Efectes de l'increment de la radiació UV-B i de l'estrés hídric sobre l'activitat fotosintètica de "*Pisum sativum*" i "*Hordeum vulgare*": aplicacions de la fluorescència clorofil.lica modulada

Salvador Nogués Mestres

EFECTES DE L'INCREMENT DE LA RADIACIÓ UV-B I DE L'ESTRÉS HÍDRIC SOBRE L'ACTIVITAT FOTOSINTÈTICA DE *PISUM SATIVUM* I *HORDEUM VULGARE*: APLICACIONS DE LA FLUORESCÈNCIA CLOROFÍL.LICA MODULADA

DEPARTAMENT DE BIOLOGIA VEGETAL

UNIVERSITAT DE BARCELONA

Departament de Biologia Vegetal Universitat de Barcelona

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Leonor Alegre Batlle
Departament de Biologia Vegetal
Universitat de Barcelona

Neil R. Baker
Department of Biology
University of Essex

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EFFECTS OF ENHANCED UV-B RADIATION AND WATER STRESS ON PHOTOSYNTHETIC ACTIVITY IN *PISUM SATIVUM* AND *HORDEUM VULGARE*: APPLICATIONS OF MODULATED CHLOROPHYLL FLUORESCENCE

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LIST OF ABBREVIATIONS

ABA	Abscissic acid
APS	Ammonium persulfate
A_{sat}	Light-saturated rate of CO_2 assimilation
a.u.	Arbitrary units
BCA	Bicinchonic acid
BSA	Bovine serum albumin
CF1	Extrinsic moiety of the coupling factor of ATPase
CFCs	Chlorofluorocarbons
CHS	Chalcone synthase
C_a	External CO_2 concentration
C_i	Intercellular CO_2 concentration
D1	The Q_B binding protein of the PSII reaction centre
DU	Dobson unit
EDTA	Ethylene diamine tetraacetate
$\phi CO2$	The quantum efficiency of CO_2 assimilation
$\phi PSII$	The relative quantum efficiency of PSII electron transport
Fm	The maximal fluorescence yield
Fo	The minimal fluorescence yield
Fs	The fluorescence yield at steady state photosynthesis
Fv/Fm	The ratio of variable to maximal fluorescence yield

HCFCs	Hydrochlorofluorocarbons
IAA	Indole-3-acetic acid
kDa	Kilodalton
LHCI, LHCII	Light-harvesting chlorophyll a/b protein complex associated with PSI and PSII respectively
NMHCs	Non-methane hydrocarbons
O_3	Ozone
PAL	Phenylalanine ammonia lyase
PAGE	Polyacrylamide gel electrophoresis
PAS300	The generalised plant action spectrum normalised to 300nm
PGA	3-phosphoglycerate
P_N	Net photosynthetic rate
PPFD	Photosynthetically-active photon flux density (400-700 nm)
PSI, PSII	Photosystems I and II respectively
Q_A, Q_B	Primary and secondary quinone electron acceptors of PSII respectively
q_P	The photochemical quenching
q_N	The non-photochemical quenching
Rubisco	Ribulose 1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose 1,5-bisphosphate
RWC	Relative water content
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylene diamine
Tris	Tris(hydroxymethyl)aminomethane

UV-A	Ultraviolet-A radiation in the wavelength band of 320-400 nm
UV-B	Ultraviolet-B radiation in the wavelength band of 290-320 nm
UV-C	Ultraviolet-C radiation below 290 nm
v/v	Volume to volume
ψ_w	Water potential
WUE	Water-use efficiency
w/v	Weight to volume

SUMMARY

Non-intrusive techniques have an important role in the detection of stress effects on photosynthetic activity. A well-established technique for monitoring the photosynthetic performance of leaves is the measurement of chlorophyll fluorescence (Kautsky and Hirsch, 1931; Schreiber and Bilger, 1987; Lichtenthaler, 1988). In this study, modulated chlorophyll fluorescence technique was used to monitor the effects of environmental stresses of water deficit and enhanced UV-B radiation on the photosynthetic performance of leaves.

Reductions in leaf water content result in reduced photosynthetic competence in many plants. In this work, modulated chlorophyll fluorescence, as well as the determination of net photosynthesis (P_N) and leaf water potential (ψ_w), were used to assess the performance of these methods in screening of the **water stress** tolerance in barley genotypes. A set of twenty genotypes of barley were tested. It is concluded from this study that these methods can be used as rapid screening tests for water stress tolerance in barley, although applied alone they are appropriate indicators for severe water stress rather than mild water stress. For genotypes growing under mild water stress conditions, it is necessary to use several screening tests simultaneously.

Further studies of water stress tolerance were pursued with only the genotypes previously selected as most tolerant and most sensitive to water stress. Water stress tolerant genotypes show smaller variations of photochemical quenching, net photosynthesis, water use efficiency and leaf temperature than water stress sensitive ones.

Over the last decades, significant decreases in stratospheric ozone concentrations have been reported above the Antarctic. These decreases have resulted in an increase in the amount of ultraviolet radiation, primarily in the UV-B region of the spectrum, and further to the UV-C region, reaching the earth surface which is potentially damaging to biological systems (Coohill, 1991). The exact consequences of this increase in ultraviolet radiation on photosynthetic activity of plants are largely unknown and this study is an attempt to estimate the effect of an

enhanced UV-B radiation on pea leaves grown under both controlled environment and field conditions.

When mature pea leaves were exposed to two levels of **UV-B** radiation, with and without supplementary **UV-C** radiation, under **controlled environment conditions**, decreases in CO_2 assimilation were observed that were not accompanied by decreases in the maximum quantum efficiency of photosystem II primary photochemistry, measured using modulated chlorophyll fluorescence techniques. Increased exposure to UV-B resulted in a further loss of CO_2 assimilation and decreases in the maximum quantum efficiency of PSII primary photochemistry, which were accompanied by a loss of the capacity of thylakoids isolated from the leaves to bind atrazine, thus demonstrating that photodamage to PSII reaction centres had occurred. Although UV-B irradiation of pea leaves will result in photodamage to PSII reaction centres, it is concluded that PSII is not the primary target site involved in the onset of the inhibition of photosynthesis in pea leaves induced by irradiation with UV-B. Such photodamage will only occur at UV-B levels considerably in excess of those currently experienced in the field. The initial decreases in the ability of leaves to assimilate CO_2 on exposure to UV-B are not accompanied by any decreases in the maximum quantum yield of PSII primary photochemistry or the ability of PSII complexes to bind quinones. Consequently, factors other than damage to the PSII reaction centre must determine the loss of ability to assimilate CO_2 during the early stages of irradiation with UV-B. It is suggested that rubisco remains as the major potential candidate for the primary site of inhibition by UV-B radiation of the photosynthetic apparatus. With increasing exposure to UV-B, when the rate of CO_2 assimilation falls below 6 μmol m^{-2} s^{-1}, a slowly reversible quenching of excitation energy in the PSII antenna occurs, which is consistent with a light-induced zeaxanthin quenching and results in a decreased quantum efficiency of PSII photochemistry. However, this quenching is almost certainly an indirect effect of UV-B inhibition of carbon assimilation mediated through a feedback inhibition. Addition of UV-C to the UV-B treatments increased markedly the rate of inhibition of photosynthesis, however the relationships between CO_2 assimilation and PSII characteristics remained the same, indicating that UV-B and UV-C inhibit leaf photosynthesis by a similar mechanism.

Enhanced UV-B treatment had no significant effect on chlorophyll fluorescence characteristics and CO_2 assimilation rate of pea plants grown under **field conditions**. These

results suggest that reduction in growth parameters of plants exposed to an enhanced UV-B radiation may be due to direct effects of UV-B on plant growth rather than a decrease in photosynthetic capacity.

It is concluded from these studies that modulated chlorophyll fluorescence is an useful, non-invasive rapid probe of the environmental stress effects on the photosynthetic performance of leaves. In particular, this method is useful for rapid screening of plants grown under various forms of environmental stresses.

RESUM

Introducció

En els darrers anys ha augmentat l'interès per conèixer quina és la resposta de les plantes quan les condicions ambientals no són favorables pel seu creixement. Les tècniques que no malmeten les plantes tenen molta importància en la detecció dels efectes dels estressos ambientals sobre l'activitat fotosintètica de les fulles. Una de les tècniques per avaluar el funcionament fotoquímic de les fulles és la mesura de la fluorescència clorofil.lica (Kautsky i Hirsch, 1931; Schreiber i Bilger, 1987; Lichtenthaler, 1988). Quan les fulles són exposades a condicions poc favorables s'observa una inhibició d'alguns dels processos fotoquímics de les corbes de la florescència clorofil.lica, de manera que aquest fet podria servir com un ràpid i senzill indicador del grau d'estrés que presenten les plantes (Smillie i Hetherington, 1983; Renger i Schreiber, 1986; Lichtenthaler i Rinderle, 1988). Aquest indicador de l'estrés ha estat utilitzat en la quantificació de la resposta de diferents espècies (per exemple, cereals) davant l'estrés hídric i permet classsificar-les segons els seu grau relatiu de tolerància a l'estrés (Havaux i Lannoye, 1985).

