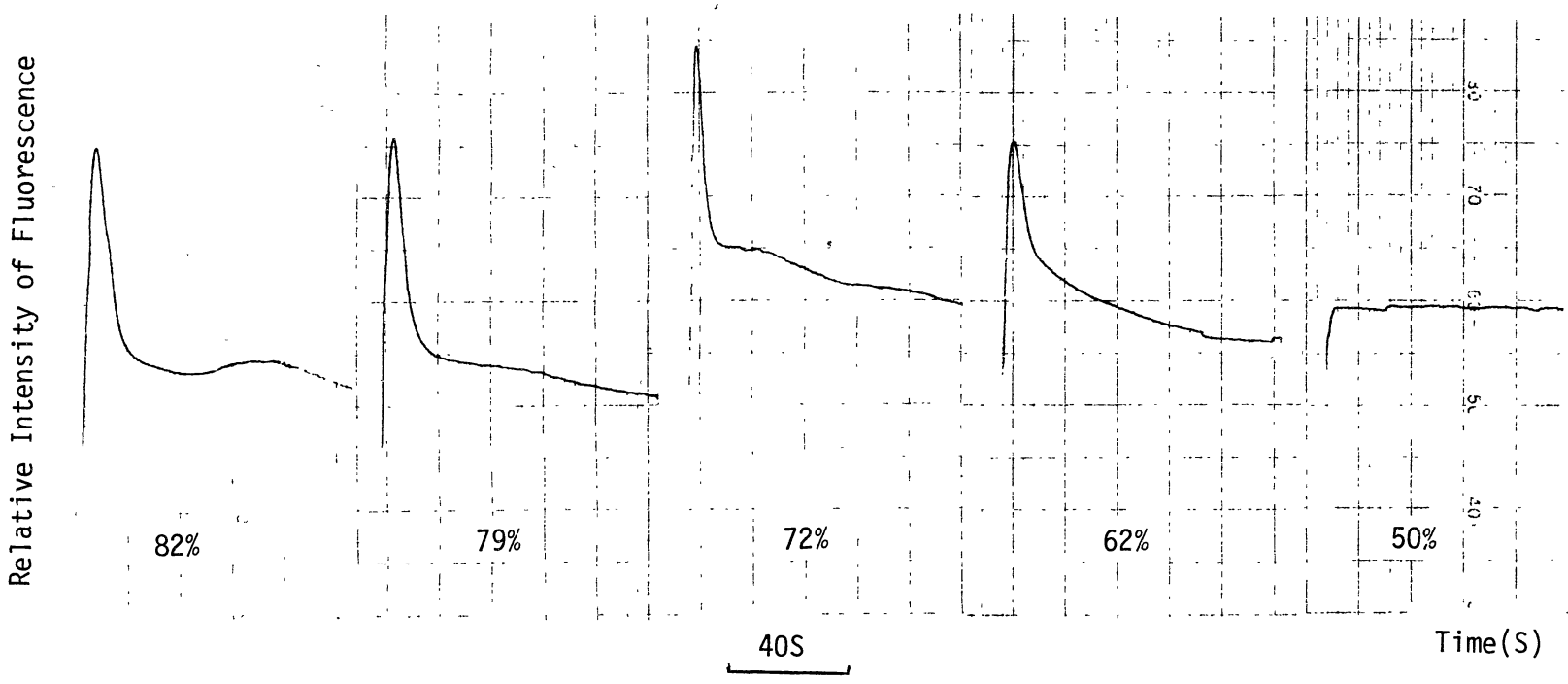

Figure 19. The Pattern Alteration of Fluorescence Induction Curves of a Attached Leaf of Cultivated Peanut EC-5 by Desiccation.

found that the variable fluorescence was always lost during leaf dehydration (Govindjee et al., 1981). In my study, no immediate loss of variable fluorescence was observed during leaf desiccation. In one experiment with young leaves of sweet pea (Lathyrus sp.) a decrease in variable fluorescence was observed when leaf water content dropped from 89% to 82% (Figure 20,A). However, in another experiment where water content was measured at 86% (between 89% and 84%), a fluorescence enhancement was found (Figure 20,B). Because this water content interval is small, such a change of fluorescence may be easily overlooked. The initial measurement of fluorescence in N. oleander leaves was made at a water potential of -9 bars and the second test was at -15 bars (Govindjee et al., 1981). Thus fluorescence enhancement might be found between these two values of water potential.

The Response of Chlorophyll Fluorescence Induction Curves to Leaf Rehydration

The ability to recover the transitory fluorescence during rehydration was an indicator of drought tolerance of marine algae. Rehydration of drought-tolerant algae resulted in rapid recovery of transitory fluorescence. However, no such recovery was found in drought-sensitive algae (Wiltens et al., 1978).

In this study, peanut leaves were used to perform the rehydration experiments. After leaf dehydration of 12 hours

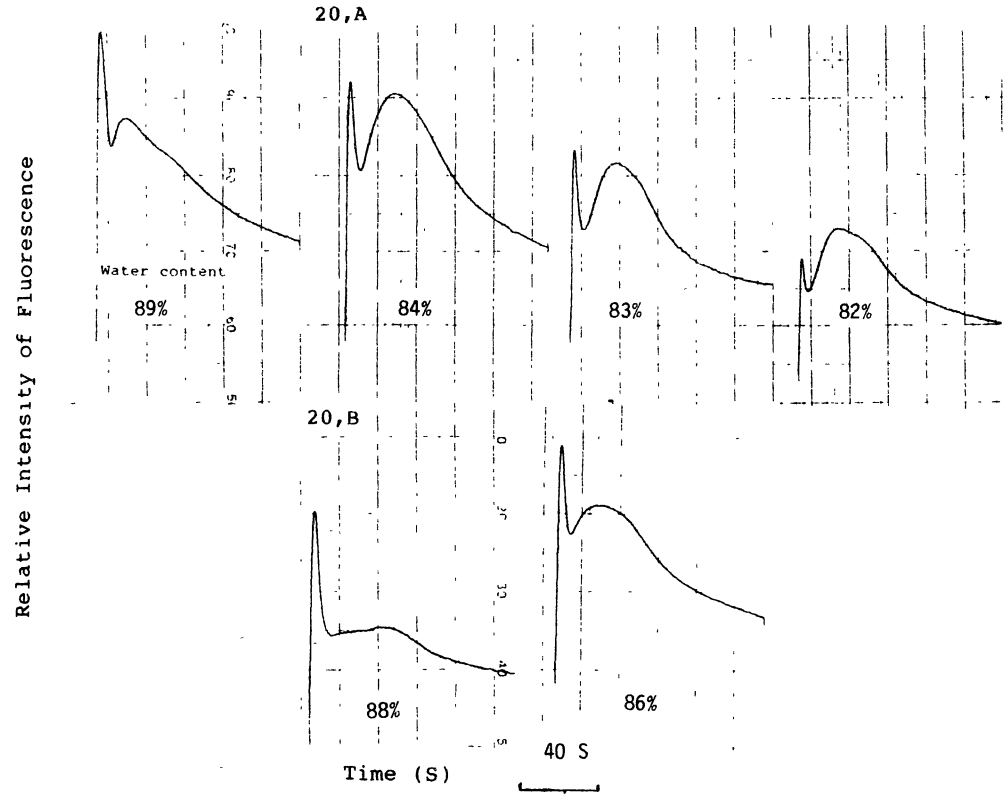

Figure 20. The Pattern Alteration of Fluorescence Induction Curves of a Detached Leaf of Sweet Pea (Lathyrus.) by Dehydration. 20,A: fluorescence yield decreased during leaf dehydration. 20,B: an enhancement of fluorescence was found between 89% and 84% of leaf water content.

the variable fluorescence was increased in both EC-5 and A. helodes leaves. Following 12 hours of rehydration the fluorescence yield was lowered to approximately the original level, while a second peak appeared (Figure 21) No essential difference was found between the cultivated and the wild species of peanut. Figure 22 also shows the diminishing of the first peak and the appearance of the second peak of fluorescence caused by leaf rehydration with a shorter time of desiccation. Dehydration often caused an increased second peak of fluorescence while subsequent rehydration did not eliminate it (Figure 23).

The effect of dehydration-rehydration cycles on the fluorescence curve is shown in Figure 24. The first drying cycle which diminished leaf water content from 87% to 84%, enhanced variable fluorescence (Figure 24, 1 & 2). The subsequent rehydration restored leaf water content to the original level (87%) and decreased fluorescence (Figure 24, 3). The second cycle of drying (from 87% to 80% of leaf water content) raised the fluorescence again (Figure 24, 4). A subsequent slow dehydration (leaf placed on wet filter paper) caused a decrease rather than increase of fluorescence (Figure 24, 5). This indicated a capacity of the leaf to adjust during the slow desiccation. The third period of drying (from 74% to 58% of leaf water content) increased the fluorescence yield once more (Figure 24, 6). These results indicate that the block initiated on the photoreducing side of PSII caused by dehydration is

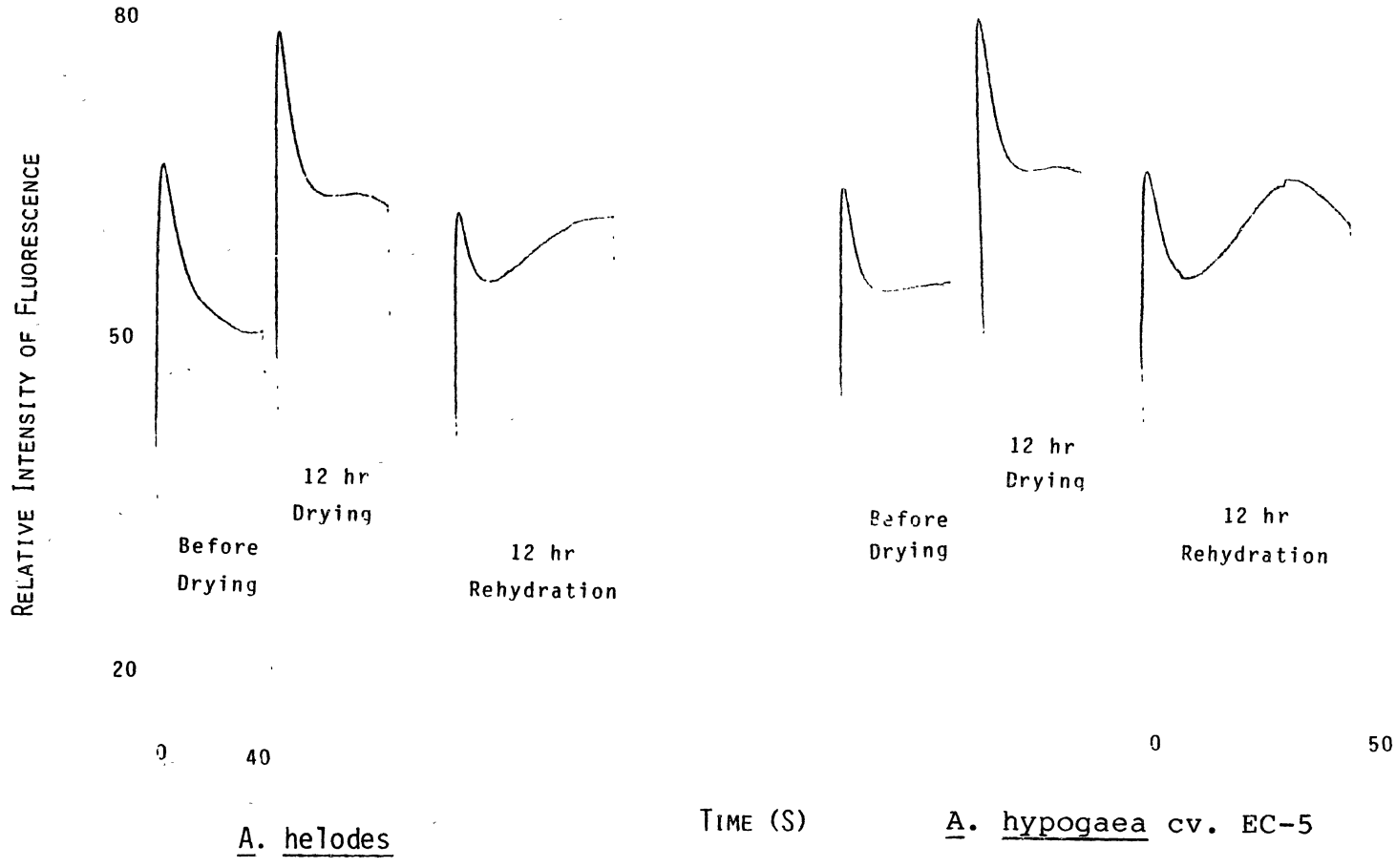

Figure 21. The Changes of Fluorescence Induction Curves of Peanut Leaves during Leaf Dehydration and Rehydration.

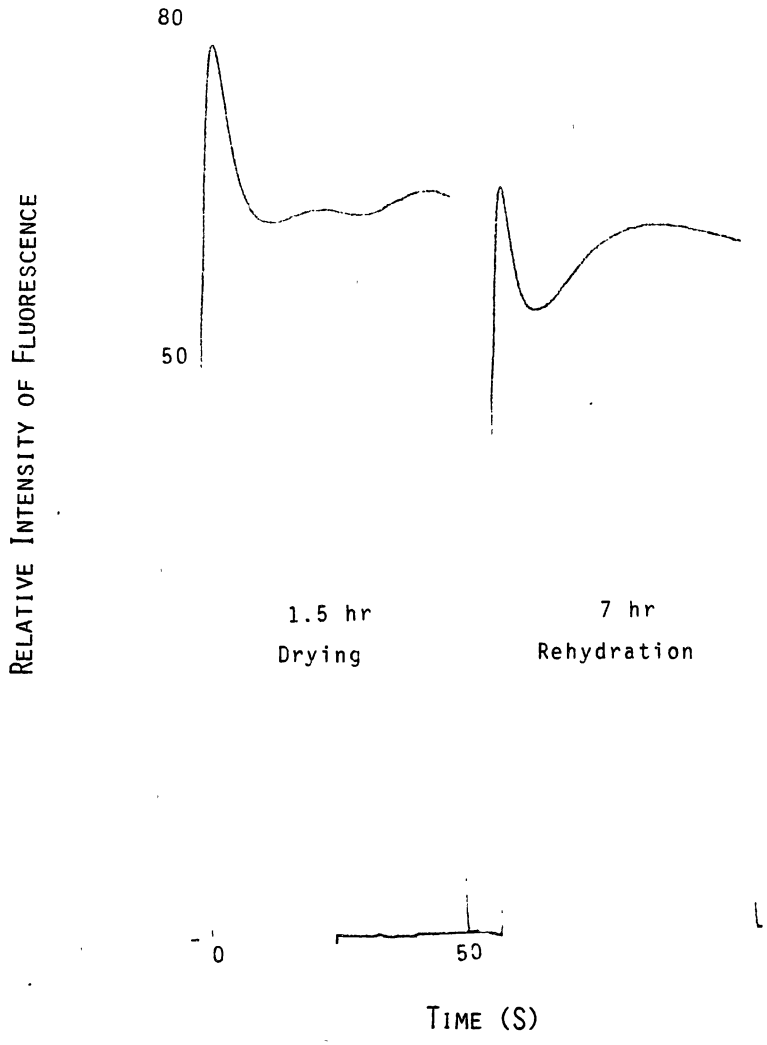

Figure 22. The Changes of Fluorescence Induction Curves of a Detached Leaf of Peanut A. helodes during Dehydration and Rehydration.