L'efecte de l'estrés hídric sobre la fotosíntesi de les fulles és un procés documentat i sobre el que existeixen articles de revisió (Bradford i Hsiao, 1982; Jones, 1983; Blum, 1989; Farquhar *et al.*, 1989; Lawlor, 1995). Els primers experiments que es van realitzar indicaven que la disminució de la taxa de bescanvi gasós era principalment el resultat d'un procés de tancament estomàtic. Però, més tard, es va demostrar els efectes no estomàtics de l'estrés hídric sobre els processos fotoquímics (Sharkey i Seemann, 1989).

Des de la darrera dècada hi ha hagut una forta disminució en la concentració de la capa d'ozó de l'estratosfera de la zona Antàrtica. Aquesta disminució, originada per l'increment de contaminats atmosfèrics, particularment dels compostos anomenats clorofluorocarbons (CFC), ocasiona un augment de la quantitat de radiació ultraviolada que arriba a la superficie terrestre (Farman *et al.*, 1985; Rowland, 1989; Frederick, 1990). Aquest augment de la radiació ultraviolada, que compren, en primer lloc, la regió UV-B de l'espectre i, després, la UV-C, pot afectar a tots els éssers vius de la terra (Coohill, 1991). La reducció de la capa

d'ozó s'espera que continui uns 20 o 30 anys més (Madronich *et al.*, 1994) i, per tant, s' arribi a reduccions, respecte als valors actuals, d'un 15% (NAS, 1979). En l'hemisferi Nord la disminució de la capa d'ozó ha estat d'entre un 4-5 % per dècada, des del començament dels anys 80 (Stolarski *et al.*, 1992; Herman *et al.*, 1993). Les conseqüències exactes d'aquesta disminució de la capa d'ozó en la capacitat fotosintètica de les plantes són desconegudes. Aquest estudi estima els efectes de l'increment de la radiació UV-B en plantes de pèsol crescudes sota condicions ambientals controlades i en el camp.

L'**objectiu** general d'aquesta Tesi és demostrar la validesa de la tècnica de la fluorescència clorofil.lica modulada per a l'estudi dels efectes de dos estressos ambientals, com són el dèficit hídric i l'increment de radiació UV-B, sobre l'activitat fotosintètica de les fulles.

Resultats i discussió

La tècnica de la fluorescència clorofil.lica modulada, junt amb la determinació de la taxa de fotosíntesi neta i el potencial hídric de les fulles, han estat utilitzats com a mètodes per classificar vint genotips diferents d'ordi (*Hordeum vulgare* L.) segons el seu grau de tolerància a la sequera. Es conclou que, en conjunt, aquests mètodes poden ser utilitzats com a tests ràpids per classificar plantes d'ordi segons el seu grau de tolerància a l'estrés hídric, però en el cas d'un estrés hídric moderat no es pot afirmar que una sola de les metodologies aplicades sigui indicadora del grau de tolerància al dèficit hídric i, per tant, és fa necessari utilitzar més d'una d'aquestes metodologies simultàniament. Aquests mètodes, si s'apliquen separadament, són millors indicadors per plantes sotmeses a estressos hídrics severs.

Els estudis de tolerància a la sequera es van continuar amb les varietats que prèviament havien estat seleccionades com més tolerants i més sensibles a la sequera. Així, els genotips més tolerants a la sequera van presentar menys variacions en el "quenching" fotoquímic de la fluorescència modulada, en la fotosíntesi neta, en l'eficiència en l'utilització de l'aigua i en la temperatura foliar, que els genotips més sensibles a la sequera.

Per altra banda, quan plantes de pèsol (*Pisum sativum* L.) van estar exposades a dos nivells de radiació UV-B, amb o sense radiació UV-C, sota condicions ambientals controlades, s'observa una disminució en l'assimilació de CO_2 que no va ser acompanyada per una disminució en la eficiència màxima per la llum del fotosistema II (PSII), mesurada utilitzant la

tècnica de la fluorescència clorofil.lica modulada de les fulles. Si augmenten l'exposició a la radiació UV-B, s'observa un augment de la disminució de l'assimilació de CO_2 i una disminució en l'eficiència màxima del PSII, acompanyada d'una pèrdua en la capacitat dels til.lacoidals dels cloroplasts per unir-se a molècules d'atrazina. Això demostra que hi ha hagut dany en els centres reactius del PSII. Malgrat que la radiació UV-B perjudica els centres de reacció del PSII, es conclou en aquest estudi que el PSII no és el primer lloc responsable de la inhibició de la fotosíntesi que s'observa en les fulles de pèsol per efecte de la radiació UV-B, ja que l'inicial disminució en l'assimilació de CO_2 que s'observa en les fulles sotmeses a radiació UV-B no esta acompanyat per una disminució en la màxima eficiència quàntica del PSII o en l'habilitat dels complexos dels PSII en unir-se a molècules d'atrazina. Per tant, altres factors que no sigui el dany als centres del PSII han d'estar implicats en la disminució de l'assimilació de CO_2 durant els primers estadis d'irradiació amb UV-B. Es suggereix que l'enzim rubisco és un dels candidats més probables, en un primer estadi, com a responsable de la disminució de l'aparell fotosintètica durant radiació amb UV-B.

A més, en el tractament a alta radiació UV-B, quan la taxa d'assimilació de CO_2 cau per sota de 6 $\mu mol\ m^{-2}\ s^{-1}$, en l'antena del PSII té lloc un "quenching" reversible, el qual es probablement degut a un "quenching" de la zeaxantina induït per la llum i resulta en una disminució en l'eficiència quàntica del PSII. Segurament, aquest "quenching" és resultat indirecte de l'efecte inhibidor de la radiació UV-B sobre l'assimilació de CO_2. Quan als tractaments amb radiació UV-B els hi afegim la radiació UV-C s'observa un fort augment de la inhibició de la taxa fotosintètica, però, la relació entre l'assimilació de CO_2 i les característiques del PSII continua essent la mateixa, indicant que les radiacions UV-B i UV-C inhibeixen la fotosíntesi foliar per un mecanisme similar.

Per últim, quan es van cultivar plantes de pèsol en condicions de camp i es van sotmetre a un increment de radiació UV-B, equivalent a un 15% d'augment de la radiació UV-B actual, no es van observar diferències significatives ni en les característiques de la fluorescència clorofil.lica ni en les taxes d'assimilació de CO_2 entre plantes control (no irradiades) i irradiades amb UV-B. Aquest estudi suggereix que les reduccions en els paràmetres del creixement de plantes exposades a un increment de radiació UV-B es pot deure més a un efecte de la radiació UV-B sobre el creixement de les plantes que a un efecte sobre la capacitat fotosintètica d'aquestes.

Es **conclou** en aquesta Tesi que la fluorescència clorofil.lica modulada és una ràpida i no destructiva tècnica per la detecció de com els estressos ambientals afecten l'activitat fotosintètica de les fulles. Concretament, aquesta tècnica es útil com a ràpid mètode per classificar plantes sotmeses a diferents tipus d'estressos.

GENERAL INTRODUCTION

CHAPTER 1:

GENERAL INTRODUCTION

1.1. THE IMPACT OF ENVIRONMENTAL STRESSES IN PLANTS

The study of plant responses to stress has been a central feature of environmental physiologist's attempts to understand how plants function in their natural environment and in particular to explain patterns of plant distribution and their performance along environmental gradients (Osmond *et al.*, 1987). In recent years, much progress has been made in understanding how stress affect plant performance and determining the ecological, physiological and biochemical responses of plants to a variety of stresses with a view to identifying common principles (Jones *et al.*, 1989).

A common approach in plant biology at the ecosystem and whole plant level is to consider as stressful any situation where the external constraints limit the rate of dry matter production of all or part of the vegetation below its "genetic potential" (Grime, 1979). The external constraints or form of stress may be biotic (*e.g.* pests or diseases) or they may be physical and related to shortages or excesses in the supply of solar energy, water or mineral nutrients or atmospheric pollutants. For agricultural systems this simple concept probably needs modifying to consider economic yield rather than dry matter production, though these two quantities will often be closely related (Jones and Jones, 1989).

Plants species or varieties differ in terms of their optimal environments and their susceptibility to particular stresses. Some workers prefer to consider as stressful only those environments that actually damage the plants and cause a qualitative change such as membrane damage or cell death, while others consider that in stressed systems, energy expenditure is increased or potential energy of the system is decreased. Both concepts seem too restrictive because mechanisms that enable plants to grow successfully in what might be expected to be sub-optical or otherwise stressful environments could be regarded as stress-tolerance mechanisms, and would certainly be of interest to plant breeders, even though no detrimental effect is necessarily observed (see Jones *et al.*, 1989).

A further difficulty is that stress factors do not usually operate alone so that interactions between and covariation of stresses are the norm in the natural environment. Stress may also have a greater effect during certain phases of the plant's life cycle than others. Seedling establishment and floral development are often particularly sensitive. Also, the

complexity of biological responses means that it is often difficult to disentangle cause and effect (Jones and Jones, 1989).

The description and quantification of the plant's response to stress is also fraught with difficulties. For example, dehydration avoidance is interpreted as the ability of genotypes to maintain a higher leaf water potential when grown under soil water deficits. Traits contributing to dehydration avoidance are leaf rolling, best adapted root system, increased pubescence of the aerial organs, increasing reflectance of incoming radiation and increased heat dissipation through decreased boundary layer resistance at the organ level. In nature, a better water balance is associated with a higher proportion of energy dissipated (Acevedo and Fereres, 1993). On the other hand, drought tolerance is summarised by Austin (1993) as a very complex character; it needs to be analysed in terms of its components, but even these have not been sufficiently well established to be useful. Some components may be: leaf glaucousness, diameter of xylem vessels in primary roots, capacity of roots and leaves to synthesize ABA and proline when stressed. Carbon isotope discrimination may, however, prove a useful integrate measure of drought tolerance (Austin, 1993).

To predict the impact of stress on plants it is necessary to know about (i) the temporal variation in stresses, (ii) the plant's potential to acclimate to stress and (iii) interactions between different stresses and the plant responses. Stresses can be very disparate in nature, even though some such as water deficits, freezing and salinity may act, at least partly, through a common mechanism. In other cases different stresses often occur together, for example high temperature and drought. Furthermore, different stresses can interact, both in their occurrence and in their effects, so that real progress in crop improvement is likely to depend on an improved understanding of these interactions and their consequences for plants and ecosystems (Jones and Jones, 1989).

1.2. THE ROLE OF CHLOROPHYLL FLUORESCENCE IN THE DETECTION OF STRESS CONDITIONS IN PLANTS

Until few years ago, ecophysiologically oriented photosynthesis research has been governed by gas-exchange measurements, mainly involving sophisticated systems for

simultaneous detection of CO_2 uptake and H_2O evaporation (Field *et al.*, 1989). With the help of these methods, fundamental knowledge on *in situ* photosynthesis has been gained. At present, progress has been made in the development of alternative practical methods for non-intrusive assessment of *in vivo* photosynthesis which have the potential of not only evaluating overall quantum yield and capacity, but also allowing insights into the biochemical partial reactions and the partitioning of excitation energy (Snel and van Kooten, 1990). As a consequence, photosynthesis research at the level of regulatory processes has been greatly stimulated, leading to important new concepts (see reviews by Demming-Adams, 1990; Melis, 1991; Allen, 1992). In particular, chlorophyll fluorescence has evolved as a very useful and informative indicator for photosynthesis electron transport in intact leaves, algae, and isolated chloroplasts (see Schreiber *et al.*, 1995). The study of the light-induced *in vivo* chlorophyll fluorescence of green plants tissue provides basic information on the functioning of the photosynthetic apparatus and on the capacity and performance of photosynthesis (Lichtenthaler and Rinderle, 1988). During the past years chlorophyll fluorescence has developed as one of the most frequently used measuring tools in plant science (Schreiber and Bilder, 1993).

Under physiological conditions fluorescence emitted from a leaf emanates primarily from pigment matrices associated with PSII. The usefulness of fluorescence measurements is that this light emission from within the PSII pigment matrices competes for excitation energy with photochemistry (*i.e.* photosynthesis) and with non-radiative decay processes. Because of this competition, it is possible to monitor changes in photochemistry and thermal deactivation by measuring florescent light emission (Baker, 1993).

In contrast to other ecophysiological methods, the advantage of measuring the different components of the chlorophyll-fluorescence signatures of intact leaves is that these fluorescence methods are very rapid. In addition, they are sensitive and non-destructive and can be performed with small leaf samples (Lichtenthaler and Rinderle, 1988). The simplicity of the method and the relatively low cost instrumentation involved are also important advantages of the fluorescence assay.

The basic observation on the applicability of fluorometric methods for practical use was made more than 100 years ago, when N.J.C. Müller (1874) discovered visually with the

aid of coloured glass filters that fluorescence changes occur in green leaves and are correlated with photosynthetic assimilation. About half a century later, Kautsky and Hirsch (1931) again visually followed the time course of chlorophyll *a* fluorescence and correlated it with that of photosynthetic CO_2 fixation. Later, Kautsky and co-workers presented detailed studies on the correlation between the time course of fluorescence emission (Kautsky curve) and photosynthetic activity (see Renger and Schreiber, 1986).

1.2.1. THE KAUTSKY CURVE

When a leaf is illuminated with continuous light following a period of darkness, fluorescence rises from a low level (Fo) via an intermediate level (I) and often a dip (D) to a peak level (P) and then decays through several phases via intermediate maxima to a value close to the original Fo-level (Govindjee, 1995). The various induction transients reflect the onset of photosynthesis as it affects the excitation density with PSII. The Fo-level, which is monitored by the weak modulated measuring beam in the preceding dark period, is a measure for the initial energy distribution to PSII and for the efficiency of excitation trapping at P680. Under normal conditions, all centres are "open" at Fo, *i.e.* all primary acceptors, Q_A, are oxidised (Schreiber and Bilder, 1987). This fluorescence rise (OIDP) reflects a gradual increase in the yield of chlorophyll fluorescence as the rate of photochemistry declines, when the pool of Q_A of the PSII reaction centres becomes increasingly reduced. Beyond this peak in fluorescence (P), electrons start to flow away from Q_A allowing photochemistry to increase, so leading to a decrease in fluorescence (Bolhar-Nordenkampf *et al.*, 1989). A secondary peak (M) usually follows P and corresponds to the initiation of CO_2 assimilation. Over minutes, fluorescence declines to a steady-state level (T) which corresponds in time to the point at which steady-state CO_2 assimilation is attained (Ireland *et al.*, 1984).

The rise to P in moderate light will typically require less than 2 seconds and is termed the fast phase of the fluorescence induction. The decline from P to T may require several minutes and is termed the slow phase of the induction (Bolhar-Nordenkampf *et al.*, 1989). This decline of fluorescence reflects the light-activation of electron transport capacity at the PSI

acceptor side, as well as the development of a number of overlapping "fluorescence quenching" processes (see below) (Schreiber and Bilder, 1987).

1.2.2. MODULATED FLUOROMETRY

Recent progress in chlorophyll fluorescence research is closely linked to the development of modulated chlorophyll fluoremeters which allow detection of fluorescescence yield in normal daylight (Ögren and Baker, 1985; Schreiber *et al.*, 1986). In principle, it had been long-term practice to select a modulated signal by frequency and phase selective amplification. However, a new generation of such instruments had been developed to satisfy the requirements of "quenching analysis" by the so-called saturation pulse method, which was conceived as the logical consequence of the original "light-doubling" technique of Bradbury and Baker (1981, 1984). Whereas Quick and Horton (1984) and Dietz *et al.* (1985) were still using mechanical choppers to produce modulated measuring light, Ögren and Baker (1985) applied light-emitting diodes, which allow much higher modulation frequencies. Furthermore, a selective pulse-amplitude-modulation system (PAM) was developed (Schreiber *et al.*, 1986) which tolerates non-modulated background signals more than 10^6 times stronger than that of the measured fluorescence yield. With such new instruments becoming commercially available (*e.g.* **PAM 101-102-103**, Heinz Walz, Mess-und Regeltechnik, Effeltrich, Germany) modulated fluorometry gained widespread use in numerous disciplines of plant science.

Very recently, an extremely miniaturised computer-controlled version of the PAM 101-102-103 fluorometer, the **PAM 2000** fluorometer (Heinz Walz, Mess-und Regeltechnik, Effeltrich, Germany) was introduced (Schreiber and Bilger, 1993). With this PAM 2000 fluorometer, all relevant fluorescence parameters are measured and the resulting values of Fv/Fm, ϕ_{PSII}, q_P, and q_N are calculated (see below). The incident light intensity can be continuously monitored in the leaf plane at the same site where fluorescence is assessed. In this way, assessment of *in situ* photosynthesis has become rather simple and efficient (Schreiber *et al.*, 1995).