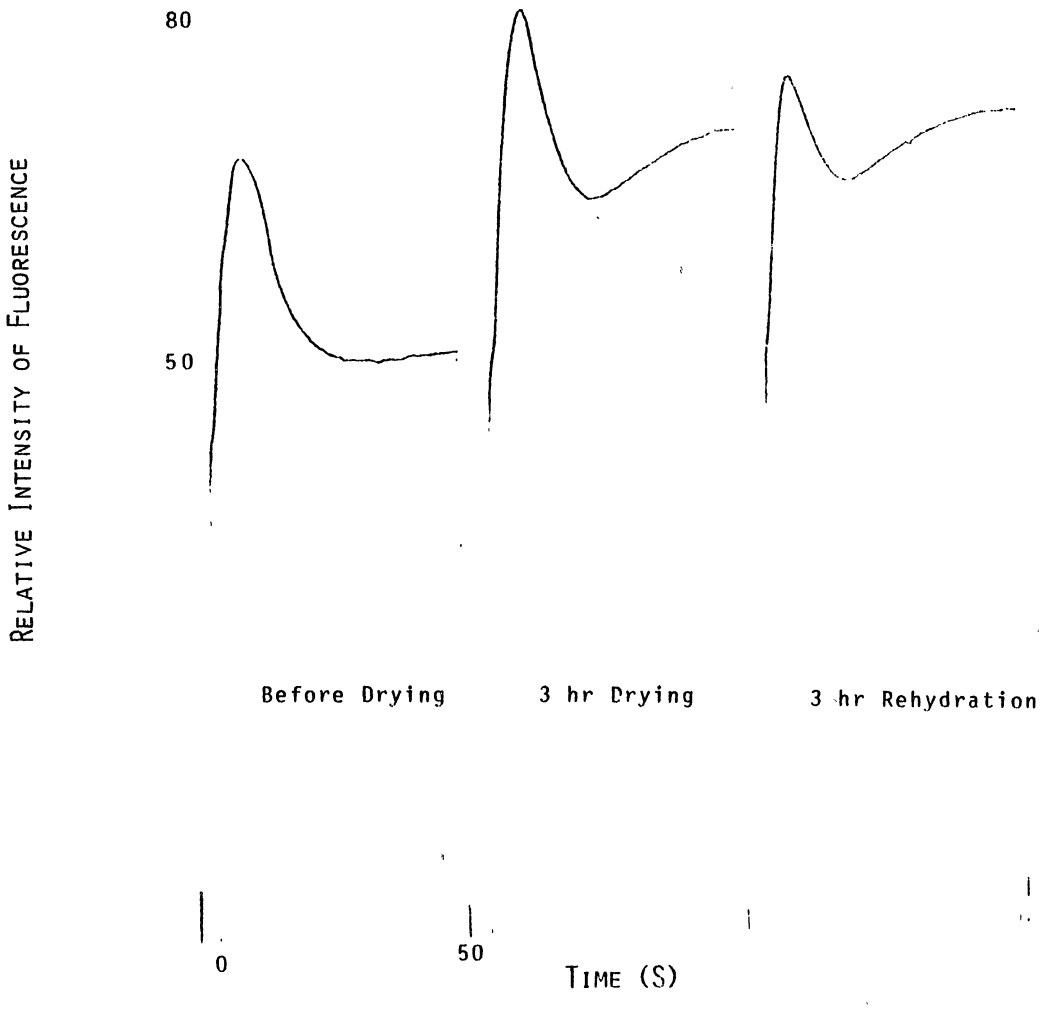

Figure 23. The Changes of Fluorescence Induction Curves of a Detached Leaf of Peanut EC-5 during Dehydration and Rehydration.

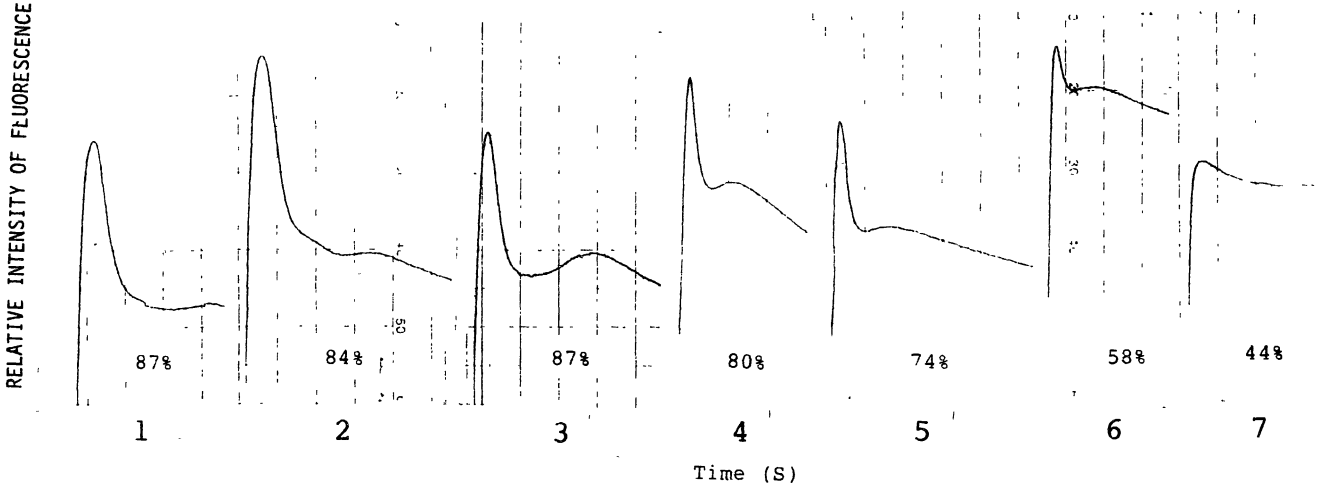

Figure 24. The Effects of Dehydration-Rehydration Cycles on Fluorescence Induction Curves of one Detached Leaf of Peanut EC-5. Before dehydration (1); followed by 2 hrs dehydration (2); then 4 hrs rehydration (3); followed by 6 hrs dehydration (4); then 12 hrs slow dehydration (leaf in petri dish) (5); followed by 6 hrs dehydration (6); followed by 4 hrs dehydration (7).

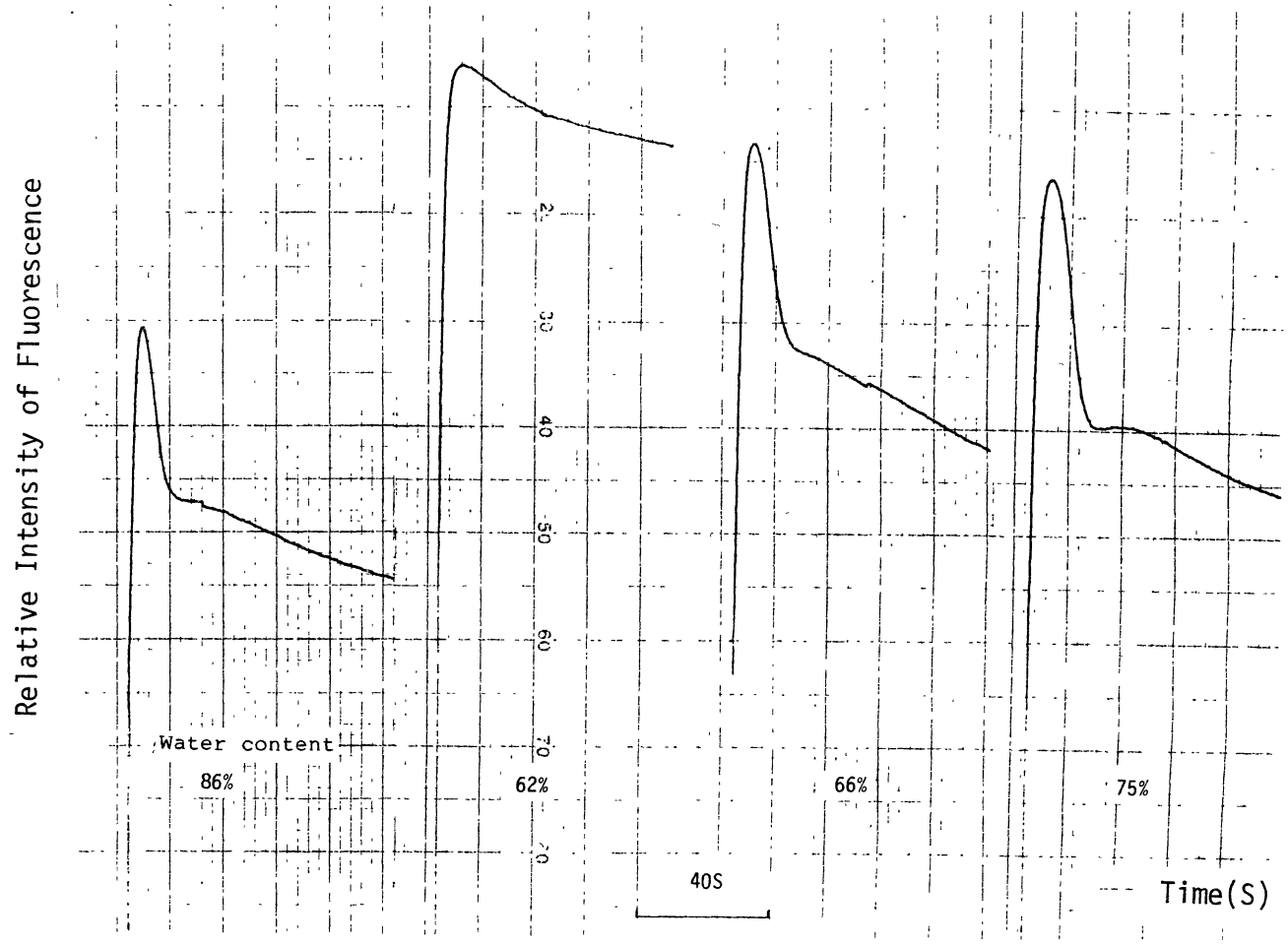

Figure 25. The Recovery of Original Pattern of Fluorescence Curve by Leaf Rehydration prior to Complete Formation of Fluorescence Plateau in Peanut EC-5 Leaves.

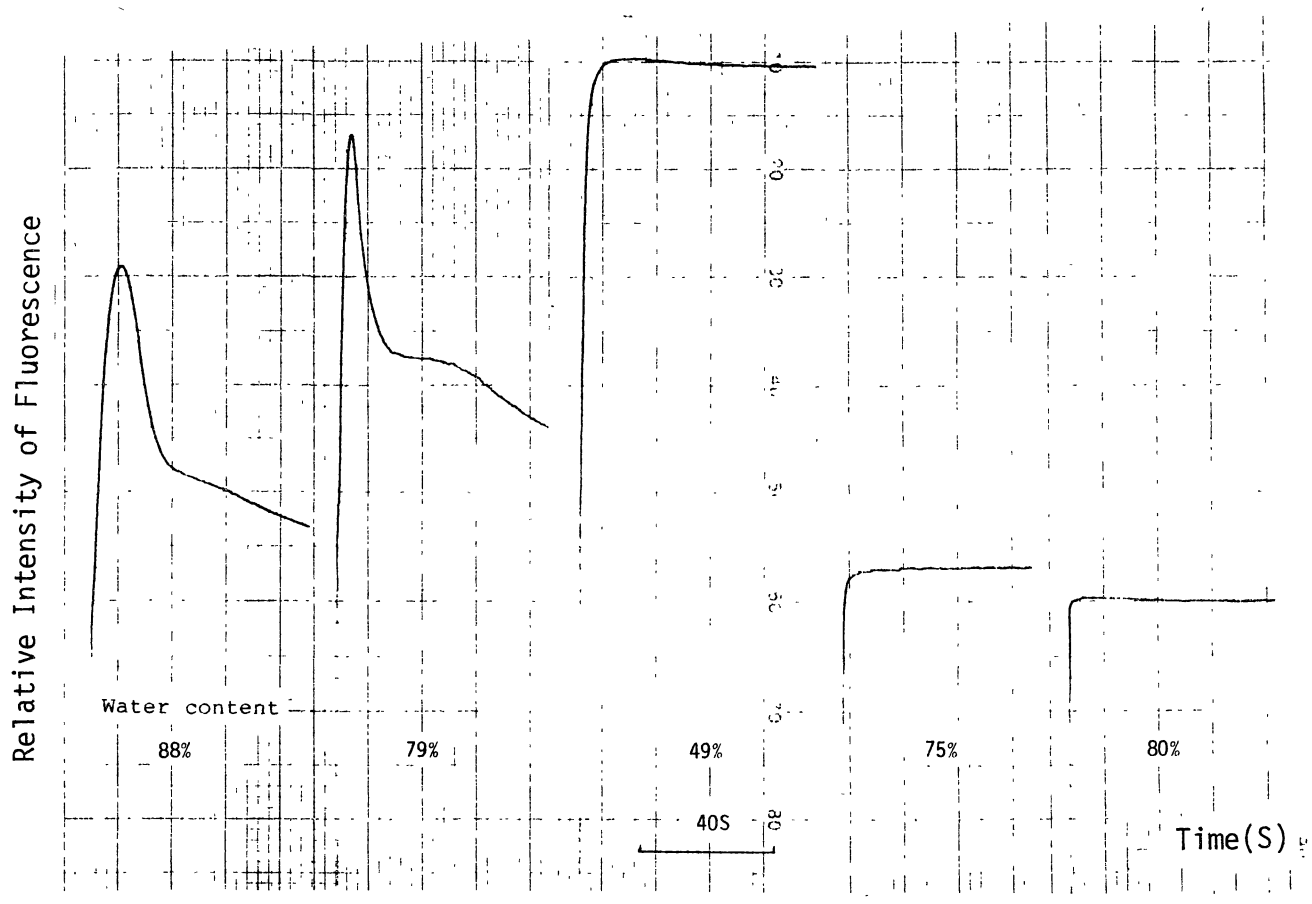

Figure 26. The Irreversible Changes in the Pattern of Fluorescence Curves by Dehydration after the Complete Formation of Fluorescence Plateau in Peanut EC-5 Leaves.

reversible. It can be relieved by leaf rehydration, and subsequent dehydration can induce the block again. Further drying below a leaf water content of 58% eventually caused a decline in fluorescence. This possibly represents an irreversible damage to the water splitting system.

The water content at the plateau of fluorescence or at the point with the highest fluorescence yield was found to be critical. Leaf rehydration usually can restore the transient fluorescence prior to this critical point. (Figure 25). This suggests that changes in photosystems in this stage are reversible. However, it was difficult to restore the original pattern of fluorescence through rehydration after this point had been reached. At this time, rehydration caused a further decrease of fluorescence (Figure 26) indicating an irreversible change occurred within the photosystems.

The Alteration of Fluorescence Induction Curves by Photosynthetic Inhibitors

Three wild species of peanut (A. helodes; A. villosulicarpa; A. paraguarensis), peanut cultivars EC-5 and peanut WH X P1541; wheat KanKing and sorghum In-2 were used in these studies. The leaves from different plant species were treated by four kinds of photosynthetic inhibitors, some of which are herbicides in common use. Based on the knowledge of action sites of these inhibitors, it is possible to correlate the alteration of patterns of

fluorescence curves with the known action sites. The results of known inhibitors can be compared with those from dehydration experiments so that action sites of water stress in distinct stages of desiccation may be inferred.

Atrazine

Atrazine is a widely used agricultural herbicide. It blocks electron transfer at the site between Q and its electron acceptor. Hence it inhibits photosynthesis and usually markedly enhances variable fluorescence. All of the tested leaves from different plants in this study displayed enhanced fluorescence after atrazine treatment. Both the variable and steady state fluorescence increased until a fluorescence plateau was attained (Figure 27). This process appeared to be similar to that observed in water stress, but it proceeded much faster. The time required for reaching the plateau was different for different plants. Three hours were needed for A. helodes. In A. villosulicarpa, WH X P1541 and sorghum one hour was sufficient (Figure 28,29 and 30). For A. paraguarensis 6.5 hours were needed to change the pattern of the fluorescence curve to a plateau (Figure 31) while only ten minutes were sufficient for KanKing wheat (Figure 32). This may be related to the rate of atrazine uptake by leaves of different plant species. Atrazine caused marked fluorescence enhancement in EC-5 leaves, but the time longer than 12 hours diminished fluorescence yield. Placing the

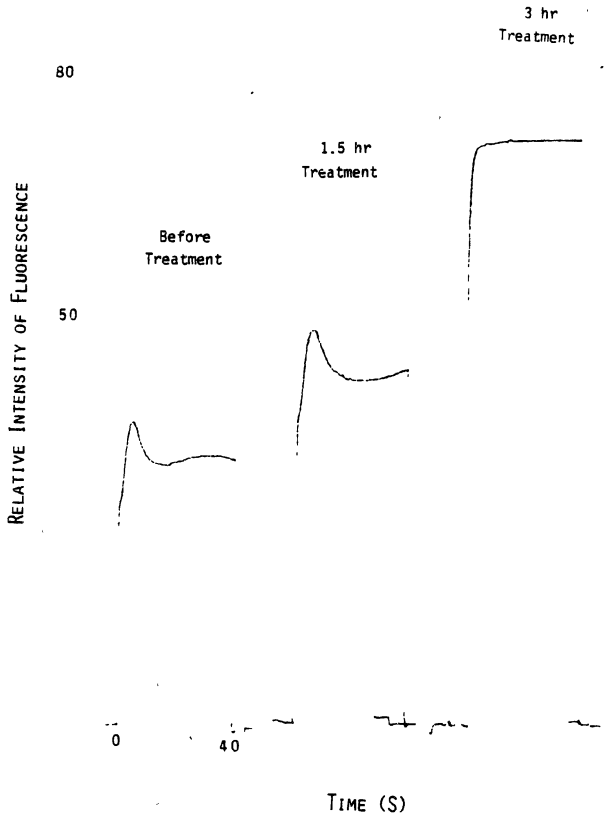

Figure 27. The Pattern Alteration of Fluorescence Induction Curves of Peanut A. helodes Leaf by the Treatment with 10^{-4} M Atrazine.