1.2.2.1. MODULATED CHLOROPHYLL FLUORESCENCE AS A PROBE OF THE QUANTUM EFFICIENCY OF PSII PHOTOCHEMISTRY

Modulated fluorescence measurements (Ögren and Baker, 1985; Schreiber *et al.*, 1986) permit the separation of fluorescence quenching into photochemical (qp) and non-photochemical (q_N) components. The principle of this method and the distinction between photochemical and non-photochemical fluorescence quenching can be illustrated simply by considering an analysis of quenching during the induction of photosynthesis in a leaf exposed to actinic light (Figure 1). When a leaf is held in the dark for an extended period of time, the quinone acceptors of PSII (Q_A and Q_B) become maximally oxidised. On exciting the leaf with modulated light of sufficiently low intensity to ensure that the primary PSII quinone acceptor (Q_A) remains oxidised (generally below 1 μmol m^{-2} s^{-1}), a minimal level of fluorescence (Fo) is generated. If the leaf is now exposed to a 1 second pulse of very intense light that fully reduces the Q_A pool (typically more than 4000 μmol m^{-2} s^{-1}), fluorescence rises to a maximal level (Fm). The rise in fluorescence from Fo to Fm is attributable to the photoreduction of Q_A in all PSII complexes; that is, the fluorescence yield increases to a maximum because the competition for excitation by photochemistry is nearly non-existent. This rise in fluorescence associated with Q_A reduction is know as variable fluorescence (Fv; Fv=Fm-Fo).

If the dark-adapted leaf is exposed to continuous actinic light then fluorescence will rise rapidly to a peak level, which will be lower than Fm if the actinic light is not sufficient to reduce maximally the Q_A pool. Thereafter, fluorescence slowly declines to a steady-state level (Fs). This quenching of fluorescence from the peak level to the steady state is coincident with the induction of CO_2 assimilation in the leaf (Ireland *et al.*, 1984) and is called **photochemical quenching (qp)**. If the leaf is exposed to the saturating 1s light pulse during this quenching from the peak level, then a rise in fluorescence is observed to a level Fm' (1), which is considerably less than the Fm level generated from the dark-adapted leaf even though Q_A is essentially fully reduced in both situations. Consequently, the difference between Fm and Fm' is attributable to a **non-photochemical quenching (q_N)** of excitation energy in the leaf exposed to actinic light.

(1) The superscript ' denotes measurements made at steady state photosynthesis.

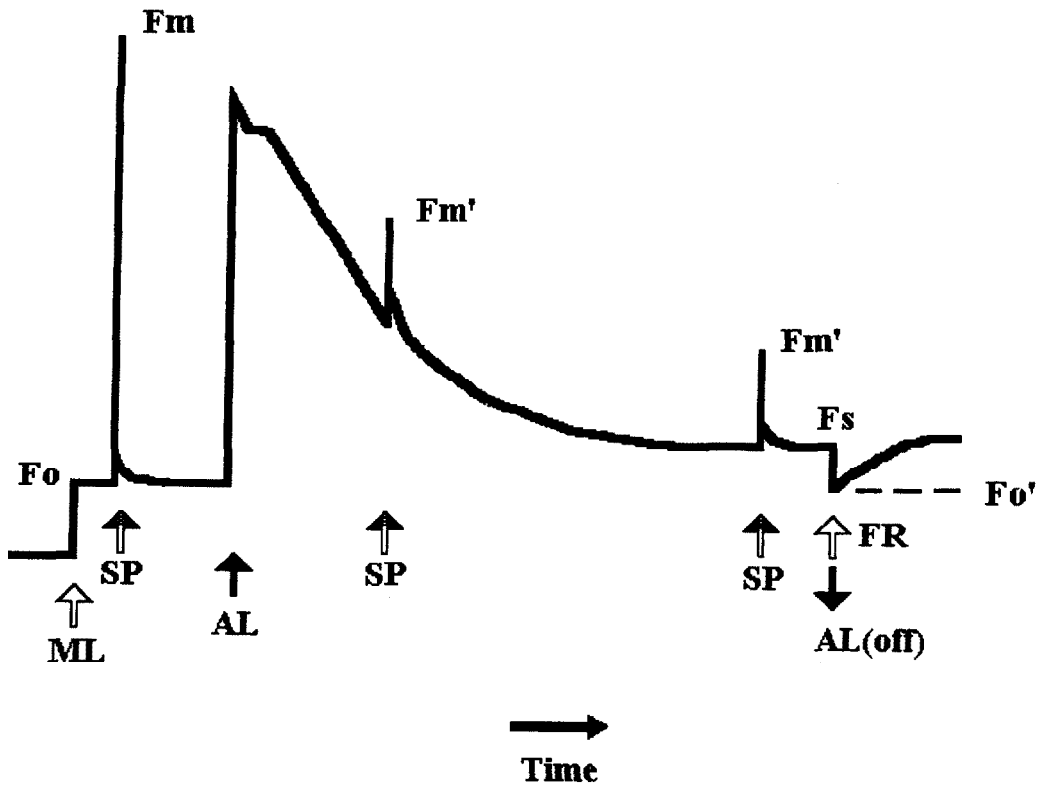


Figure 1. Changes in fluorescence yield of a dark-adapted leaf when exposed to actinic light (AL) and saturating pulses of light (SP). Fluorescence yield was measured with a pulse-amplitude modulated fluorimeter (PAM). Fo is the minimal fluorescence yield produced on exposure of the dark-adapted leaf to the weak modulated measuring light (ML). Fm is the maximal fluorescence yield produced from the dark-adapted leaf by a short, saturating light pulse (SP; PPFD = 10000 μmol m^{-2} s^{-1} for 1s). Fm' is the maximal fluorescence yield produced by the short, saturating light pulse when the leaf is exposed to actinic light. The magnitude of Fm' is dependent upon the period of exposure of the leaf to the actinic light. Fo' is the fluorescence yield obtained in a leaf exposed to actinic light when PSII reaction centres have been maximally oxidised; this is generally achieved by removing the actinic light and simultaneously exposing the leaf to far-read light (FR, PPFD of *ca.* 100 μmol m^{-2} s^{-1} at wavelengths above 700 nm). Fv is the variable fluorescence yield achieved when non-photochemical quenching processes are minimal in a dark-adapted leaf (Fv=Fm-Fo). Fv' is the variable fluorescence yield after exposure of the leaf to actinic light (Fv'=Fm'-Fo'). Fs is the fluorescence yield when the leaf is at steady-state photosynthesis. The PPFD of actinic light used in this example was 600 μmol m^{-2} s^{-1} (from Baker, 1993).

Non-photochemical processes of excitation energy dissipation are involved because this quenching is independent of the redox state of Q_A. This non-photochemical quenching may be calculated from (Fm-Fm')/Fm' = Fm/Fm'-1. This is clearly light-induced in that it does not occur in dark-adapted leaves and the magnitude of the quenching is associated with the rate of electron transport during the induction of photosynthesis. Increases in non-photochemical quenching reduce the quantum yield of PSII photochemistry (ϕ_{PSII}), and consequently diminish the quantum efficiency of non-cyclic electron transport, which in turn will reduce the quantum efficiency of CO_2 assimilation (ϕ_{CO2}) (Baker, 1993).

Recently it has been demonstrated that simple fluorescence parameters can be used as a rapid and easy measure of ϕ_{PSII}, which depends upon (i) the efficiency with which an absorbed photon can reach a reaction centre to perform photochemistry; and (ii) the proportion of reaction centres which are capable of transferring an electron to an acceptor at that point in time, *i.e.* photochemically "open" reaction centres (Genty *et al.*, 1989). Since the efficiency of excitation energy capture by "open" PSII reaction centres is defined by the ratio of variable to maximal fluorescence (Fv/Fm), and the photochemical quenching coefficient (qp) is a measure of the proportion of "open" PSII reaction centres, Genty *et al.* (1989) noted that the quantum efficiency of PSII photochemistry (ϕ_{PSII}) in a leaf is defined by:

$$\phi_{PSII} = Fv'/Fm' \times qP$$

where qp is determined from (Fm'-Fs/Fm'-Fo'). In practice the relative ϕ_{PSII} for leaves under any given environmental conditions can be made by monitoring the steady-state fluorescence level (Fs) under the conditions in interest, exposing the leaves to a saturating flash and measuring the maximal fluorescence level (Fm') that is attained. The parameter (Fm'-Fs')/Fm' equates to [(Fv'/Fm') x qp] for the leaf under the given conditions and thus is a measure of ϕ_{PSII} (Genty *et al.*, 1989).