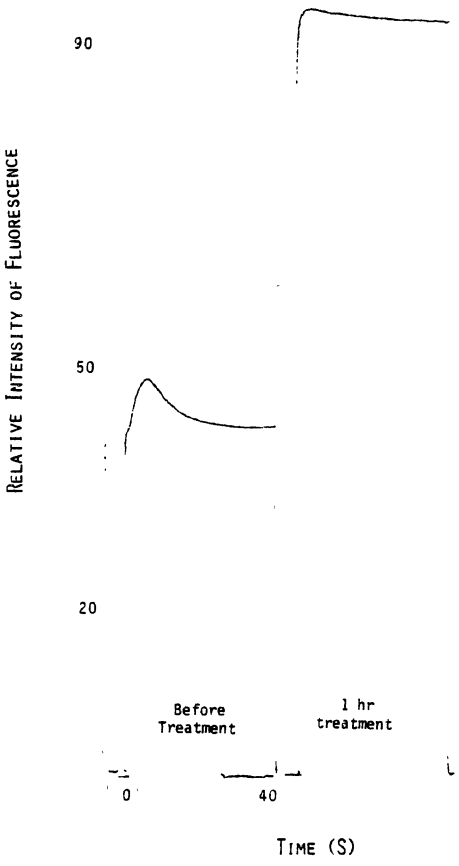

Figure 28. The Pattern Alteration of Fluorescence Induction Curves of a Wild Peanut A. villosulicarpa Leaf by the Treatment with 10^{-4}M Atrazine.

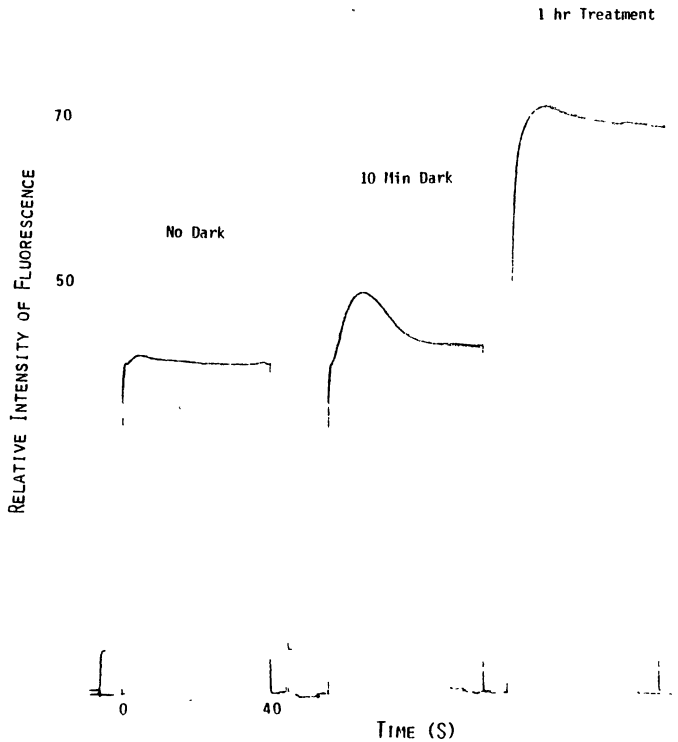

Figure 29. The Pattern Alteration of Fluorescence Induction Curves of Peanut (WH X P 1541) Leaf by the Treatment with 10^{-4}M Atrazine.

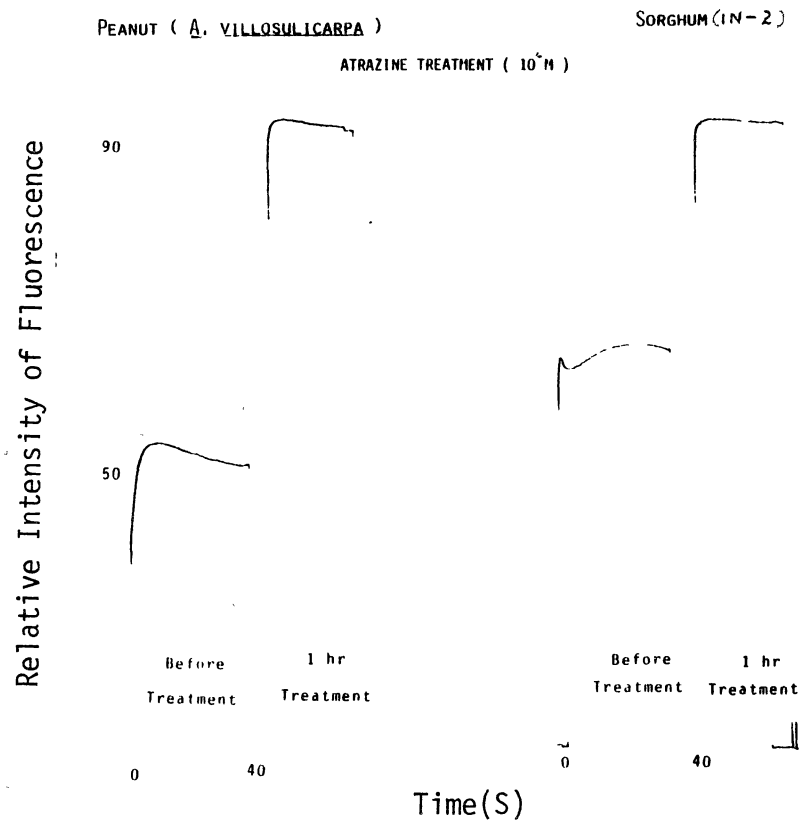

Figure 30. The Pattern Alteration of Fluorescence Induction Curves of Leaves of Wild Peanut A. villosulicarpa and Cultivated Sorghum IN-2 by the Treatment with 10^{-4}M Atrazine.

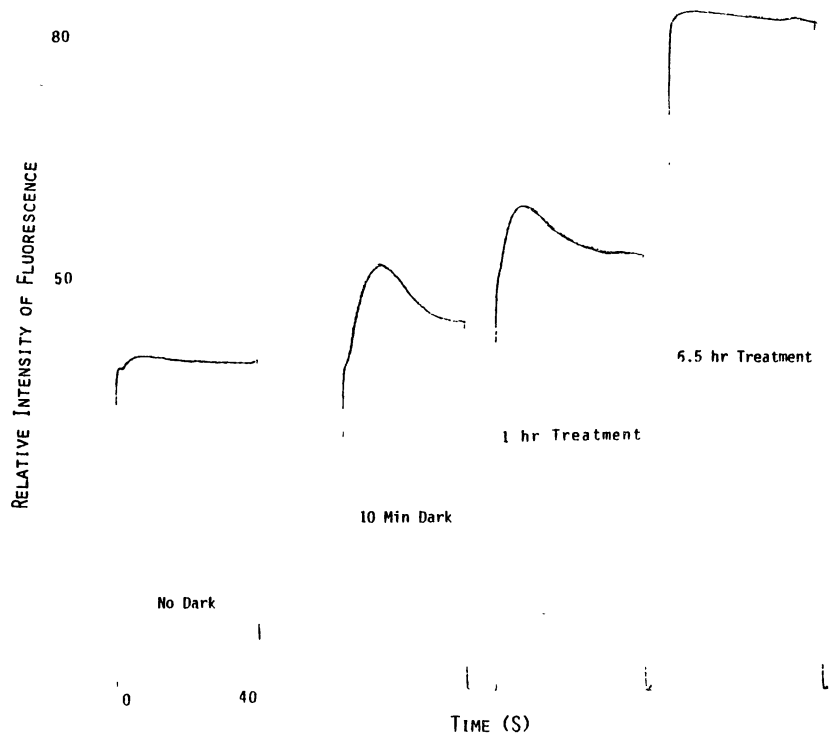

Figure 31. The Pattern Alteration of Fluorescence Induction Curves of a Wild Peanut A. paraguarensis Leaf by the Treatment with 10^{-4}M Atrazine.

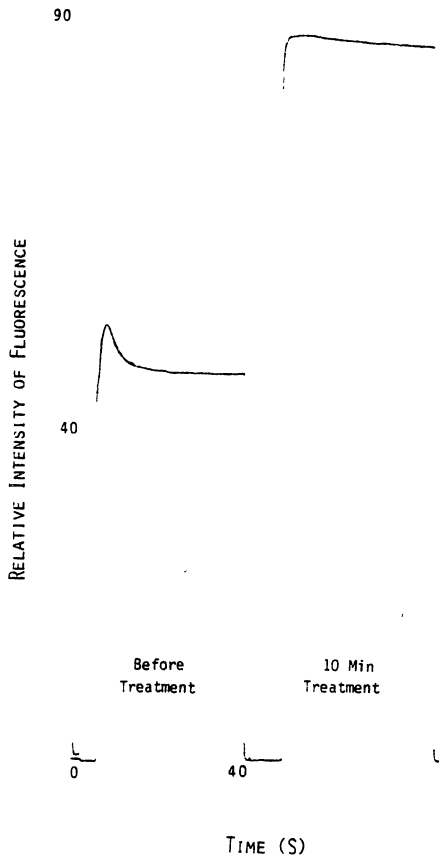

Figure 32. The Pattern Alteration of Fluorescence Induction Curves of KanKing Wheat Leaf by the Treatment with 10^{-4}M Atrazine.

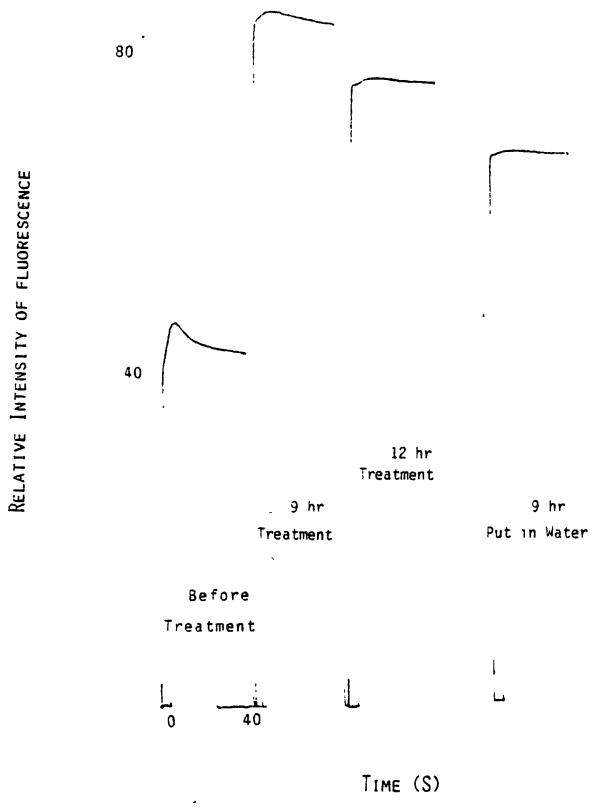

Figure 33. The Pattern Alteration of Fluorescence Induction Curves of Cultivated Peanut EC-5 Leaf by the Treatment with 10^{-4} M Atrazine.

leaf in water did not restore the original curve of fluorescence indicating an irreversible damage to the photosystems (Figure 33).

Paraquat

As an electron acceptor, paraquat affects photosynthesis by causing electron diversion from PSI. After receiving one electron from PSI, the paraquat radical can be generated (Moreland, 1976; Dodge, 1982). Paraquat radical can induce superoxide as well as other highly active agents which attack cell membrane systems (Mead, 1976).

The effect of paraquat on the pattern of fluorescence curves was different from those of atrazine. No fluorescence enhancement was observed. On the contrary, the fluorescence yield declined by paraquat treatment. The second peak of fluorescence appeared and then a platform was reached (Figure 34, 35). Since paraquat accepts electrons and is immediately regenerated, it may enhance electron transport and thus increase the rate of oxidation of Q, as a consequence, fluorescence yield decreases.

2,4 Dichlorophenol (DCP) and N,N'-Dicyclohexylcarbodimide (DCCD)

As an uncoupler, DCP dissociates the electron transfer from the formation of ATP (Moreland and Hilton, 1976). Under the effect of DCP, electron transfer may

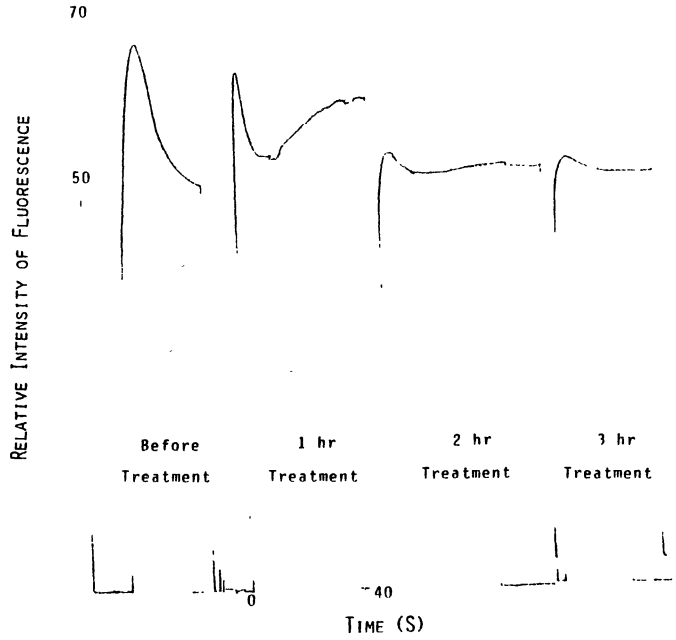

Figure 34. The Pattern Alteration of Fluorescence Induction Curves of Cultivated Peanut EC-5 Leaf by the Treatment with 10^{-2} M Paraquat.

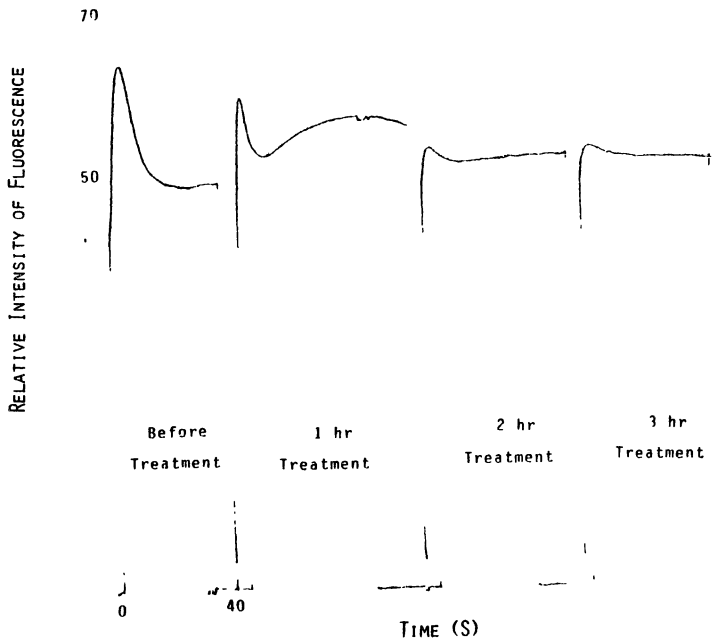

Figure 35. The Pattern Alteration of Fluorescence Induction Curves of Peanut A. helodes by the Treatment with 10^{-2}M Paraquat.

proceed even at an increased level, but there is no ATP formation in photosynthesis.

The effect of DCP on the pattern of the fluorescence curve in EC-5 is shown in Figure 36. Only 30 minutes after treatment, the first peak of fluorescence declined and particularly, the second peak was enhanced. It appears possible that the decreased level of ATP is correlated with the appearance of the second peak of fluorescence.

DCCD, as known to be an inhibitor of proton secretion through ATPases and other enzymes such as cytochrome c oxidase and the cytochrome b-c_1 complex, should limit ATP formation. Figure 37 shows that only 30 minutes treatment with DCCD changed the pattern of the fluorescence curve into a platform in both A. helodes and EC-5 leaves. It is very similar to the pattern displayed by leaves at the final stage of desiccation. However, in the case of leaf desiccation more than twenty hours of drying were needed to reach the platform. Avron (Berry et al., 1983) noted that a primary site of damage occurring with water stress is the structure of the ATP-synthase system on the surface of the chloroplast membranes. Therefore, the pattern alteration of the fluorescence curve during water stress should be related to the limitation of the ATP level in leaf cells.

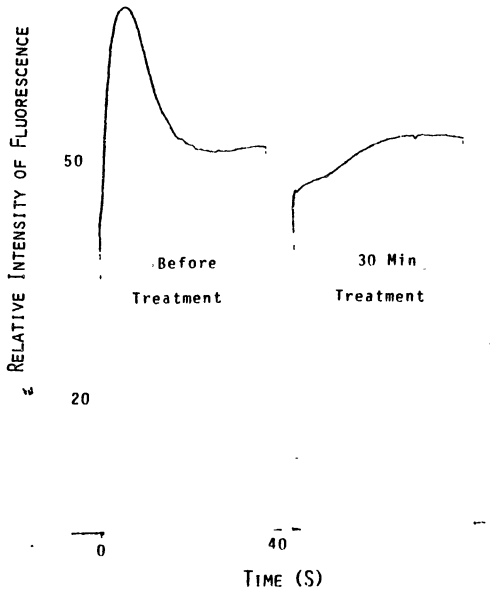

Figure 36. The Pattern Alteration of Fluorescence Induction Curves of Cultivated Peanut EC-5 Leaf by the Treatment with 10^{-4} M DCP.

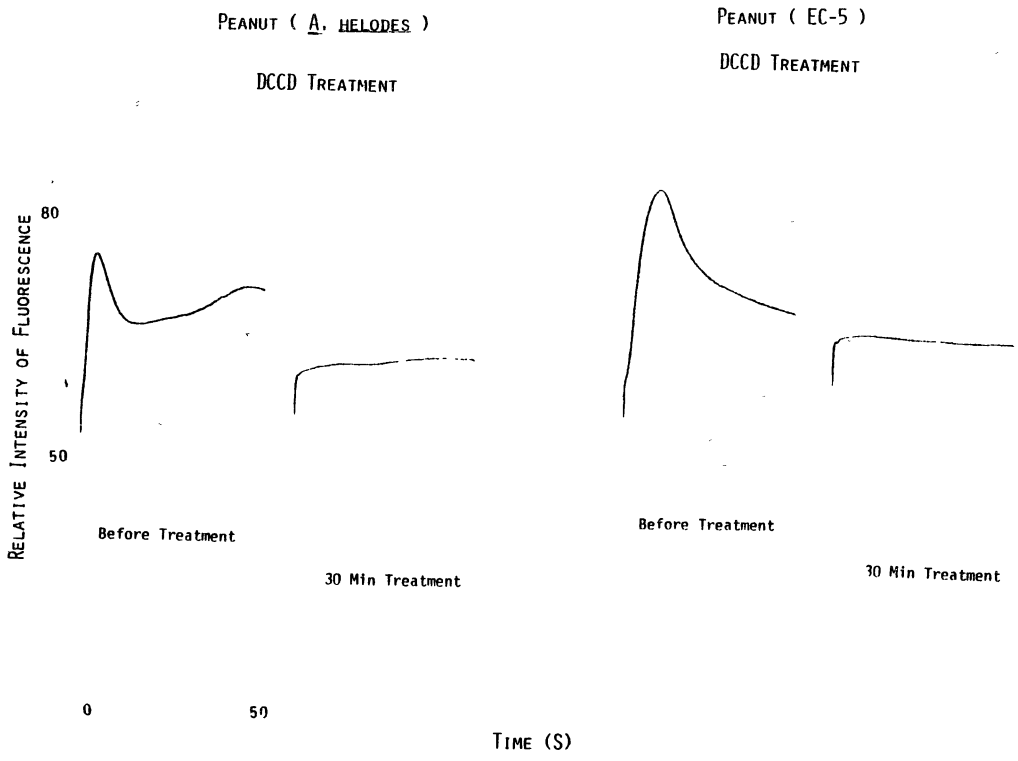

Figure 37. The Pattern Alteration of Fluorescence Induction Curves of Leaves of a Wild Peanut A. helodes and a Cultivated Peanut EC-5 by the Treatment with 5×10^{-5} M DCCD.

CHAPTER V

SUMMARY AND CONCLUSIONS

1. The pattern of fluorescence curves is usually species dependent. It can be altered dramatically by leaf water loss. The major factor which determines the pattern of fluorescence curve is leaf water content in leaves. However, other factors, such as dark adaptation period, the age and the physiological state of the plant, and the time of leaf removal during the experiment cannot be ignored.

2. For peanuts, wheat, sorghum as well as other plant species examined in this study, progressive leaf water loss caused a successive increase in variable fluorescence yield (OP). This indicated that a block was initiated on the photoreducing side of PSII as leaf dehydration started. At the same time, the level of the steady state of fluorescence (S) was also raised by leaf dehydration without exception. This may represent a progressive prevention of electron transfer between Q and the next electron acceptor.

3. With the loss of leaf water, the pattern of the fluorescence curve was changed to a fluorescence plateau at which time the maximum yield of fluorescence was usually attained. The leaf water content at which the

fluorescence plateau was first completely formed was found to be critical. It was species dependent and it may be used as an indicator for testing the plant tolerance to drought.

4. Leaves may or may not show the ability to adjust during the dehydration process. Leaves without the ability to adjust followed a three-stage model of pattern alteration of the fluorescence curve during desiccation. The pattern alteration for leaves having this ability was more complex.

5. The enhanced variable fluorescence by desiccation can be restored by leaf rehydration before the fluorescence plateau was completely formed. The second cycle of drying can again cause an increase of fluorescence. It indicates that the block on the photoreducing side of PSII is reversible. However, after the formation of a fluorescence plateau the original pattern of the fluorescence curve usually cannot be restored by rehydration. This suggests that irreversible damage occurred to the photosystems.

6. A strong second peak of fluorescence was often observed in sorghum leaves. A second peak also appeared during dehydration or rehydration, and can be induced by DCP treatment. These changes probably relate to ATP levels in the plant tissue.

7. Four kinds of photosynthetic inhibitors affected fluorescence curves in different ways. Atrazine enhanced fluorescence yield while paraquat diminished it. DCP

mainly caused the appearance of the second peak of fluorescence. The treatment of DCCD caused the formation of fluorescence platform which usually appeared at the final stages during leaf desiccation. So water stress may affect ATPases which are located on chloroplast thylakoid membrane. Fluorescence tests might be used as an indicator to evaluate plant resistance to herbicides acting as photosynthetic inhibitors.

PART II

ACTIVITIES AND ISOZYMES OF SUPEROXIDE DISMUTASE AS AFFECTED BY CYANIDE AND PARAQUAT IN PEANUT AND WHEAT LEAVES

CHAPTER V

LITERATURE REVIEW

Superoxide, an oxygen free radical, is formed in living cells as a common metabolic intermediate. It is deleterious to organisms. Moreover, it can give rise to other highly reactive species like singlet oxygen and hydroxyl free radicals (Halliwell, 1978). By initiating the peroxidation of lipid *in vivo*, these toxic agents may result in damage to cell membrane systems (Mead, 1976).

Superoxide dismutase (SOD) catalyzes the dismutation of superoxides to molecular oxygen and hydrogen peroxide, and hence protects cells from attack by this free radical. (McCord and Fridovich, 1969). SOD enzyme has been found in numerous prokaryotic and eukaryotic organisms and seems to be ubiquitous (Fridovich, 1975, 1976, 1978). Many works provide evidence for the protective role of SOD enzyme under conditions where oxygen radicals are produced.

An oxygen-resistant mutant of the green alga *Chlorella sorokiniana* contained more SOD than the wild type (Pulich, 1974). A high level of SOD in the blue-green alga *Plectonema boryanum* conferred resistance toward photooxidation death (Stenitz et al., 1979). SOD enzyme may play a key role in restricting the damage caused by lipid peroxidation that occurrs in soybean seeds during storage

(Stewart and Bewley, 1980). Leaf senescence was correlated with diminishing of SOD level (Dhindsa et al., 1980). The peroxidation of lipid in two types of mosses caused by dehydration was found to correlate with SOD activity (Dhindsa and Matowe, 1980). SOD enzyme played a role in protecting plant tissue from ozone damage (Lee and Bennett, 1982; Pauls and Thompson 1982) as well as SO injury (Tanaka et al., 1982). SOD enzyme, by scavenging superoxides, appeared to supplement the protective action of carotenoids against photo-oxidative injury in ripening tomato fruits (Rabinowitch et al., 1982). A positive correlation between SOD level and dehydration tolerance in wheat seedlings was reported (Wu and Todd, 1985).

In view of its important role in organisms the activities and isozymes of SOD in higher plants are being widely investigated. Activities of SOD in corn, oat and pea plants have been reported. It was found that shoots contained more enzyme than roots. A high SOD level was found in the embryo. The specific activity of SOD in corn and oat was two to three times higher than those of pea plant (Giannopolitis and Ries, 1977). In wheat seedlings it was also found that the shoot had a higher SOD activity than the root. The level of SOD was changed with the process of growth and development of seedlings (Wu and Todd, 1985). The relative amounts of SOD isozymes of pea plant were found to be different in leaves at different stages of plant growth and development (Lastra et al., 1982).

growth and development (Lastra et al., 1982).

Superoxide dismutases occur as several metalloproteins in which the Cu-Zn-containing SOD is cyanide-sensitive and mainly found in eukaryotic organisms (McCord and Fridovich, 1969), Mn- and Fe-containing SOD are cyanide-insensitive and are mostly restricted to prokaryotes (Keele et al., 1970; Vance et al., 1972) and to mitochondria of eukaryotes (Weisiger and Fridovich, 1973). In recent years SOD isozymes have been characterized in various monocotyledonous and dicotyledonous plants. Both acrylamide gel electrophoresis and isoelectricfocusing electrophoresis were used to analyze SOD isozymes. The results varied depending on the experimental procedures used since different numbers of isozymes were reported for the same plant species. Wheat germ showed three electrophoretic bands. Two bands distributed on the lower side of the gel were completely inhibited by 0.2 mM cyanide. The third one on the upper side of the gel was cyanide-insensitive and was identified as Mn-SOD located in mitochondria, only one band was found in wheat leaves (Beauchamp and Fridovich, 1973). A single band was obtained by running the chloroplast fraction of spinach leaves on gels. The purified SOD had a molecular weight of 32,000 daltons and was composed of two subunits. No Mn-SOD was detected (Asada et al., 1973). However, Mn-SOD was found later in the mitochondria of spinach leaves (Jackson et al., 1978). Mn-SOD and Fe-SOD were found in blue-green algae. The Fe-SOD contained two atoms of iron

were insensitive to cyanide, only Fe-SOD was inactivated by hydrogen peroxide (Asada et al., 1975). Mutiple forms of SOD exist in leaf extracts of corn, oat and pea seedlings. A total of ten bands were identified by polyacrylamide gel electrophoresis. Corn extracts contained seven bands while oat and pea had only three for each species. Nine bands were cyanide-sensitive, only one band from corn was cyanide-insensitive and identified as Mn-SOD. It was found that the total SOD activity in three species was completely inhibited by 3 mM KCN (Giannopolitis and Ries, 1977). Later the plastidic, cytosolic and mitochondrial forms of SOD were also reported in corn (Baum and Scandalios, 1981). For pea plants, one cyanide-insensitive SOD was reported (del Rio et al, 1978). Later work done on pea plants distinguished three distinct SOD isozymes including one Mn-SOD in leaf extracts. The Mn-SOD had a molecular weight of 94,500 daltons and was composed of four subunits (Sevilla et al, 1982). Three isozymes of SOD were found in kidney bean leaves. Two of them were inhibited by cyanide hence they were Cu-Zn-SOD. Another isozyme was cyanide-insensitive and not inactivated by hydrogen peroxide, indicating that it was Mn-SOD (Kono et al., 1979). Four distinct bands were observed on both polyacrylamide gel and isoelectricfocusing gel (pH 4-6) in etiolated seedlings of barley (Jaaska and Jaaska, 1982). The major bands were strongly inhibited by DIECA (diethyldithiocarbamate), hence presumably designated as Cu-Zn-SOD. Two other minor bands were DIECA-

insensitive and might be Mn-SOD. SOD isozymes were found in leaf cells of C_3 plants (pea and wheat) and C_4 plants (maize and sorghum). The SOD isozymes within leaf cells were characterized as chloroplastic, cytoplasmic and mitochondrial SOD enzymes. Chloroplastic SOD exhibited a single cyanide-sensitive band which had the highest mobility and accounted for 50-80% of the total soluble SOD. Pea and wheat had only one cytoplasmic SOD which was cyanide-sensitive. Maize and sorghum had two cytoplasmic SODs. A single cyanide-insensitive SOD was present in both C_3 and C_4 plant leaves which was located in the mitochondria (Foster and Edwards, 1980).

Compounds such as a methyl viologen (paraquat) can be reduced by electrons leaked from photosystem I of the photosynthetic apparatus to produce a monocation radical which, in turn, reduces oxygen rapidly to superoxide. So the action of this kind of herbicide depends upon the diversion of electrons to dioxygen. Because of the function of SOD enzyme in scavenging superoxide, it is interesting to find a correlation between plant resistance to paraquat and the SOD level *in vivo*.

A bacterium, *Escherichia coli*, was shown to adapt to sublethal levels of paraquat by increasing SOD biosynthesis (Hassan and Fridovich, 1977). The increased level of SOD rendered the cells relatively resistant to paraquat (Hassan and Fridovich, 1978). The mutants of three species of higher plants, which were resistant to paraquat, contained

a higher SOD activity (Harper and Harvey, 1978; Youngman and Dodge, 1981). In the unicellular green alga Chlorella sorokiniana, SOD enzyme was proved to provide a defense against the herbicidal action of paraquat. C. sorokiniana contained three electrophoretically distinct SOD isozymes. Two bands represented Fe-SOD. The other one was Mn-SOD. None of them were cyanide-sensitive. The authors reported that paraquat can induce a new SOD band identified as Mn-SOD. Paraquat (>10 μM) added in the illuminated cultures diminished the growth rate but elevated SOD level in cells. Paraquat in cultures also increased cell protein content but decreased carbohydrates (Rabinowitch et al., 1983).

SOD levels in wheat seedlings in relation to their dehydration tolerance has been studied (Wu and Todd, 1985). In this study, new data that give insight into SOD enzyme, both its activities and isozymes, particularly the effects of paraquat as well as cyanide on SOD isozymes in peanut and wheat leaves will be presented.

CHAPTER VI

MATERIALS AND METHODS

Assay of the Activity of Superoxide Dismutase

Preparation of crude SOD Enzyme

Leaves were thoroughly ground in a mortar with pH 7 50 mM cold phosphate buffer solution (50mg leaf/ml buffer). The homogenate was centrifuged at 11000 x g for 25 minutes. All of the procedures were conducted in the cold room ($4^{\circ}C$). The supernatant was used for the assay.

Assay of SOD Activity

The photochemical method established by Beauchamp and Fridovich (1971) and modified by Stewart and Bewley (1980) for SOD activity was used in this study. The principle of the assay is based on the inhibition of the photoreduction of nitro blue tetrazolium (NBT) by SOD enzyme in a sample mixture. A 3 ml sample mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 100 nM ethylenediaminetetraacetic acid (EDTA) and 0-100 μl SOD enzyme extracts. Riboflavin was photoreduced in the mixture under illumination. The reoxidation of reduced riboflavin induced the generation of

superoxide free radicals which can reduce NBT to form the blue formazan, which can be measured by the absorbance at 560 nm. SOD enzyme inhibits this rection by scavenging superoxides, hence it can be quantitated by a spectrophotometric method. During the assay riboflavin was added to the mixture last. Three 15 W fluorescence tubes were arranged about 15 cm from test tubes. Tubes were shaken well before turning on the light. The photoreaction proceeded for 6 to 9 minutes under the experimental conditions depending on the speed of color development. The reaction was terminated by turning off the light and immediately covering the tubes with aluminum foil. The absorbance was read at 560 nm using a Beckman Model DU (Gilford) spectrophotometer. A sample mixture without added enzyme was used as a control which showed a deep blue-gray color amoung samples after illumination. The color declined with increasing amounts of the enzyme. One unit of SOD was defined as the amount of enzyme which caused a 50% inhibition of the reaction rate of the control. The percent inhibition of NBT photoreaction by different amounts of SOD added to the mixture was calculated by the equation as follows:

$$\text{Percent Inhibition} = \frac{A_{560(\text{without enzyme})} - A_{560(\text{with enzyme})} \times 100}{A_{560(\text{without enzyme})}}$$

A_{560} = Absorbance at 560 nm

Based on the percent inhibition versus SOD concentration an experimental curve was constructed. The volume of SOD required for reaching of 50% inhibition of the rate of reaction in control (equivalent to one SOD unit) was read on the abscissa. From this volume the activity of SOD enzyme based on fresh weight was calculated.

Protein Estimation

The protein content in crude SOD extracts and in concentrated samples for electrophoresis was determined by the method of Lowry et al., (1951) and modified by Hartree (1972). Bovine serum albumin was used as the reference standard. The absorbance was measured at 650 nm by a Beckman Model DU (Gilford) spectrophotometer.

Paraquat Treatment And Cyanide Inhibition

Leaves were placed in a petri dish with 0.01 M paraquat solution under 230 $\mu E\ m^{-2}\ s^{-1}$ light intensity and at 31° C. Leaf controls were under the same conditions but placed in distilled water. After different time lengths of treatment, leaves were taken for the enzyme assay or the determination of protein and chlorophyll content, as well as the identification of SOD isozymes on slab gels.