1.2.2.2. RELATIONSHIP BETWEEN THE QUANTUM EFFICIENCIES OF PSII PHOTOCHEMISTRY AND CO_2 ASSIMILATION

In maize leaves, modifications of ϕ_{CO2} in response to changes in light intensity and atmospheric CO_2 concentration or during the induction of photosynthesis in a dark-adapted leaf were observed to be directly proportional to the changes observed in ϕ_{PSII}. Linear relationships between ϕ_{CO2} and ϕ_{PSII} were also observed in barley and ivy leaves exposed to a range of light intensities under conditions in which photorespiration had been minimised by reducing the atmospheric O_2 concentration to 2% (Genty *et al.*, 1989). Similar relationships have also been observed between ϕ_{CO2} (Krall and Edwards, 1990), the quantum yield of O_2 evolution (Keiler and Walker, 1990; Seaton and Walker, 1991; van Wijk and van Hasselt, 1990) and ϕ_{PSII} in a leaves of a range of species. It should be noted that some non-linearity was found at very low light intensities, probably because of differences in the chloroplasts populations being monitored for fluorescence and CO_2 assimilation and differences in their excitation rates. Light-induced modifications to the organisation of the photosynthetic apparatus, such as a state transition, could also account for non-linearity (Baker, 1993). As would be expected for leaves of C_3 plants under conditions favourable for photorespiratory activity where the products of electron transport are apportioned between carboxylation and oxygenation reactions, the strict linear relationship between ϕ_{CO2} and ϕ_{PSII} breaks down (Genty *et al.*, 1990).

Simultaneous measurements of ϕ_{PSII} and the quantum yield of PSI photochemistry (ϕ_{PSI}) made over a wide range of light intensities on leaves under both photorespiratory and non-photorespiratory conditions have shown a good correlation between these parameters (Genty *et al.*, 1990). This observation, together with the linear relationship found between ϕ_{PSII} and ϕ_{CO2}, suggests that ϕ_{PSII} is a good measure of the quantum efficiency of non-cyclic electron transport. The conserved linearity between ϕ_{PSI} and ϕ_{PSII} under a wide range of physiological conditions implies that the rate of PSI-mediated cyclic electron transport, if it occurs *in vivo*, is always proportional to the rate of non-cyclic electron transport (Baker, 1993).

1.3. GENERAL AIMS

The aim of this thesis was the use of modulated chlorophyll fluorescence techniques to monitor the effects of the environmental stresses of water deficit and enhanced UV-B radiation on the photosynthetic performance of leaves.

In the following chapters, modulated chlorophyll fluorescence technique was assessed as a rapid screening method for water stress tolerance in barley plants (chapter 2) and this technique was used to evaluate the effects of an enhanced UV-B radiation on pea plants grown under both controlled environment and field conditions (chapters 3 and 4 respectively).

MODULATED CHLOROPHYLL FLUORESCENCE AND PHOTOSYNTHETIC GAS EXCHANGE AS RAPID SCREENING METHODS FOR WATER STRESS TOLERANCE

CHAPTER 2:

MODULATED CHLOROPHYLL FLUORESCENCE AND PHOTOSYNTHETIC GAS EXCHANGE AS RAPID SCREENING METHODS FOR WATER STRESS TOLERANCE

2.1. ABSTRACT

Determination of modulated chlorophyll fluorescence coupled to measurements of net photosynthesis and leaf water potential has been assessed as a screening method to score a set of twenty genotypes of barley (*Hordeum vulgare* L.) for drought tolerance. The results show that these methods can be used as rapid screening tests for water stress tolerance in barley, although applied alone they are appropriate indicators for severe water stress rather than mild water stress. For genotypes growing under mild water stress conditions, it is necessary to use several screening tests simultaneously. Further studies of drought tolerance were pursued with only the genotypes previously selected as most tolerant and most sensitive to drought. Drought tolerant genotypes show smaller variations of photochemical quenching, net photosynthesis, water use efficiency and leaf temperature than drought sensitive ones.

2.2. INTRODUCTION

Drought limits plant growth and crop productivity more than any other single environment factor (Boyer, 1982). Water supply is restricted in many parts of the world and productivity in these environments can only be increased by the development of crops that are well adapted to dry conditions. It is clear that the potential for biotechnological improvement of crop performance cannot be realised until we have identified genes and gene products which are responsible for the desired characteristics of drought tolerance. This in turn cannot occur without a thorough understanding of the biophysical, biochemical and physiological perturbations that are induced by a restricted water supply (Sharp and Davies, 1989).

Although plant growth rates are generally reduced when soil water supply is limited, shoot growth is often more inhibited than root growth and in some cases the absolute root biomass of plants in drying soil may increase relative to that of well-watered controls. It is also commonly observed that the roots of unwatered plants grow deeper into the soil than roots of plants that are watered regularly. Clearly, increases in the density and depth of rooting can help sustain a high rate of water extraction in drying soil and may promote substantial improvement in yield in dry years (see Sharp and Davies, 1989).

2.2.1. WATER STATUS OF CELLS

Water potential (*i.e.* the chemical potential of water in a system compared with the chemical potential of pure water at atmospheric pressure and at the same temperature) is the most widely used measure of water status and provides a thermodynamically based description of water in the plant. The water potential gradient is the driving force for water movement. The water potential of cells is determined by two major components: osmotic potential, determined by the intracellular concentration of osmotically active solutes, and turgor pressure. Turgor pressure provides the driving force for cell expansion where it causes tensions in the primary wall and a viscous (irreversible) flow of wall material (Steudle, 1993). Osmotic potential is not sensed directly by the cell contents but the solutes contributing to it have direct effects on metabolism. The other commonly used measure of water status is water content. This is usually expressed as relative water content (RWC) which is the water content as a proportion of that at full saturation. RWC is therefore a measure of relative cell volume and shows the changes in cell volume which could affect interactions between macromolecules and organelles (Lawlor, 1995).

2.2.2. INHIBITION OF PHOTOSYNTHESIS BY WATER STRESS

The effects of water stress on leaf photosynthesis are well documented and have been critically summarised in several reviews (Bradford and Hsiao, 1982; Jones, 1983; Blum, 1989; Farquhar *et al.*, 1989). Early experiments indicated that the decrease in net leaf CO_2 uptake was mainly the result of stomatal closure. However, it was also shown non-stomatal effects of drought stress on photosynthetic processes (Sharkey and Seemann, 1989).

Water stress decreases the rate of CO_2 assimilation per unit leaf area (A) with constant incident radiation, temperature and vapour pressure deficit, independent of the CO_2 supply outside the leaf (C_a). Lawlor (1995) states possible reasons for the decreases in A are:

(i) limited CO_2 diffusion to the intercellular spaces of the leaf as a consequence of reduced stomatal conductance (g_s);

(ii) impaired metabolism by direct inhibition of biochemical processes caused by ionic, osmotic or other conditions induced by loss of cellular water.

Reductions in leaf water content result in reduced photosynthetic competence in many plants. Under mild drought stress, decreases in photosynthesis are generally considered to be the result of reduced availability of CO_2 due to stomatal closure (Kaiser, 1987; Schulze, 1986a). It is possible that leaf water deficits can also have effects on chloroplast biochemistry that would contribute to depressions in photosynthetic performance. Studies on intact leaves subjected to moderate drought stresses have demonstrated that the potential for thylakoid photochemical activity is not decreased (Ben *et al.*, 1987; Cornic *et al.*, 1989; Genty *et al.*, 1987). Consequently, the effects of low leaf water potentials on the photochemical activities of isolated thylakoids are unlikely to be representative of the situation occurring *in vivo* and are probably due either to artefacts caused by the procedures for isolation of thylakoids from stressed leaves or by photoinhibition when electron transport is constrained in the isolated membranes (Cornic and Briantais, 1991). Reductions in intercellular CO_2 concentration and chloroplasts biochemical dysfunctions will reduce the rate of CO_2 assimilation in a leaf and would be expected to reduce the quantum efficiency of non-cyclic photosynthetic electron transport (Baker, 1993).

Many studies have shown that A decreases progressively at both saturating and sub-saturating C_i as water deficit develops (for review see Lawlor, 1995). The interpretation is that CO_2 assimilation mechanism is inhibited by processes not dependent upon the maintenance of C_i, *i.e.* biochemical processes are made less effective or damaged in some way. However, the problem of non-uniform distribution of stomatal closure across leaf surfaces (*i.e.* 'patchy' stomatal apertures) during water stress is of potential importance (Farquhar *et al.*, 1987). Stomatal patchiness is not a universal effect of stress, and it depends on the type of water deficit, rate of application and species (Lawlor, 1995).

By increasing C_a, it should be possible to overcome stomatal limitations and return A to the unstressed value if metabolic processes are not affected. Very large concentrations of CO_2 have been used to overcome the stomatal limitation but, in general, with moderate to severe stress, increasing C_a does not increase A to the rate in the non-stressed leaves (Graan and Boyer, 1990).

Other approaches suggest that A is regulated by g_s because at mild stress C_i does decrease (Thomas and André, 1982). Vassey and Sharkey (1989) concluded that decreased photosynthesis in *Phaseolus vulgaris* was due to decreased g_s, despite evidence from the A/C_i curves. In *Lupinus albus*, photosynthesis is not affected by decreasing RWC from 100 to 80%, but below this A decreases almost linearly with decreasing RWC (Chaves, 1991). In contrast to the view that g_s regulates A, a number of analyses show that metabolism is affected by water deficit. Increasing C_a does not increase A, despite the fact that doubling C_a at constant g_s should increase C_i and thus stimulate A (Matthews and Boyer, 1984), even when patchy stomatal distribution was not considered a factor (Gimenez *et al.*, 1992).

Analysis of CO_2 compensation concentration indicates the relative changes in photosynthesis and respiration in stressed leaves. Generally, respiration increases with decreasing water potential. In unstressed or mildly stressed leaves, CO_2 is mainly derived from photorespiration as the compensation concentration is reduced to a few μmol CO_2 mol^{-1} by decreasing the atmospheric oxygen from 21 to 2 % (Lawlor, 1995). However, the marked increase in compensation concentration at low water potential is not prevented by low oxygen, so dark respiration occurring in the light is the probable cause (Lawlor, 1976).

2.2.3. WATER STRESS EFFECTS ON THE PARTIAL PROCESSES OF PHOTOSYNTHESIS

Water stress frequently decreases the number of photons captured by leaves simply because wilted leaves are at a steeper angle to the sun's rays. Changes in absorption characteristics of leaves occur due to cell shrinkage. Initial stress may increase chlorophyll concentration but prolonged stress causes pigment loss and senescence. However, the changes at the chloroplast and thylakoid level are relatively small (Lawlor, 1995). Genty *et al.* (1987) did not observe changes in photon capture or energy distribution between the two photosystems in cotton. The fluorescence component Fo remains relatively constant, showing that stress does not alter the pigment bed substantially. There are indications that more severe stress damages both photosystems (Genty *et al.*, 1987; Meyer and de Kouchkowsky, 1993). Meyer and de Kouchkowsky (1993) observed that severe stress decreased maximum rates of

PSII, possibly caused by loss of active PSII centres, although the antenna and proteins of the complexes are not damaged by such severe stress.

Electron transport to the normal physiological acceptors has not been well examined in water stressed tissues. There is evidence that the pools of reduced pyridine nucleotides are little affected (NADPH) or increased (NADH) in stressed leaves and that $NADP^+$ increased and then decreased with progressive stress and NAD^+ decreased, suggesting that both the supply of electrons for reduction of acceptors and the supply of oxidised nucleotides is adequate and that the process is efficient even under severe stress (Lawlor and Khanna-Chopra, 1984; Stuhlfauth *et al.*, 1991). Other redox components between Q_A and $NADP^+$ are presumably also maintained in a reduced state, together with ferrodoxin. Electron transport therefore is considered to continue at a considerable rate even in severely stressed tissues and the electrons are passed to the normal physiological acceptors $NADP^+$ and ferredoxin and to alternative acceptors such as oxygen (Mehler reaction) and eventually in photorespiration (Lawlor, 1995). The reduction of oxygen in the Mehler reaction leads to the formation of superoxide and hydrogen peroxide. These reactive forms of oxygen, collectively known as active oxygen, are potentially dangerous. Additionally, adsorption of excess light by chlorophyll results in formation of triplet chlorophyll which can then pass excitation energy to oxygen resulting in formation of singlet oxygen. This is more reactive that ground-state oxygen and can lead to peroxidation and breakdown of thylakoid lipids. Limitation of carbon dioxide fixation by water deficit appears to increase formation of active oxygen and is a potential means of damage (Björkman and Demming-Adams, 1994; Smirnoff, 1993).

The efficiency of energy conversion is not affected by mild stress but decreases with severe stress, particularly if the thylakoids have been subjected to bright light which causes photoinhibition of the photosystems. Decreased Fv/Fm and Fv'/Fm' reflect this. There are probably large differences between species and within species resulting from differences in growth conditions and exposure to bright light and water deficit (Lawlor, 1995). Cornic and Briantais (1991) have demonstrated that decreases in ϕ_{PSII} occur during desiccation of a bean leaf, which are accompanied by decreases in Fv'/Fm' and qp. They also estimated the rate of electron flow to O_2 from the measurements of ϕ_{PSII} and CO_2 assimilation, and argued that the allocation of electrons to O_2 increased as the rate of CO_2 assimilated decreased with

dehydration of the leaf. Reduction of the O_2 concentration in the atmosphere of a droughted leaf from 21% to 1% resulted in large decreases in ϕ_{PSII} and the estimated rate of non-cyclic electron transport, which were reversible on returning the O_2 concentration to 21%. Water stress decreased q_P with in bean but in sunflower it increased in 20% O_2 but decreased with stress in 2% O_2, indicating differences in response to photorespiration and in the state of Q_A (Scheuerman *et al.*, 1991; Stuhlfauth *et al.*, 1988). Also, q_P and q_N were unaffected by stress over the water potential range -0.7 to -2.5 MPa during steady-state photosynthesis in *Digitalis lanata*, although in the dark to light transition q_N increased with stress, indicating increased thylakoid membrane energization possibly as a consequence of inhibition of ATP generation or of ATP consumption (Stuhlfauth *et al.*, 1988).

Photophosphorylation under water stressed conditions is therefore of great importance but poorly understood. There is good evidence of decreased ATP content and energy charge in stressed tissues and of inhibition of photophosphorylation by damage to CF in relatively and severely stressed plants, but recent *in vivo* studies on field-grown plants have contradicted this. The difference may reflect the operation of defence mechanisms protecting ATP synthesis during slow stress (Lawlor, 1995).

In general, mild water deficits have little effect on metabolism (Quick *et al.*, 1989) but greater deficits have more substantial effects (Sharkey and Seemann, 1989). As the central process in photosynthetic carbon metabolism, the behaviour of the Calvin cycle is important for understanding the decrease in photosynthetic rate under water stress. Water stress decreases the pools of metabolites in the Calvin cycle (PGA and RuBP) and products (sucrose and starch) (Krampitz *et al.*, 1984). Gimenez *et al.* (1992) suggest that inhibition of CO_2 fixation is related to the decreased RuBP pool, not to the supply of CO_2, and so factors regulating the RuBP pool are prime candidates for the limitation of photosynthesis under stress. A marked sigmoidal relationship between photosynthesis and the RuBP content of the tissue at constant C_i was observed by Gimenez *et al.* (1992) The k_m of the enzyme for RuBP is 25-40 mmol m^{-3} and the estimated concentration of RuBP is 8 mol m^{-3} in unstressed leaves decreasing to 1 mol m^{-3} in stressed leaves.

Rubisco is one of the best studied Calvin cycle enzymes in water stressed leaves and there is a degree of agreement on its response. Stress does not substantially affect the k_{cat} of

the enzyme extracted rapidly and measured *in vitro*, its carbamylation state, the amount of Rubisco per unit area or its proportion to total soluble protein (Gimenez *et al.*, 1992; Sharkey and Seemann, 1989). Other enzymes of the Calvin cycle have not been analysed as extensively as Rubisco. Fructose-1,6-bisphosphatase (FBPase) has been suggested as a likely site of inhibition. The increased acidification of the chloroplasts stroma which occurs with drought could inhibit the enzyme. This would decrease RuBP synthesis, but the evidence suggests that mild stress does not affect the activity of FBPase (Vassey and Sharkey, 1989). The content of fructose 6-phosphate did decrease with mild stress, suggesting that the FBPase activity might be decreased (Sharkey and Seemann, 1989).

The supply of ATP and NADPH is a very important aspect of metabolism. ATP pools decrease and ADP pools increase to a lower energy charge (Lawlor, 1995). Stuhlfauth *et al.* (1991) observed similar changes in the adenylates and the free phosphate pool dropped markedly, but the energy charge increased. In general, NADPH content decreased little or only slightly, so the ratio ATP/NADPH dropped substantially and the reduced to oxidised nucleotides and reductant to ATP ratios increased greatly (Lawlor, 1995).

The pool of sucrose decreased substantially and rapidly in severely stressed leaves of sunflower (Lawlor and Fock, 1977). Sucrose is consumed by respiration and serves as a substrate for organic acid synthesis in the tricarboxylic acid cycle and eventually for the synthesis of such stress metabolites as proline (Larher *et al.*, 1993).

Photorespiration consumes energy as NADPH and ATP in the synthesis of RuBP and as NADH in the glycolate pathway. Photorespiration increases relative to photosynthetic CO_2 fixation with increasing water stress so expending relatively more reductant than ATP, particularly as the NAD(P)H produced may be oxidised by the mitocondrial respiratory chain and result in ATP synthesis (Lawlor, 1995).