In assays of enzyme activity under cyanide inhibition, 3 mM KCN was placed in the sample mixture before adding enzyme. For electrophoresis, the mixture of the protein

sample and the sample buffer contained 3 mM KCN. After concentration by using Centricon 10, this KCN treated sample was run on a slab gel in comparison with the control in which KCN was not added.

Chlorophyll Estimation

Chlorophyll was extracted from the leaf samples obtained from different species of peanut. Eight leaf discs (0.5 cm diameter) were ground in a cold mortar with 5 ml 80% acetone. The absorbance of the extract was measured at 652 nm using a Beckman Model DU (Gilford) spectrophotometer. The equation listed below was followed to calculate chlorophyll concentration (Arnon, 1949).

$$\frac{\text{Chlorophyll (mg)}}{\text{Fresh Weight (g)}} = 1000 \times \frac{A_{652}}{34.5} \times \frac{\text{Volume of Extract}}{\text{Leaf Material (mg)}}$$

Preparation of Chloroplast Fraction For Isoelectricfocusing Electrophoresis

Leaves of Kanking wheat and EC-5 peanut were used to isolate chloroplasts. A 15 g quatity of fresh washed leaves were used in the preparation. The procedure for chloroplasts isolation was that of Moreland (1977) without the addition of ascorbic acid. The isolated intact chloroplasts were transferred into 0.05 M Tris buffer (pH 8.8) hence disrupted by osmotic shock. The chloroplast fraction obtained was centrifuged at 1000 x g for 10 minutes

in the cold room. The supernatant was used as the sample for electrophoresis.

Isoelectric Focusing Electrophoresis

Preparation of the Slab Gel

A 14 x 14 cm^2 slab gel was composed of 7.5% acrylamide, 0.2% N,N-methylene bisacrylamide and 2% ampholyte (pH 4-6) (Jaaska and Jaaska, 1982). The mixing solution was aerated for 15 minutes, then ammonium persulfate and Temed were added into the solution. Polymerization began in 5 minutes. The solution was swirled gently and immediately poured into the space between two glass plates. After polymerization the prepared gel was covered with Saran wrap and stored in the cold room until needed. Sample buffer was composed of distilled water, glycerol and ampholyte buffer (pH 4-6).

The Sample Preparation

Leaf samples of 250-300 mg were thoroughly ground in 0.05 M Tris buffer (pH 8.8; 150mg/ml). The homogenate was centrifuged under 5000 x g for 25 minutes. The supernatant was added to an equal volume of the sample buffer. Two ml of the mixture was obtained. The mixture was placed in centricon-10 filter container and concentrated by centrifugation at less than 5000 x g until about 0.3 ml of concentrated protein sample was obtained. All the procedures were conducted at 4°C.

Running of Slab Gel

Two kinds of buffer were used. The buffer in the upper side (cathode) was 0.02 M ethylene diamine (pH 10-11), whereas the buffer in the lower side (anode) was 0.02 M phosphoric acid (pH 2-3). Bovine SOD enzyme was used as the standard reference. A voltage of 500 V was applied (Bio-Rad) and at the begining the electric current was usually within 10-15 mA per slab gel. Electrophoresis was performed at 4^{o}C. When the electric current dropped close to zero all proteins in the sample had reached their isoelectric points. Six hour period was usually required.

pH Determination And Gel Staining

After completing the development procedures the gel was carefully removed from the glass plate. The pH values at the different distances from the top edge of the gel were measured immediately by using a surface electrode. Thus, R_f value for a given pI could be calculated. The gels were stained for SOD activity by the photochemical procedure described previously. A gel was placed in 400 ml Tris buffer (pH 8.8) containing 32 mg NBT and 68 mg riboflavin and shaken in the dark for 35 minutes at room temperature. Then the gel was illuminated by a 25 W fluorescent tube for about 15 minutes until the achromatic zone appeared.

CHAPTER VII

RESULTS AND DISCUSSION

SOD Activities And Isozymes in Peanut And Wheat Leaves

SOD Activities in Peanut And Wheat Leaves

According to data accumulated in more than two years, it was found that the leaf SOD activity based on fresh weight varied widely. It was not only dependent on plant species, but also related to leaf age and growth conditions and to the individual plant from which leaf samples were obtained. Compared with SOD activity, leaf protein content was found to be less variable. SOD activity and the leaf protein content for two wild peanut species (A. helodes; A. villosulicarpa) and cultivated peanut (EC-5) are listed in Table I. The wild species showed higher enzyme activity statistically than the cultivated species. It was found that the content of water soluble protein in leaves of both wild peanut species was much higher than the cultivar EC-5. The ratio of leaf protein contents among the three species of peanut was approximately 1 : 2.1 : 2.8 (A. hypogaea cv. EC-5 : A. villosulicarpa : A. helodes). So the specific activity of SOD in leaves of wild peanut was not higher than that in leaves of peanut cultivar.

TABLE I

THE ACTIVITIES OF SOD ENZYME AND THE PROTEIN CONTENT IN PEANUT LEAVES

Plant species	SOD activity (Units/100mg Fr.Wt.)	Protein content (mg/ml leaf extract)
A. hypogaea cv. EC-5	20 to 65 ** 41 + 13	0.59 + 0.07 *
A. villosulicarpa	50 to 77 *** 63 + 10.6	1.25 + 0.13 *
A. helodes	50 to 83 *** 64 + 13.4	1.63 + 0.12 *

* Mean of four experiments with standard error.
** Mean of twenty-six experiments with standard error.
*** Mean of eight experiments with standard error.

The activity of SOD enzyme in leaves of Kanking and Ponca wheat was less variable than in peanuts. The average SOD activity in Kanking leaves was found to be higher than that in Ponca leaves. The leaf protein content for these two varieties was similar, thus the specific activity of SOD enzyme in KanKing leaves was higher than that in Ponca leaves (Table II). SOD activity in wheat leaves was lower than in peanut leaves, especially compared with wild species.

SOD Isozymes And Their Localizations

SOD isozymes in leaf extracts of wheat and peanut plants have been characterized by isoelectric focusing electrophoresis on slab gels in this study. Four major bands of bovine SOD were found and had pI values of 5.0, 5.1, 5.25 and 5.3 respectively (Figure 1). Electrophoretically distinct bands of SOD were also found in leaf extracts of KanKing and Ponca wheat. Four isofocusing bands were discovered and were designated as SOD-A and SOD-B in the order of their decreasing electrophoretic mobility. SOD-A, a single intense band with higher mobility, had a pI value 3.9 under our experimental conditions. SOD-B, which had an intermediate mobility, included three subbands designated as SOD-B_1,-B_2 and -B_3. They had pI values of 5.0, 5.1 and 5.2 respectively. SOD-B was a weak band. No qualitative differences in patterns of isofocusing bands were found between KanKing and Ponca (Figure 1). When

TABLE II

THE ACTIVITIES OF SOD ENZYME AND PROTEIN CONTENT IN WHEAT LEAVES

Wheat plants	SOD activity (Units/100 mg Fr. Wt.)	Protein content (mg/ml)	SOD activity (Units/mg protein)
KanKing	44 + 7.5 *	0.90 + 0.02 *	24
Ponca	29 + 5.9	0.92 + 0.02	16

* Mean of four experiments with standard error.

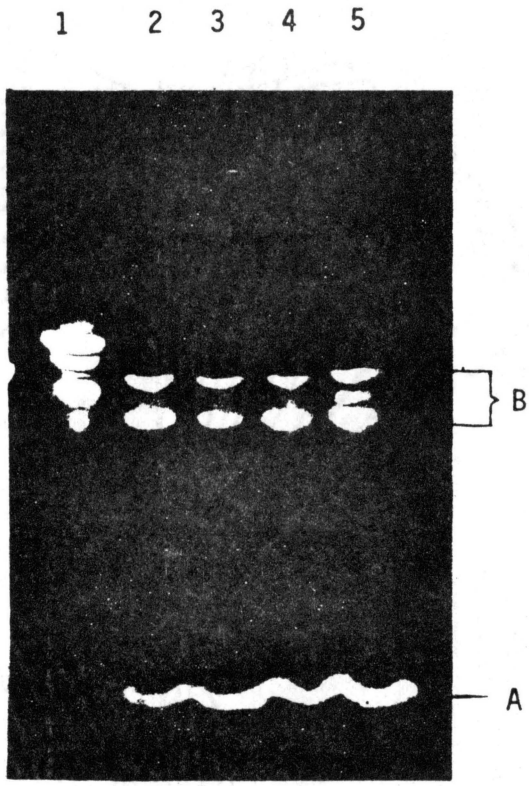

Figure 1. Isoelectric Focusing Electrophoresis of SOD Isozymes in Wheat Leaves (Shown by photographs). 1. Bovine SOD; 2. KanKing; 3. Ponca; 4. KanKing; 5. Ponca.

polyacrylamide gel electrophoresis was used, only one band of SOD isozyme was found in wheat leaves (Beauchamp and Fridovich, 1973). Two bands in wheat leaves were reported by Foster and Edwards (1980). Analysis by isoelectric-focusing electrophoresis in this study, has revealed four bands.

Different patterns of SOD isoelectricfocusing bands were found between the wild species and cultivated peanut plants. In wild peanut leaves, the SOD isofocusing bands were distributed in three regions on slab gel and designated as SOD-A, SOD-B and SOD-C (Figure 2). A single band of SOD-A with the highest mobility amoung the three, had pI values of 3.95 (A. helodes) and 4.0 (A. villosulicarpa). For A. helodes SOD-B included three subbands labelled as SOD-B_1, -B_2 and -B_3, which had pI values of 5.0, 5.1 and 5.25 similar to bands found in wheat leaves. However, the three bands were equally intense. In A. villosulicarpa, SOD-B was usually a single strong band and had a pI value of 5.0. Sometimes SOD-B_2 and -B_3 were not found in A. helodes and this rendered the pattern of isoelectric-focusing bands similar to the pattern of A. villosulicarpa. The reason for this was not clear. Three isoelectric-focusing bands in this region were also reported in barley (Jaaska and Jaaska, 1982). SOD-C in this study was a single minor band which had a pI value of 6.0 for A. villosulicarpa and about 5.9 for A. helodes. SOD-C in A. villosulicarpa was stronger than it was in A. helodes. The pattern of

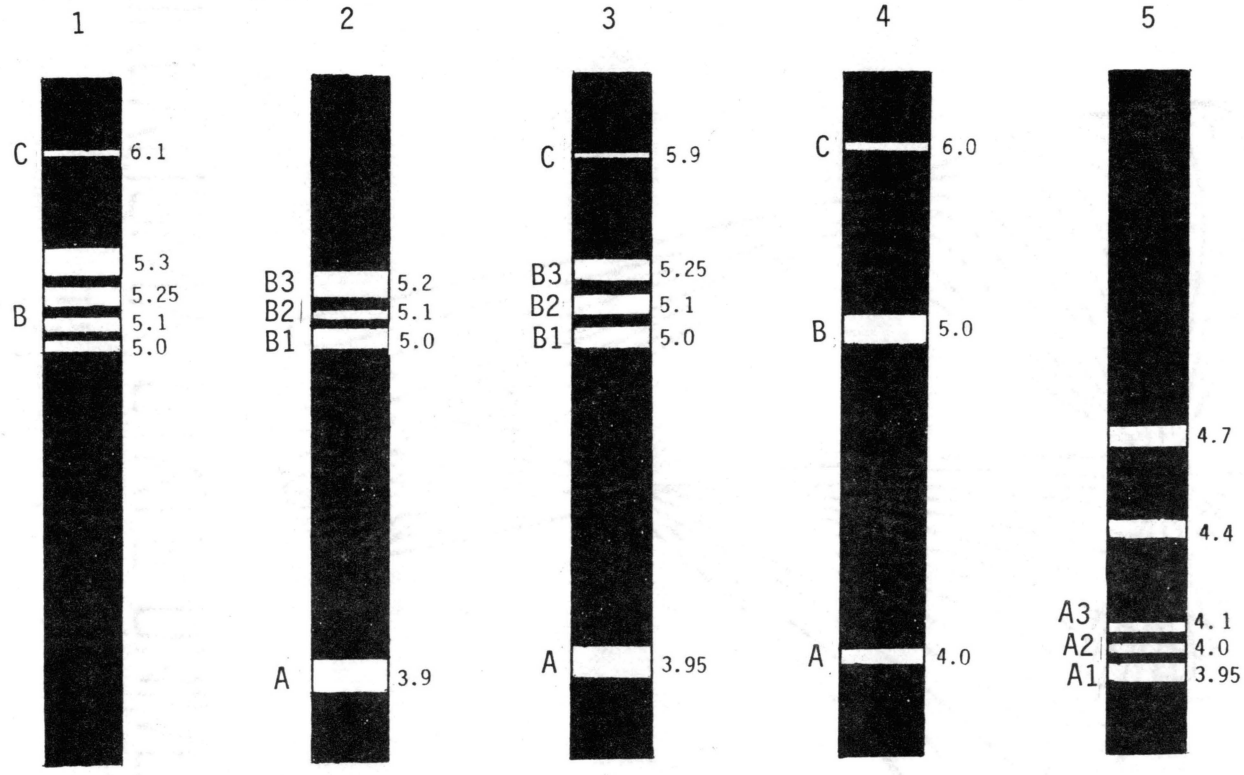

Figure 2. Isoelectric Focusing Electrophoresis of SOD Isozymes of Wheat and Peanut 1. Standard bovine SOD; 2. Wheat (KanKing or Ponca); 3. Peanut A. helodes 4. Peanut A. villosulicarpa; 5. Cultivated peanut EC-5.

isoelectricfocusing bands for peanut cultivars EC-5 and Tamnut were different from those in wild species. Five bands were distinguished on the gel. The pI values for all of these bands were found to be lower than 5.0. They were 3.95, 4.0, 4.1, 4.4 and 4.7. The lower closely spaced three bands were labelled as SOD-A_1, -A_2 and -A_3. SOD-A_1 and the bands at pI 4.4 and 4.7 were stronger than SOD-A_2 and -A_3 Electrophoretic enzymograms of SOD for wheat and peanut leaves are shown in Figure 2. To my knowledge, no other reports on SOD isozymes in peanut plants have appeared in the literature. Patterns of isofocusing bands for some other plant species are shown in the Appendixes. In the literature, the predominant single band found in many plant species, which is equivalent to SOD-A in this study, proved to be the chloroplastic SOD isozyme (Jackson et al., 1978; Foster and Edwards, 1980). By runing the chloroplast fraction extracted from wheat leaves, SOD-A was proven to be the chloroplastic isozyme (Figure 3). Four bands were exhibited on enzymograms of leaf extracts while only a single band of SOD-A was obtained by running of chloroplast fraction. A similar situation was found in EC-5 leaves. Therefore, SOD-A should be located in chloroplasts, whereas SOD-B may be the cytoplasmic SOD isozymes. The activities of SOD-A and SOD-B in both wheat and peanuts were inhibited by 3 mM KCN. They were cyanide-sensitive and should be the Cu-Zn-SOD. For intensively expressed bands (pI 3.9, 5.0 in wheat; pI 3.95, 5.0, 5.1, 5.25 in A. helodes ; pI 5.0 in

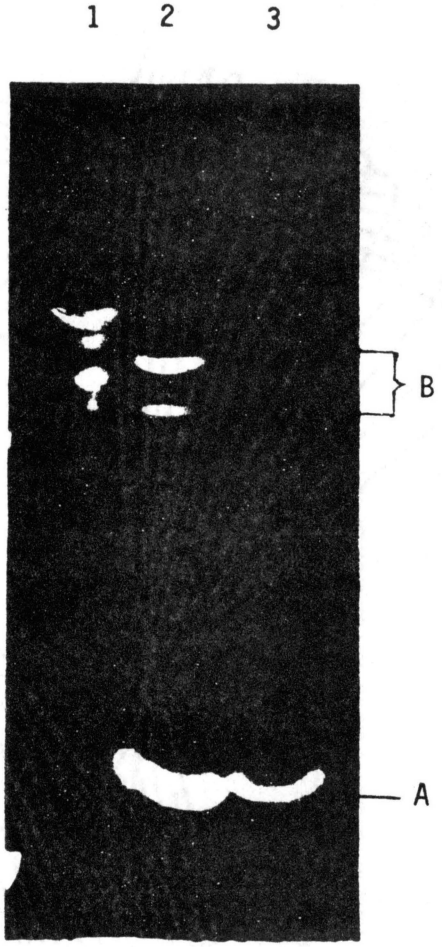

Figure 3. Isoelectric Focusing Electrophoresis of SOD Isozymes of KanKing Wheat Leaves. (Shown by photograph). 1. Bovine SOD; 2. KanKing leaf extracts; 3. KanKing chloroplast fraction.