2.2.4. CONVENTIONAL BREEDING METHODS FOR WATER STRESS TOLERANCE: A BARLEY CASE

Over the past 30 years major efforts have been made by plant physiologists and breeders to improve the drought tolerance of a wide range of agricultural and horticultural

crops. There are many processes that affect the 'fitness' of a plant in water-limited situations, but those such as survival that may be appropriate in natural ecosystems are often not applicable to agricultural crops, where productivity is usually of the greatest interest (Jones, 1993).

Critical evaluation of progress in plant breeding over a period of several decades has demonstrated a genetic improvement in yield under both favourable and stress conditions. The yield improvement under drought stress occurred before many of the physiological issues of drought resistance were understood and resulted partly from the genetic improvement of yield potential and partly from the improvement of stress resistance. The improvement of yield under stress must therefore combine a reasonably high yield potential with specific plant factors which would buffer yield against a severe reduction under stress. On the other hand, potentially lower yielding genotypes occasionally have been found to perform very well under drought stress conditions, especially under severe drought stress (see Blum, 1989).

The development of better varieties for dry conditions conventionally involves extensive selection and testing for yield performance over diverse environments using various biometrical approaches (Hanson and Robinson, 1963). These empirical methods have led to the development of drought resistant cultivars of many crops such as barley (Blum, 1989).

Drought is the single most important factor limiting barley yield. Yet, compared to many other cereals, barley is well adapted to this condition, as it is shallow and poorly fertile soils and high temperatures, thanks to its better water use efficiency (WUE). Moreover, it can even escape drought and high temperatures because it is able to complete its biological cycle before the onset of these adversities (Cattivelli *et al.*, 1994).

It appears that in barley there are three main critical stages at which this stress affects productivity, The first occurs in approximate coincidence with floral initiation. At this stage stress causes a reduction in the number of spikelets and florets per spike as well as of grain per unit of area through the suppression of tillering. The second critical moment is anthesis, when stress again expresses itself as a decrease in grain number per spike through negative pressure on reproductive efficiency. The third stage is at initial milk ripening, when the stress elicits a decline in grain weight (Weltzien and Srivastava, 1981).

Most of the progress in breeding barley for drought tolerance has been achieved through the application of empirical methods. A drought-tolerant cultivar is in actual practice

defined as one that produces the highest yield under limited soil moisture (Cattivelli *et al.*, 1994).

Although drought-resistant varieties have been developed by the use of empirical breeding methods that employ yield as a selection index, these methods are too costly and require a long period of testing and evaluation. A direct reference to some physiological attributes in the selection for drought resistance would allow us to address the underlying factors of stability, to the same extent that selection for example disease resistance addresses the specific plant interaction with the pathogen rather than yield. When physiological attributes are addressed, the genetics involved should become simpler, as a function of the lower level of plant organisation. Physiological selection criteria must therefore supplement yield evaluations (Blum, 1989).

2.2.5. PHYSIOLOGICAL SELECTION CRITERIA FOR WATER STRESS TOLERANCE

According to Blum (1989) the physiological approach to selection depends on two conditions:

(i) an understanding of the physiological parameters of plant water relations which support plant productivity under stress, and

(ii) a technical ability for a rapid and simple measurement of the relevant attributes in large breeding populations.

Crop plant responses to drought stress in an agricultural ecosystem and the respective physiologies can be classified into two categories. The first category is when water stress exists but a positive carbon balance is maintained by the plant. Resistant genotypes achieve a relatively greater net gain of carbon as compared with susceptible ones, with a subsequent effect on yield. The second category is when stress is severe and a net loss of carbon takes place so that plants are merely surviving stress. Resistant genotypes survive and subsequently recover better upon rehydration, compared with susceptible ones (Blum, 1989).

Cellular and subcellular attributes that are directly associated with the maintenance of carbon gain under stress are not commonly used as selection indices, mainly because of methodological difficulties even thought, for example, the performance of the photosynthetic apparatus under conditions of cellular stress is of primary importance (Blum, 1989). The measurement of gas exchange in various genotypes subjected to the same level of water deficit is a very slow technique, but use of chlorophyll fluorescence as a rapid probe of PSII responses may hold a potential in selection for drought resistance (Havaux and Lannoye, 1985a).

When leaves are exposed to unfavourable environmental constraints, inhibition of some of the photochemical processes controlling the fluorescence yield *in vivo*, results in substantial changes in the characteristics of the chlorophyll fluorescence curves, which can then serve as easy and rapid indicators of stress conditions in plants (Smillie and Hetherington, 1983; Renger and Schreiber, 1986; Lichtenthaler and Rinderle, 1988). These fluorescence indicators have been used to quantify the response of different species (*e.g.* cereal plants) to environmental stress and hence to rank them according to their relative stress tolerance (Havaux and Lannoye, 1985a). Belkhodja *et al.* (1994) also pointed out that chlorophyll fluorescence could be an excellent tool for salinity tolerance screening since it is easy to measure and may allow for the screening of large numbers of genotypes in a short time. It has been shown that it is possible to make use of *in vivo* chlorophyll fluorescence in developing rapid and sensitive screening methods to select higher plants for tolerance to other environment stresses such chilling, freezing, heat and salinity (see Havaux and Lannoye, 1985b). None of these citations have used modulated chlorophyll fluorescence parameters (like we do) for water stress tolerance screening. There is a need for screening test that is a more specific indicator of drought tolerance than yield itself, that will measure the response of the plant integrated over a substantial part of its life cycle, is non-destructive and can be used for testing large numbers of plants (Austin, 1989).

2.3. AIMS

The present study had two aims: (1) to evaluate whether the methods used to measure chlorophyll fluorescence and gas exchange are applicable as rapid screening tests for drought

tolerance in barley, and (2) to examine some physiological responses of barley to drought such as photochemical quenching, photosynthetic gas exchange and leaf temperature.

2.4. MATERIALS AND METHODS

2.4.1. PLANT MATERIAL AND GROWTH CONDITIONS

Twenty genotypes of barley (*Hordeum vulgare* L.) were used; details of the genotypes used and their row numbers in the spike, vegetative cycle and source are given in Table 1. Ten of them have two rows on the spike and the others have six rows. Nine genotypes have winter vegetative cycles, three genotypes have alternative and the others have spring vegetative cycles. Plants were grown in a greenhouse at the Université Libre de Bruxelles, Belgium. Maximum photosynthetic photon flux density was *ca.* 1200 μmol m^{-2} s^{-1}, day/night air temperature 27/14 °C and relative humidity about 78%. Seeds of barley were sown in 13 x 13 cm plastic pots filled with a Terofor compost-sand mixture (1:1, v:v). Plants were watered daily until third leaf emergence.

2.4.2. WATER STRESS TREATMENT

Water stress was developed gradually after withholding of water. The development of water stress was monitored by regular measurement of leaf water potential. For all studies, the third fully expanded non-senescing leaves were used and detailed measurements were made every day at the same time. Leaf water potential was measured using a hydraulic press (Havaux *et al.* 1988a). Experiments were started early in the morning when ψ_w was -0.4±0.1 MPa for control plants and about -1.0±0.2 MPa for stressed plants, which was achieved about 12 days after starting water stress treatment. In some cases, plants were rewatered at the end

Table 1. List of barley genotypes used, their row numbers on the spike, vegetative cycle and source. CIMMYT = International Maize and Wheat Improvement Centre. ICARDA = International Centre for Agricultural Research in the Dry Areas. B = British. F = French. G = German. S = Spanish.

GENOTYPES	ROW	VEGETATIVE CYCLE	SOURCE
ALBADA	2	ALTERNATIVE	CIMMYT
ALPHA	2	WINTER	EUROPEAN(F)
MOGADOR	2	WINTER	EUROPEAN(F)
ALEXIS	2	SPRING	EUROPEAN(G)
BEKA	2	SPRING	EUROPEAN(S)
EV1-89-213	2	SPRING	EUROPEAN(S)
EV1-89-215	2	SPRING	EUROPEAN(S)
ZAIDA	2	SPRING	EUROPEAN(S)
KYM	2	SPRING	EUROPEAN(B)
CAMEO	2	SPRING	EUROPEAN(B)
DOBLA	6	SPRING	EUROPEAN(S)
PLAISANT	6	WINTER	EUROPEAN(F)
BARBARROSA	6	WINTER	EUROPEAN(F)
ID-4016	6	ALTERNATIVE	CIMMYT
HATIF	6	WINTER	EUROPEAN(F)
ALBACETE	6	WINTER	EUROPEAN(S)
DACIL	6	WINTER	EUROPEAN(S)
PANE	6	WINTER	EUROPEAN(S)
ROXANA	6	ALTERNATIVE	ICARDA
AD-8	6	WINTER	EUROPEAN(S)

of the ninth (EV1-89-213) or thirteenth (Dobla) day after the beginning of the water stress treatment and measurements were carried on the following day to monitor the recovery.