A. villosulicarpa; pI 4.7 in EC-5) the 3 mM KCN weakened the enzyme activities dramatically but did not eliminated them. The weak bands (pI 4.0 in A. villosulicarpa; pI 3.95, 4.0, 4.1, 4.4 in EC-5) disappeared from the gel after cyanide treatment (Figure 4). An elimination of the activity of isoelectricfocusing bands by 3 mM KCN was observed in A. villosulicarpa. Both SOD-A and SOD-B were eliminated by cyanide treatment. However, no effect on the minor band of SOD-C was observed (Figure 5). Therefore, SOD-C isozymes found in leaves of two wild peanut species was cyanide-insensitive and might be associated with mitochondria. The Mn-SOD was evaluated in the similar region of gels from many plant species (Jackson et al., 1978; Kono et al., 1979; Foster and Edwards, 1980; Baum and Scandalios, 1981). So far Fe-SOD was only found in two species of higher plants (Salin and Bridges, 1980 and 1982).

It was reported that wheat contained 1% Mn-SOD, maize and sorghum contained 2-5% Mn-SOD, whereas 20% Mn-SOD was found in pea plant (Foster and Edwards, 1980). The percent inhibition of total SOD activity by cyanide can be used to evaluate the content of Cu-Zn-SOD versus Mn-SOD (Beauchamp and Fridovich, 1973). It was found that 3 mM KCN completely inhibited SOD activities in enzyme extracts of corn, oat and pea plants (Giannopolitis and Ries, 1977). In this study, 3 mM KCN was used to inhibit SOD activity in enzyme extracts of different peanut leaves. KCN inhibited SOD activity in leaf extracts of cultivar EC-5 by 69% approximately, whereas

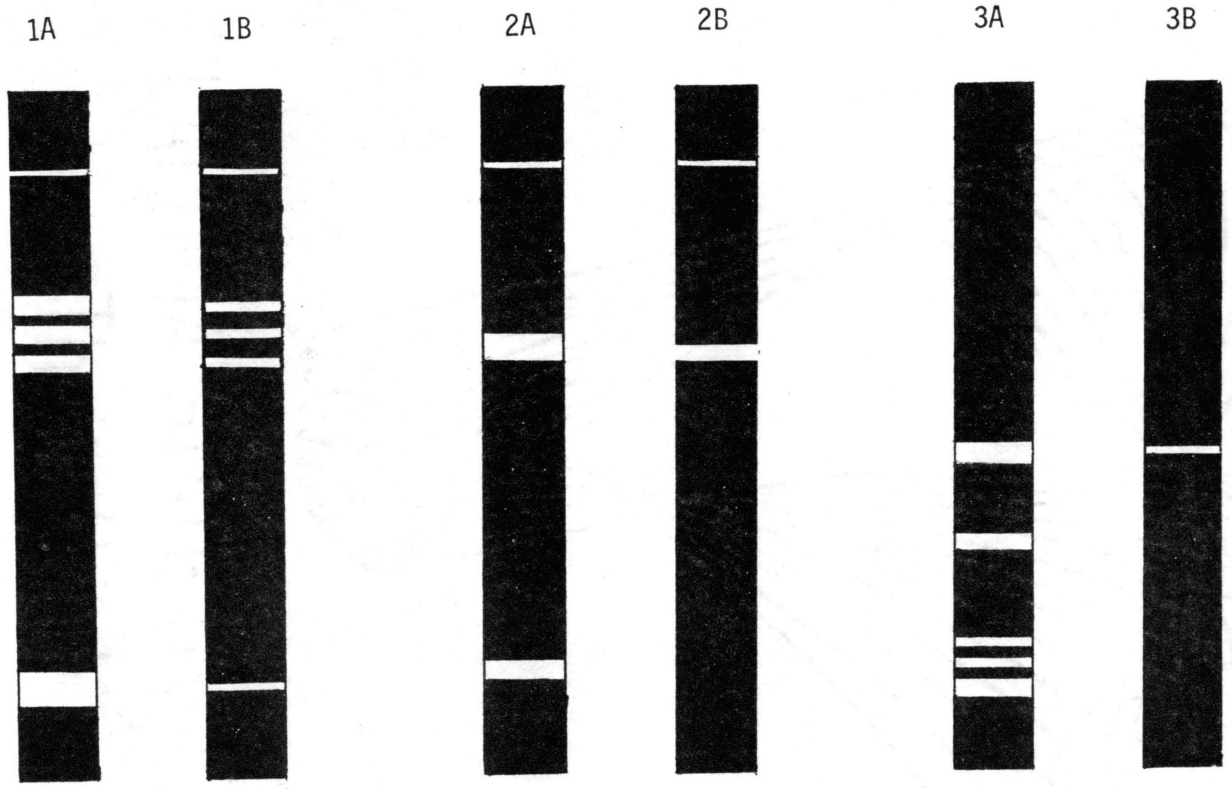

Figure 4. The Effect of KCN on Peanut SOD Isozymes
1A— A. helodes control; 1B— A. helodes treatment; 2A— A. villosulicarpa control; 2B— A. villosulicarpa treatment; 3A— EC-5 control; 3B—EC-5 treatment.

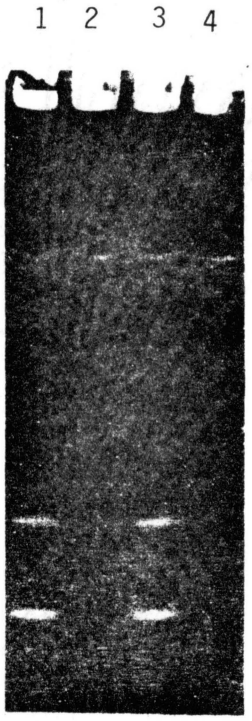

Figure 5. Isoelectric Focusing Electrophoresis of SOD Isozymes in Leaves of Peanut A. villosulicarpa Affected by 3 mM KCN. (Shown by photograph). 1. Control; 2. KCN treatment; 3. Control; 4. KCN treatment.

only about 50% of the activity was eliminated by cyanide in wild species (Table III). Thus it is concluded that the relative content of Cu-Zn-SOD as compared to Mn- and Fe-SOD in leaves of peanut cultivar EC-5 is significantly higher than those in leaves of two wild species.

Effects of Paraquat on SOD Activities And Isozymes in Leaves of Wild And Cultivated Peanut Plants

Both paraquat and the fungal toxin cercosporin can cause leaf damage through a similar mechanism in which the production of superoxide is accelerated in vivo (Moreland, 1976; Daub and Hangarter, 1983). As the scavenger of oxygen radicals, SOD enzyme protects plants from being damaged by these toxic agents. At the begining of this study, both paraquat and cercosporin were used to treat peanut leaves. No apparent effect of cercosporin on SOD activity was confirmed. However, when paraquat was used to treat leaves, a dramatic decrease of SOD activity was observed, particularly in the leaves of peanut cultivars. After 12 hours treatment, more than 60% of the leaf SOD activity was lost in EC-5 as well as in Tamnut (Figure 6 and 7), but the percent loss of SOD activity by paraquat treament in wild peanut leaves can be less than 30% (Figure 8 and 9). Data showing the average of percent loss of SOD activity measured in several repeated experiments are listed in Table IV. The data indicate that the percent loss of enzyme activity in

TABLE III

THE PERCENT INHIBITION OF SOD ACTIVITY BY CYANIDE IN WILD SPECIES AND CULTIVAR OF PEANUT

Peanut species	The percent inhibition of SOD activity by 3 mM KCN
A. hypogaea cv. EC-5	69 + 2.4 *
A. helodes	54 + 3.3
A. villosulicarpa	50 + 3.1

* Mean of four experiments with standard error.

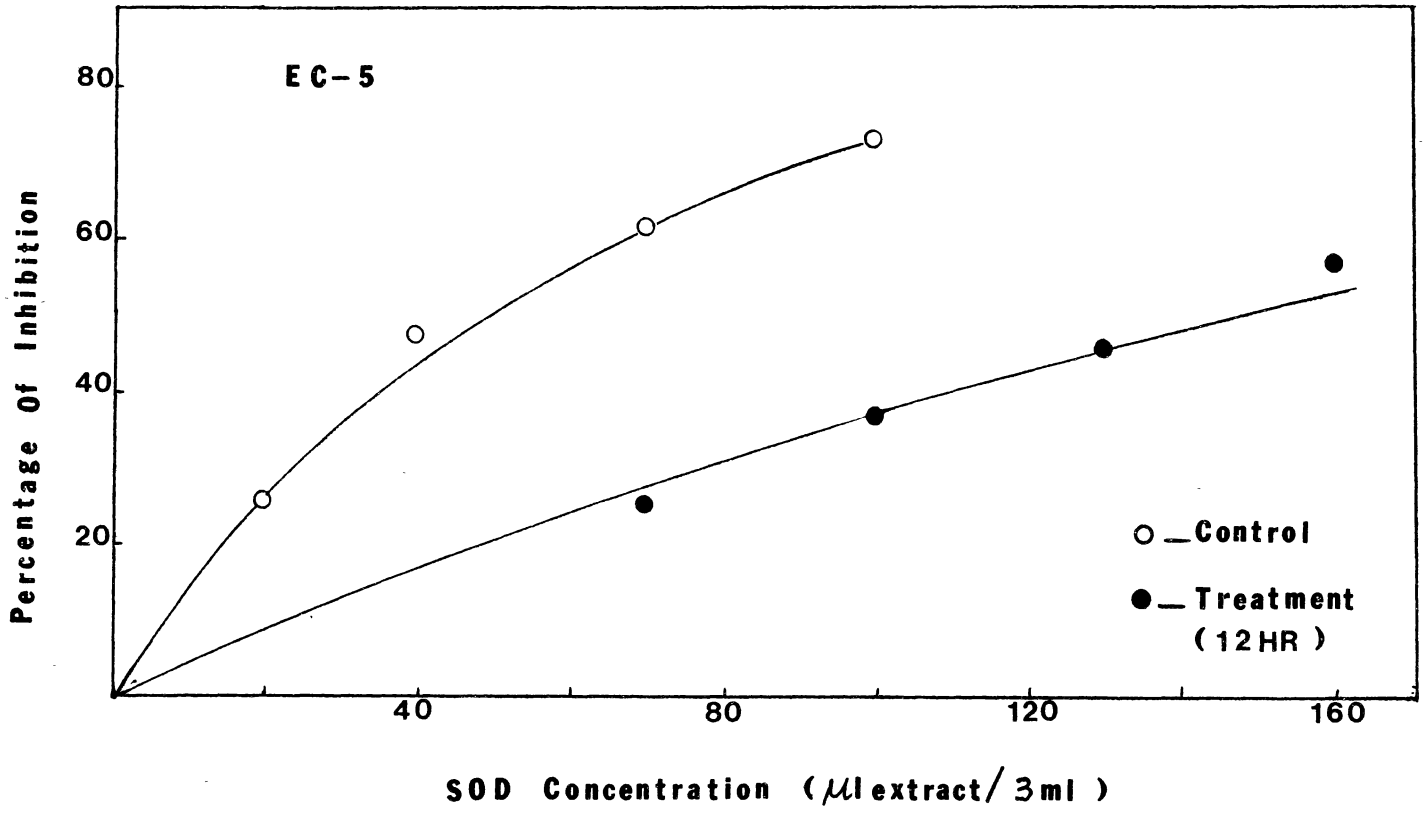

Figure 6. Paraquat Effects on SOD Activity in Leaves of Peanut A. hypogaea cv. EC-5.

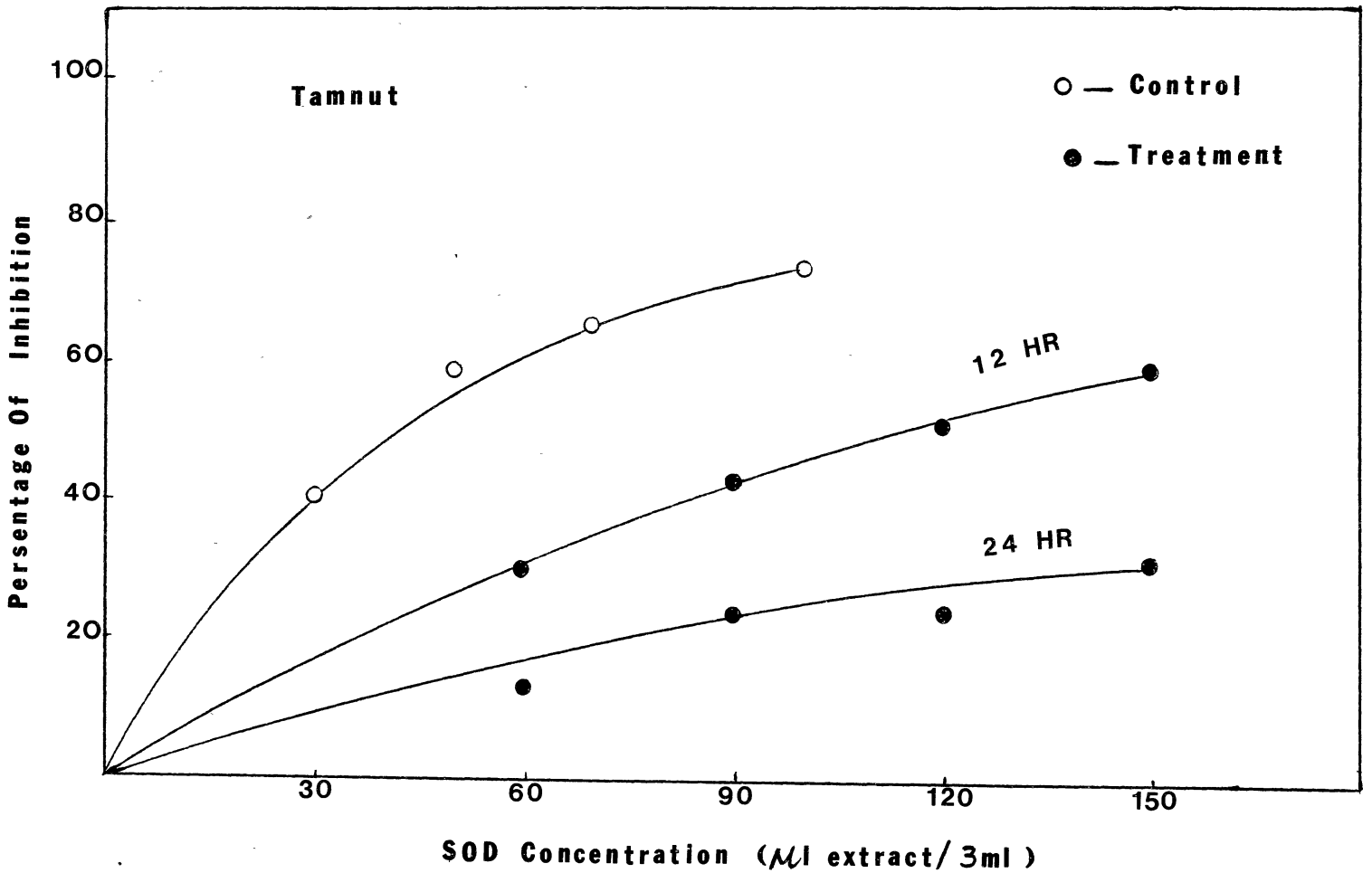

Figure 7. Paraquat Effects on SOD Activity in Leaves of Peanut A. hypogaea cv. Tamnut.

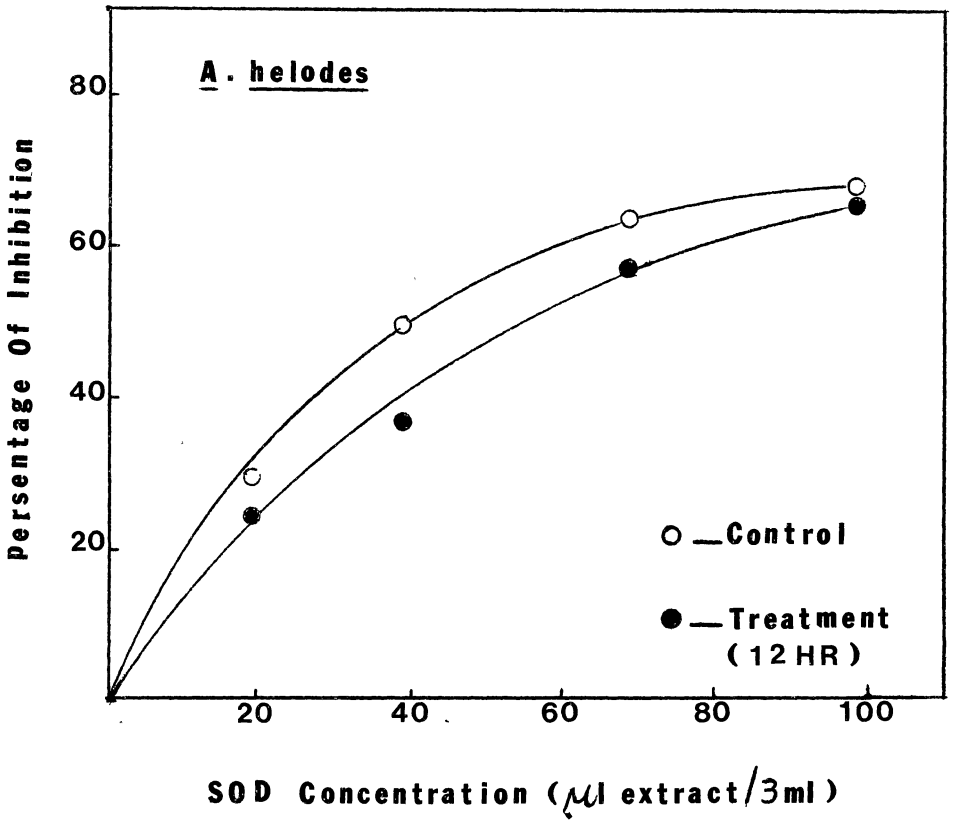

Figure 8. Paraquat Effects on SOD Activity in Leaves of the Wild Peanut A. helodes.

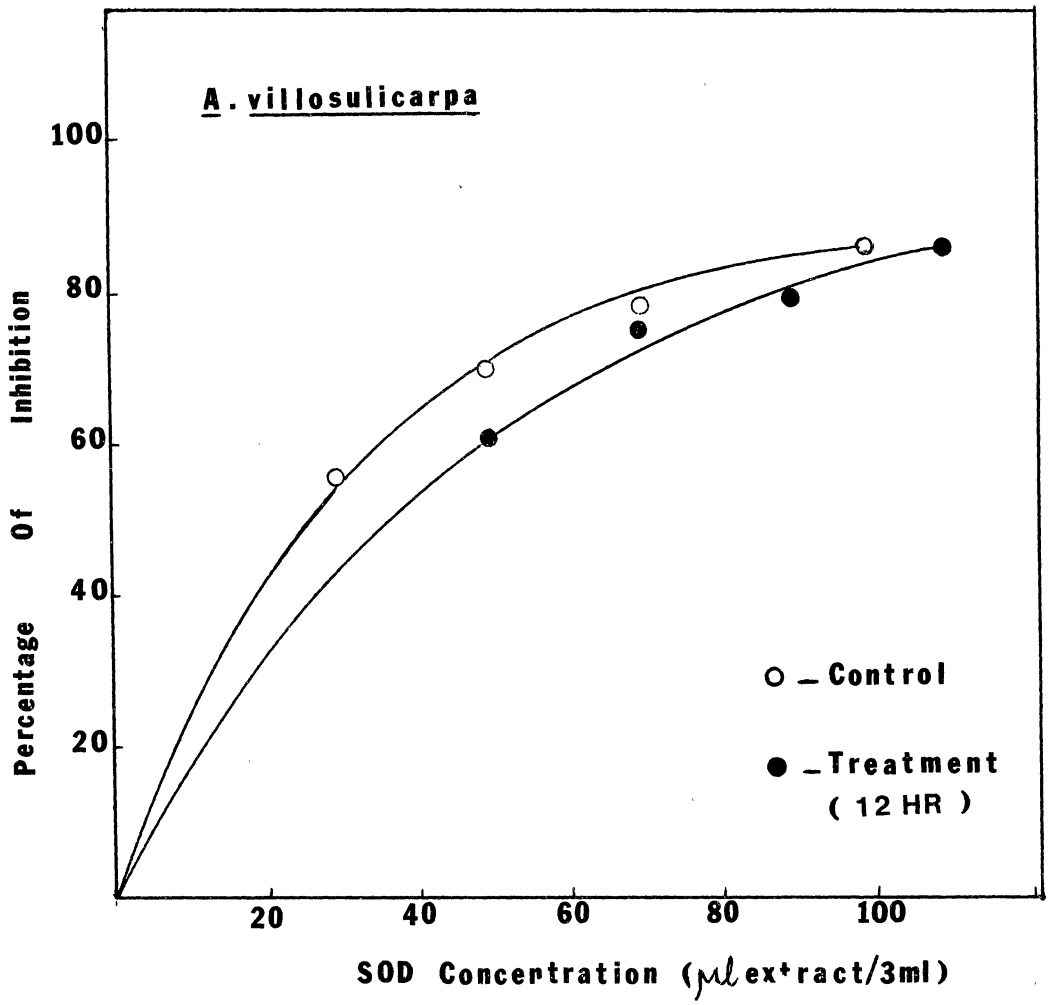

Figure 9. Paraquat Effects on SOD Activity in Leaves of the Wild Peanut A. villosulicarpa.

TABLE IV

THE EFFECT OF PARAQUAT ON SOD ACTIVITIES IN THE LEAVES OF PEANUT PLANTS

Peanut species	The percent loss of SOD activity by paraquat treatment
A. hypogaea cv. EC-5	64 + 7.1 *
A. helodes	38 + 7.1
A. villosulicarpa	33 + 8.4

* Mean of four experiments with standard error.

the two wild species was significantly lower than those in cultivars. The higher enzymatic tolerance may be associated with a higher paraquat resistance of intact plants because of the protective role of this enzyme. There may be two reasons that could account for the decline of enzyme activity by the paraquat treatment. This herbicide may inhibit SOD enzyme *in vivo*. However, even 0.02 M paraquat did not show any inhibition of standard bovine SOD enzyme. The other possibility is that paraquat may cause protein decomposition as well as the destruction of SOD enzyme *in vivo*. Data on protein content and specific activity of SOD enzyme are given in Table V. Leaf protein content dropped markedly following paraquat treatment in leaves of three peanut species. From 59% to 76% of the protein, depending on the species, was lost after treatment. However, the specific activity in *A. villosulicarpa* leaves was increased more than two times after treatment. In *A. helodes* it was increased by about 39% while only a slight change of SOD specific activity was found in *A. hypogaea* cv. EC-5 leaves.

Paraquat effects on SOD isofocusing bands are shown in Figure 10. In two wild species, SOD-B, which may be located in cytosol, was insensitive to paraquat. SOD-A located in chloroplasts was decreased by paraquat markedly. In contrast, all of the isofocusing bands in EC-5 leaves were eliminated or diminished by paraquat treatment, although in some cases the band with 4.7 of pI value still remained. Because paraquat preferentially attacks chloroplasts and the

TABLE V

THE CHANGES OF THE SPECIFIC ACTIVITIES OF SOD ENZYME IN THE WILD SPECIES AND THE CULTIVAR OF PEANUT

Peanut species and paraquat treatment	Protein content (mg/ml)	SOD activity Units/mg protein	Percent increase of SOD activity
A. hypogaea cv.EC-5			
0.0 mM	0.68	27	
10.0 mM	0.29	28	3%
A. helodes			
0.0 mM	1.70	18	
10.0 mM	0.70	25	39%
A. villosulicarpa			
0.0 mM	1.20	20	
10.0 mM	0.29	49	145%

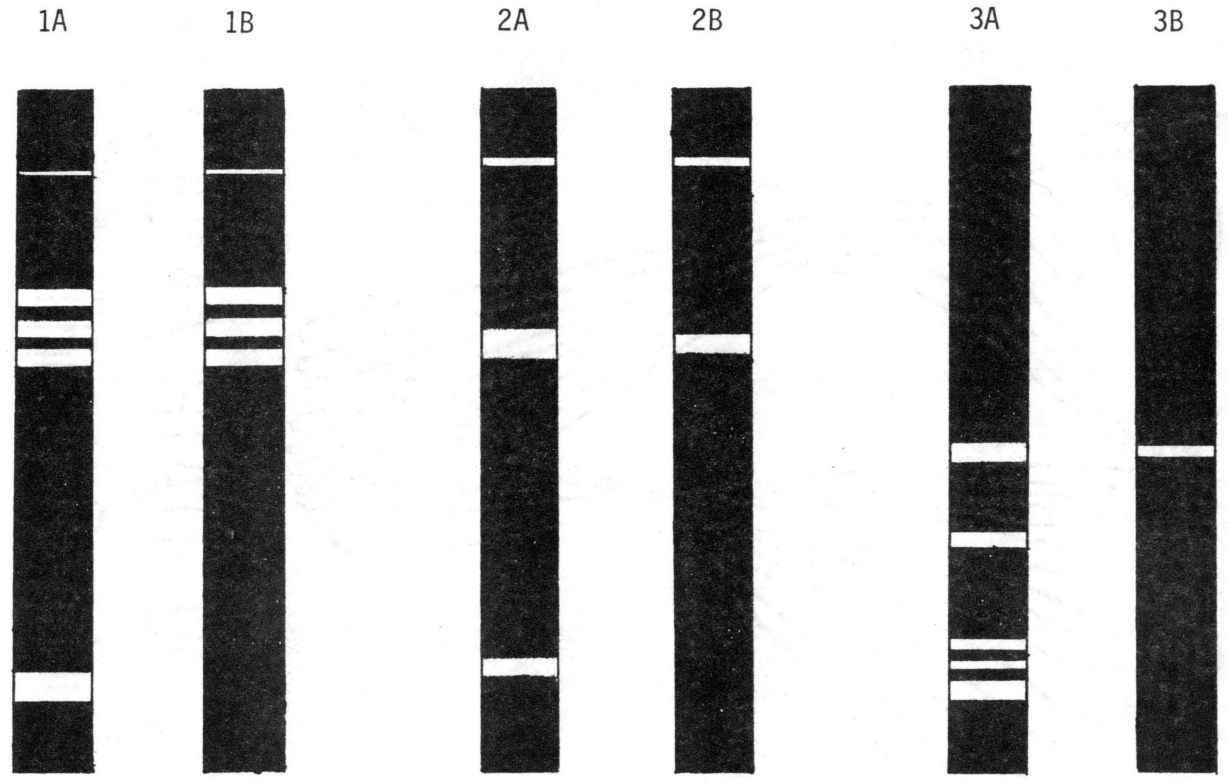

Figure 10. The Effect of Paraquat (0.01 M) on Isoelectricfocusing Bands of SOD Enzymes. 1A-- A. helodes control; 1B-- A. helodes treat-ment; 2A-- A. villosulicarpa control; 2B-- A. villo-sulicarpa treatment; 3A--EC-5 control; 3B-- EC-5 treatment.

leaf cells of wild peanuts contained considerable SOD activity which located outside chloroplasts. After paraquat treatment this part of the activity may still remain their function and contribute to the tolerance of wild peanuts to paraquat.

Chlorophyll as A Marker to Evaluate Leaf Tolerance to Paraquat

Chlorophyll content in leaves was often used as a marker to test the effects of herbicides on plants. In this study detached peanut leaves were treated with 0.01 M paraquat. After 12, 24 and 36 hours leaf chlorophyll content was determined. Results are shown in Figure 11. The process of chlorophyll loss was slower in A. villosulicarpa leaves than in leaves of EC-5. This process was also slower in A. helodes than in EC-5 after 12 hours treatment. After 36 hours treatment the content of chlorophyll in cultivar EC-5 leaves dropped to zero, whereas a significant amount of chlorophyll still remained in leaves of the two wild peanut species. A higher paraquat tolerance by wild peanut leaves is suggested.

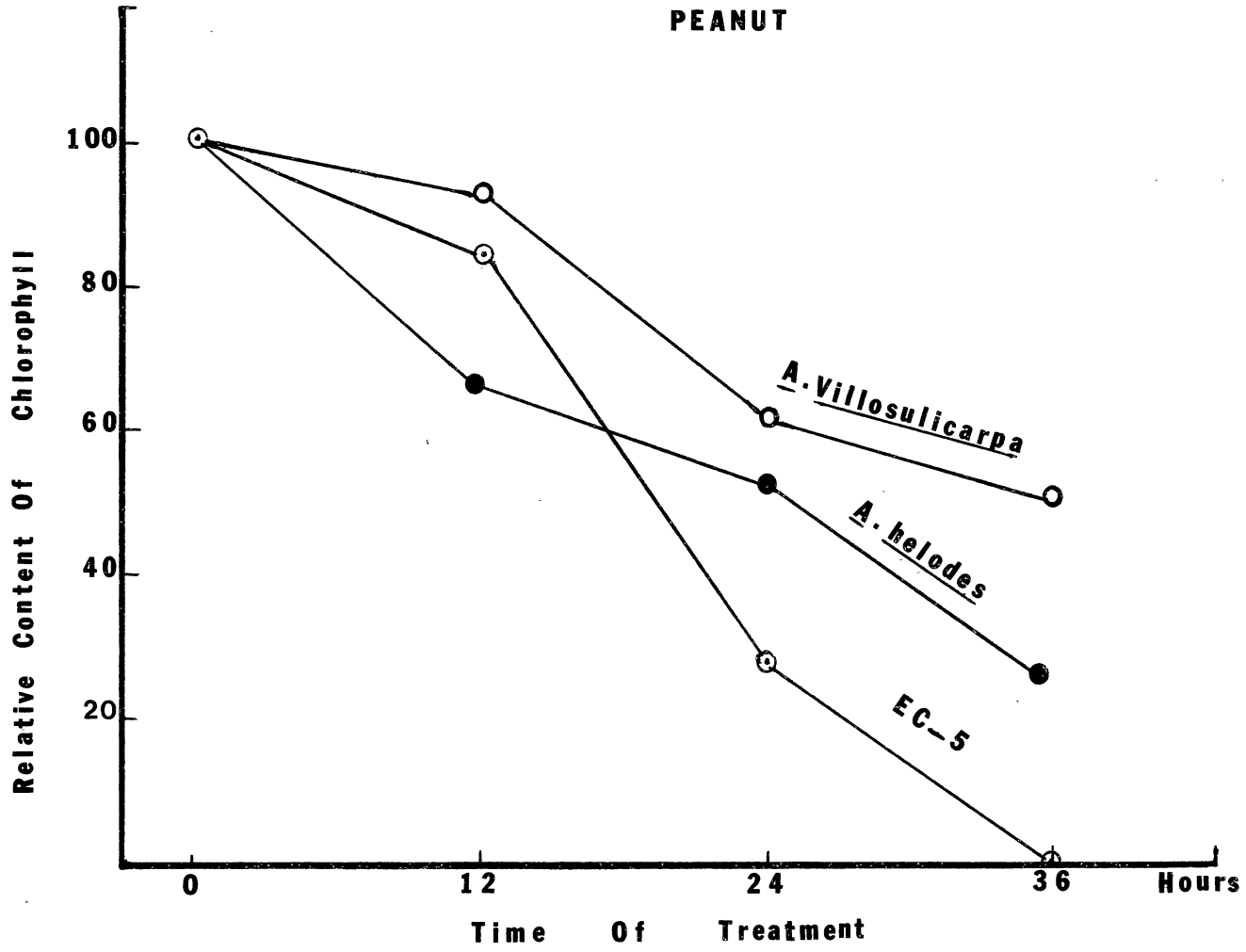

Figure 11. The Decline of the Chlorophyll Content in Detached Peanut Leaves Caused by 10^{-2} M Paraquat Treatment.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

1. SOD activities in leaf extracts of the wild species of peanut (A. helodes and A. villosulicarpa) based on fresh weight were significantly higher than those in A. hypogaea cultivar EC-5. However, the leaves of wild peanut contained much more protein than leaves of the peanut cultivar, so their specific SOD activities was found to be lower than in EC-5. Wheat cv. KanKing leaves possessed higher SOD activity than cv. Ponca on either a fresh weight or unit protein basis.

2. Wheat cv. Kanking and Ponca exhibited similar patterns of isoelectricfocusing bands which included four bands located in chloroplasts and in the cytosol. However, the patterns of isoelectricfocusing bands shown by leaves of wild and cultivated peanuts were different. Both cyanide-sensitive and cyanide-insensitive SOD isozymes were found in wild peanut leaves, which may be located in chloroplasts (SOD-A), cytosol (SOD-B) as well as in mitochondria (SOD-C). In contrast, the isoelectricfocusing bands of SOD in peanut cultivar EC-5 were mainly chloroplastic isozymes which were cyanide-sensitive and all five isoelectricfocusing bands had pI values lower than 5.0.

3. Paraquat reduced SOD levels markedly in peanut leaves.

This was mainly due to the destruction of enzyme protein. The specific activity SOD in leaves of wild peanut species, particularly in A. villosulicarpa, was enhanced dramatically by paraquat treatment.

4. SOD enzyme in leaves of two wild peanut species exhibited higher paraquat tolerance than the peanut cultivar. Detached leaves of wild peanuts also showed higher tolerance to paraquat than peanut cultivar. Wild peanut species rather than cultivar would be predicted to have greater paraquat resistance than cultivated peanuts.

5. Analysis of the isoelectricfocusing bands of SOD from paraquat-treated leaves of wild peanut species indicated that SOD-B and SOD-C were paraquat-insensitive, these isozymes may be located in the cytosol and mitochondria. However, the major activity of SOD in cv. EC-5 leaves was mainly chloroplastic SOD isoenzymes which are sensitive to paraquat.

6. Three mM KCN inhibited SOD activity by 50% in leaves of the wild species of peanut, whereas as high as 70% of enzyme activity in cv. EC-5 leaves was eliminated by cyanide treatment. Therefore, the relative Cu-Zn-SOD content in cv. EC-5 leaves was significantly higher than those found in leaves of two wild species, whereas wild species contain more Mn- or Fe-SOD than the cultivar.

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APPENDIXES

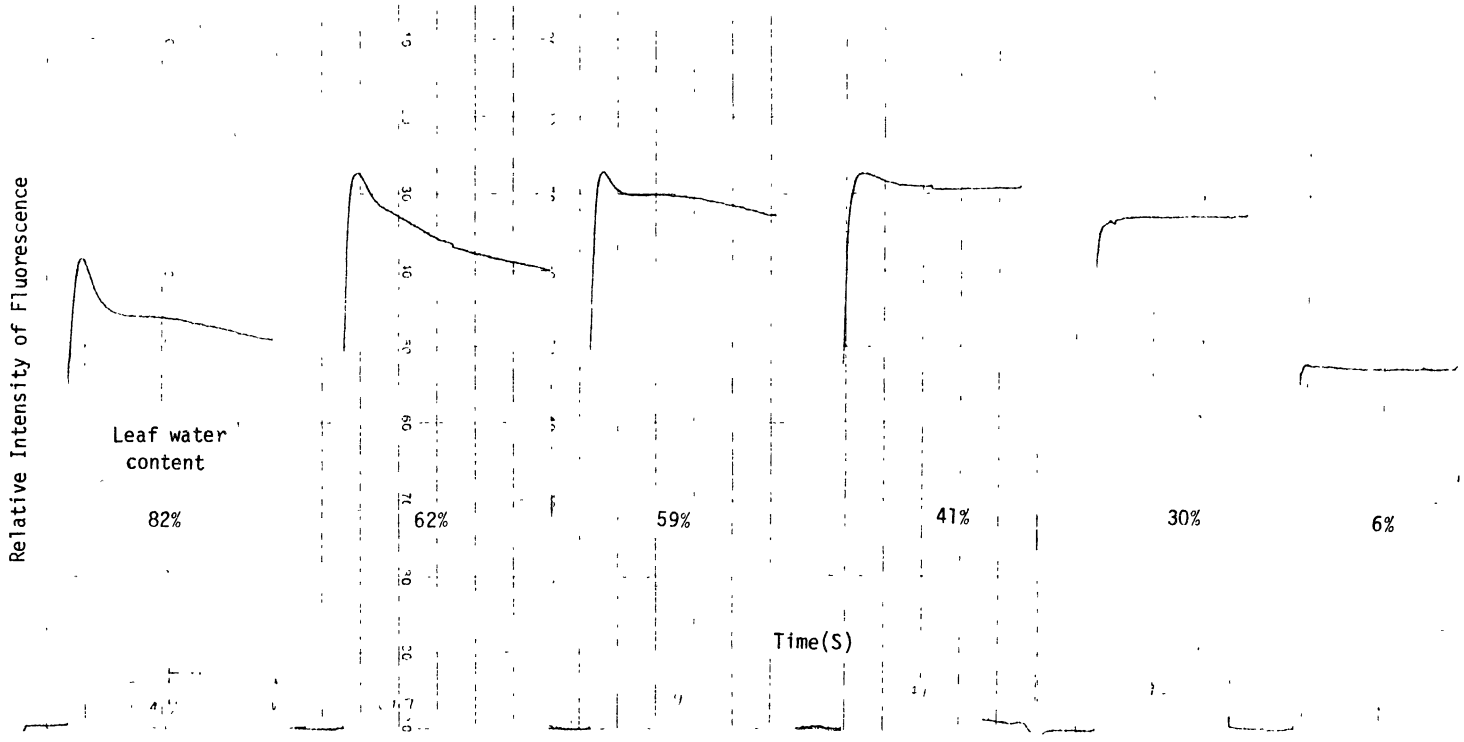

Figure 38. (Part I). The Pattern Alteration of Fluorescence Induction Curves of a Detached Leaf of Wild Peanut A. villosulicarpa by dehydration.

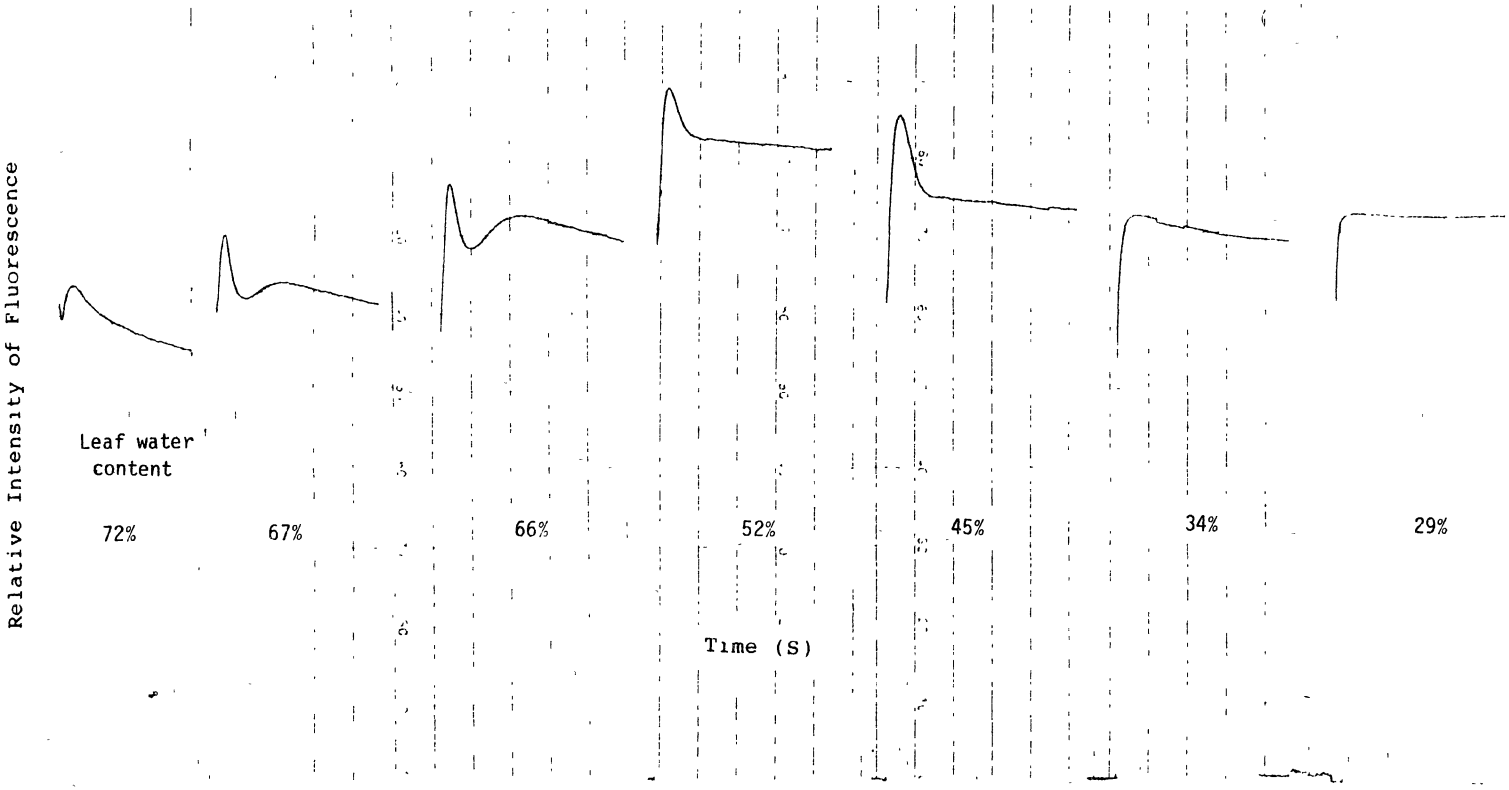

Figure 39. (Part I). The Pattern Alteration of Fluorescence Induction Curves of a Detached Leaf of Cotton Plant Gossypium hirsutum by Dehydration.

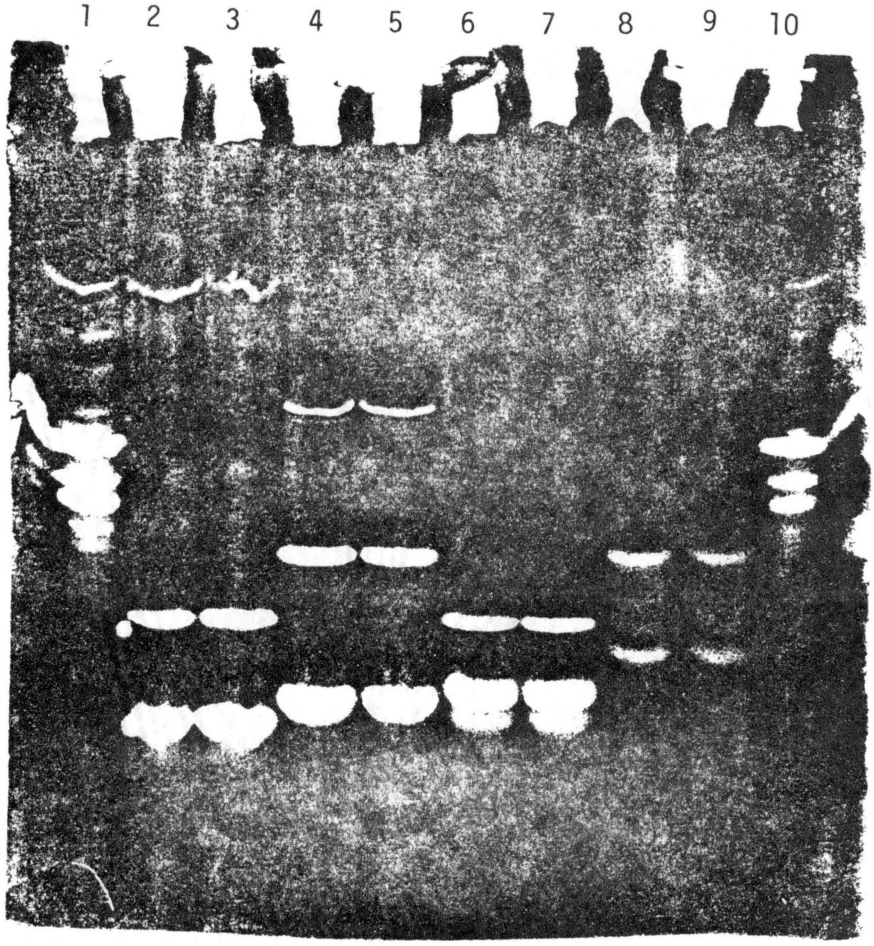

Figure 12. (Part II). Patterns of SOD Isoelectric-focusing Band from Leaves of Distinct Plants.(Shown by Photographs). 1. Bovine SOD; 2 & 3. Wild peanut (A. paraguarensis); 4 & 5. Bean (Phaseolus vulgaris);; 6 & 7. Alfalfa (Medicago sp.) 8 & 9 Euphorbia sp. 10. Bovine SOD.

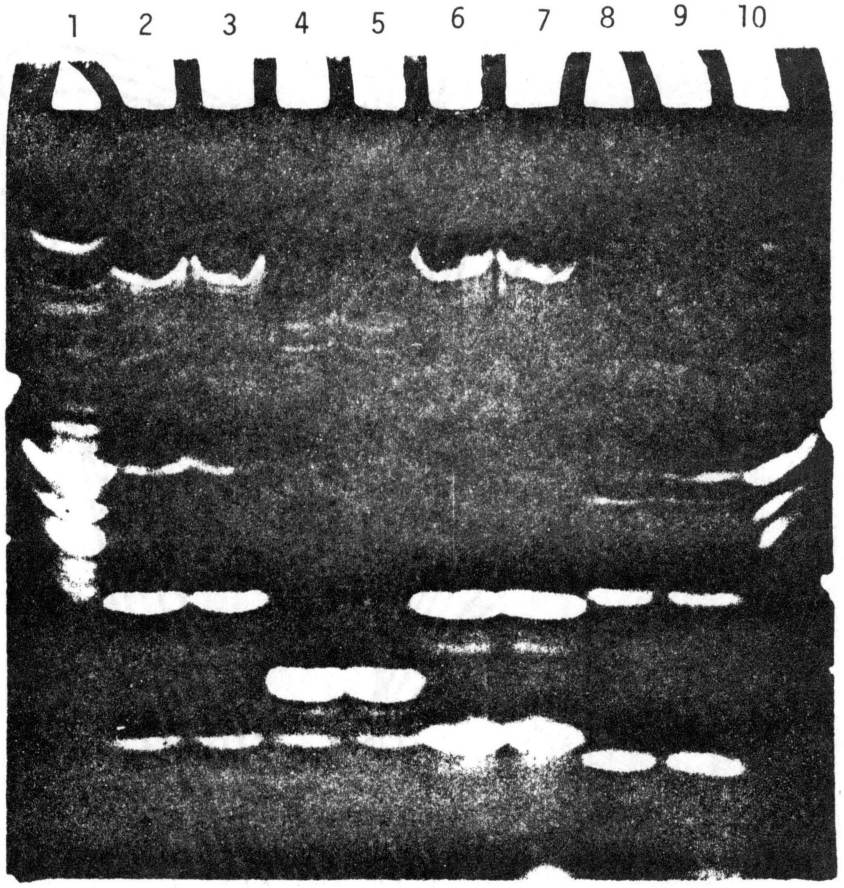

Figure 13. (Part II). Patterns of SOD Isoelectric-focusing Band from Leaf and Callus of Different Plants (Shown by Photographs). 1. Bovine SOD; 2 & 3. A. helodes (callus); 4 & 5. A. hypogaea L. cv. EC-5 (callus); 6 & 7. Wild peanut WA401 x P1541; 8 & 9. Sorghum S. bicolor cv. IN-2; 10. Bovine SOD.

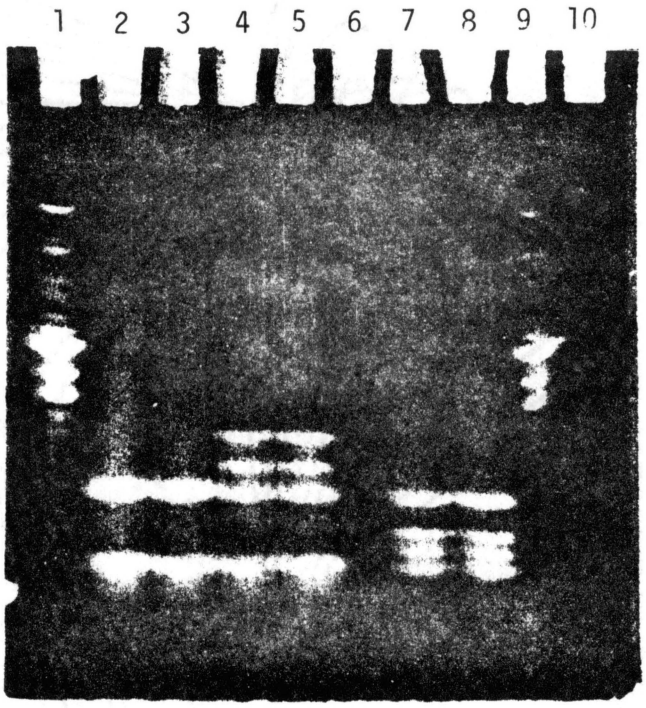

Figure 14. (Part II). Patterns of SOD Isoelectric-focusing Band from Leaves of Peanut Plants. (Shown by Photographs). 1. Bovine SOD; 2 & 3. Wild peanut 33 A; 4 & 5. Wild peanut 48 O; 7 & 8. A. hypogaea cv. EC-5; 9. Bovine SOD.

VITA

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Doctor of Philosophy

Thesis: CHLOROPHYLL A TRANSIENT FLUORESCENCE IN DETACHED LEAVES OF PEANUT, WHEAT AND SORGHUM UNDER DESICATION; ACTIVITIES AND ISOENZYMES OF SUPEROXIDE DISMUTASE AS AFFECTED BY CYANIDE AND PARAQUAT IN PEANUT AND WHEAT LEAVES

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