2.4.3. LEAF GAS EXCHANGE

Gas exchange measurements were made on third leaves using an open gas exchange system (ADC LCA-2, Analytical Development Co. Ltd., Hoddeston, Herts, UK) which has already been described (Winner *et al.* 1989). Three Philips lamps provided a irradiance of 1000 $\mu mol\ m^{-2}\ s^{-1}$. A water-filled cooling circuit placed between the ADC and the lamps controlled the temperature of the leaf chamber analyser. With the Data Processor DL-2 of ADC, net photosynthesis, transpiration rate and leaf temperature was calculated from measurements of CO_2 uptake, % relative humidity, leaf chamber temperature and light photon flux using equations developed by von Caemmerer and Farquhar (1981).

Chlorophyll *a* fluorescence induction: Chlorophyll *a* fluorescence induction-kinetics of intact third leaves dark adapted for 30 min. was measured at room temperature using a PAM 101-102-103 modulated fluorescence system (Heinz Walz, Mess-und Regeltechnik, Effeltrich, Germany), as described in the general introduction (see section 1.2.2.1.). With the PAM, F_O was excited by a weak red light modulated at 1.6 kHz and F_S was induced by a non-modulated white light (1000 $\mu mol\ m^{-2}\ s^{-1}$) supplied by an *Osram Xenoplot HLX* light source.

According to Fischer and Maurer (1978), drought susceptibility index (SI) for each genotype was calculated for each of the parameters measured (qp, P_N and ψ_W) as follows:

$$SI\ (\%)=(Xs/Xc) \times 100$$

where Xs is the mean value of the parameters measured under water stress conditions, and Xc is the mean value of the parameters measured under irrigated conditions.

2.4.4. STATISTICS

The analysis of variance was performed using Statgraphics (v. 2.1). Following the LSD test, the Tukey multiple range test was used.

2.5. RESULTS

2.5.1. SCREENING TEST OF BARLEY GENOTYPES RESPONSE TO WATER STRESS

The genotypes tested show different patterns of modulated chlorophyll fluorescence. A gradual reduction in q_P was observed in plants subjected to water stress, *e.g.* q_P was 0.76± 0.03 and 0.46±0.08 in stressed leaves of the genotypes Plaisant and EV1-89-213 respectively. A range of sensitivity among genotypes from approximately 90% for Plaisant and Dobla to 57% for the EV1-89-213 was observed (Table 2). The differences observed in net photosynthesis between control and stressed plants were higher in the genotypes EV1-89-213, EV1-89-215 and Alexis than in Plaisant and Dobla, since, *e.g.*, P_N was 9.22±2.38 and 0.31± 0.85 in stressed leaves of the genotypes Dobla and Alexis respectively, whereas in control (non-stressed) leaves P_N was about 20 [$\mu mol(CO_2)\ m^{-2}\ s^{-1}$] for both genotypes.

No correlation was observed between the screening by modulated fluorescence or net photosynthesis and the screening by water potential. With data obtained from the drought susceptibility index (SI), a score (S) of drought tolerance was made for each of the parameters (q_P, P_N and ψ_W). Higher S data means more drought tolerance than lower S data. With respect to drought tolerance Alpha, Dobla, Plaisant, Mogador and AD-8 were the most tolerant genotypes to water stress and EV1-89-213, EV1-89-215, Albada, Barbarrosa and Alexis were the most sensitive (Fig. 2).

Further one genotype (Dobla) selected as tolerant, and one genotype (EV1-89-213) selected as sensitive were studied in detail.

2.5.2. EFFECT OF WATER STRESS AND IRRADIANCE ON THE PHOTOCHEMICAL QUENCHING, PHOTOSYNTHETIC GAS EXCHANGE AND LEAF TEMPERATURE

The tolerant genotype (Dobla) and the sensitive (EV1-89-213) showed lower values of qp (or higher values of 1-qp) at increasing irradiance. Since qp is a measure of the proportion of oxidised or 'open' PSII reaction centres, 1-qp is a measure of the proportion of reduced or 'close' PSII reaction centres. Sensitive stressed plants showed a higher response of 1-qp to irradiance than unstressed plants (Fig. 3). These curves of tolerant genotypes were similar irrespective of whether they were grown under unstressed or stressed conditions. Furthermore, the ratio of maximum fluorescence to minimal fluorescence Fm/Fo, representing a measure of the photochemical efficiency of PSII (Krause and Weiss 1984), decreased by water stress in the sensitive genotype (EV1-89-213), whereas no significant differences were found in the tolerant genotype (Dobla) under water stress (Table 3).

Table 3. Changes in Fm/Fo ratio in two barley genotypes: Dobla and EV1-89-213. Statistically significant differences between control and water stress treatments (evaluated by Student *t*-test for 4 replications at P=0.95) are marked by asterisk.

	DOBLA		EV1-89-213	
	Control	Water stress	Control	Water stress
Fm/Fo	1.94	2.03	2.05	1.46*

The response of net photosynthesis to photon flux density under well watered conditions ($\psi_W \approx$ -0,4 MPa) of the genotype Dobla was not different from that of EV1-89-213 (Fig. 4). In addition no difference was observed in the CO_2 uptake rate between control and stressed plants in the tolerant genotype Dobla, while water stressed plants of the sensitive

Table 2. Drought Susceptibility Index (SI) for modulated chlorophyll fluorescence, net photosynthesis and leaf water potential of the twenty genotypes of barley. S = Score of drought tolerance. HG = Homogeneous Groups from Multiple Range Analysis of Tukey's Method (α= 0.05). The genotypes with same letter were not significantly different. Values of SI are average of three observations. Plaisant $q_{P\ control}$ = 0.84±0.01 and $q_{P\ stress}$ = 0.76± 0.03, EV1-89-213 $q_{P\ control}$ = 0.80±0.02 and $q_{P\ stress}$ = 0.46±0.08. Dobla $P_{N\ control}$ = 20.49±2.51 and $P_{N\ stress}$ = 9.22±2.38, Alexis $P_{N\ control}$ = 20.30±0.43 and $P_{N\ stress}$ = 0.31± 0.85 $\mu mol(CO_2)\ m^{-2}\ s^{-1}$. Alpha $\psi_{w\ control}$ = -0.40±0.10 and $\psi_{w\ stress}$ = -0.50±0.20, Barbarrosa $\psi_{w\ control}$ = -0.35±0.05 and $\psi_{w\ stress}$ = -1.30±0.1 MPa.

	MODULATED FLUORESCENCE			NET PHOTOSYNTHESIS			WATER POTENTIAL		
S	GENOTYPES	SI	HG	GENOTYPES	SI	HG	GENOTYPES	SI	HG
20	PLAISANT	90	a	DOBLA	45	d	ALPHA	125	j
19	DOBLA	87	ab	ALPHA	28	de	ID-4016	150	jk
18	ALPHA	87	ab	PLAISANT	25	ef	MOGADOR	175	jkl
17	AD-8	86	ab	KYM	22	efg	DOBLA	200	jklm
16	ALBACETE	84	abc	MOGADOR	22	efg	PLAISANT	200	jklm
15	DACIL	83	abc	AD-8	20	efgh	AD-8	200	jklm
14	MOGADOR	82	abc	ROXANA	15	efghi	EV1-89-213	200	jklm
13	ROXANA	82	abc	BEKA	15	efghi	PANE	225	jklm
12	CAMEO	82	abc	ALBACETE	13	efghi	ROXANA	225	jklm
11	PANE	81	abc	ZAIDA	10	efghi	BEKA	250	jklm
10	EV1-89-215	81	abc	ALBADA	9	fghi	ZAIDA	250	jklm
9	HATIF	81	abc	PANE	8	fghi	CAMEO	275	klm
8	ALEXIS	80	abc	HATIF	8	fghi	HATIF	275	klm
7	BARBARROSA	80	abc	BARBARROSA	5	ghi	ALBACETE	275	klm
6	BEKA	79	abc	DACIL	5	ghi	ALEXIS	275	klm
5	ZAIDA	79	abc	ID-4016	5	ghi	EV1-89-215	275	klm
4	ALBADA	75	abc	EV1-89-213	4	ghi	KYM	275	klm
3	KYM	67	abc	EV1-89-215	3	hi	ALBADA	300	lm
2	ID-4016	62	bc	CAMEO	2	hi	DACIL	300	lm
1	EV1-89-213	57	c	ALEXIS	1	i	BARBARROSA	325	m

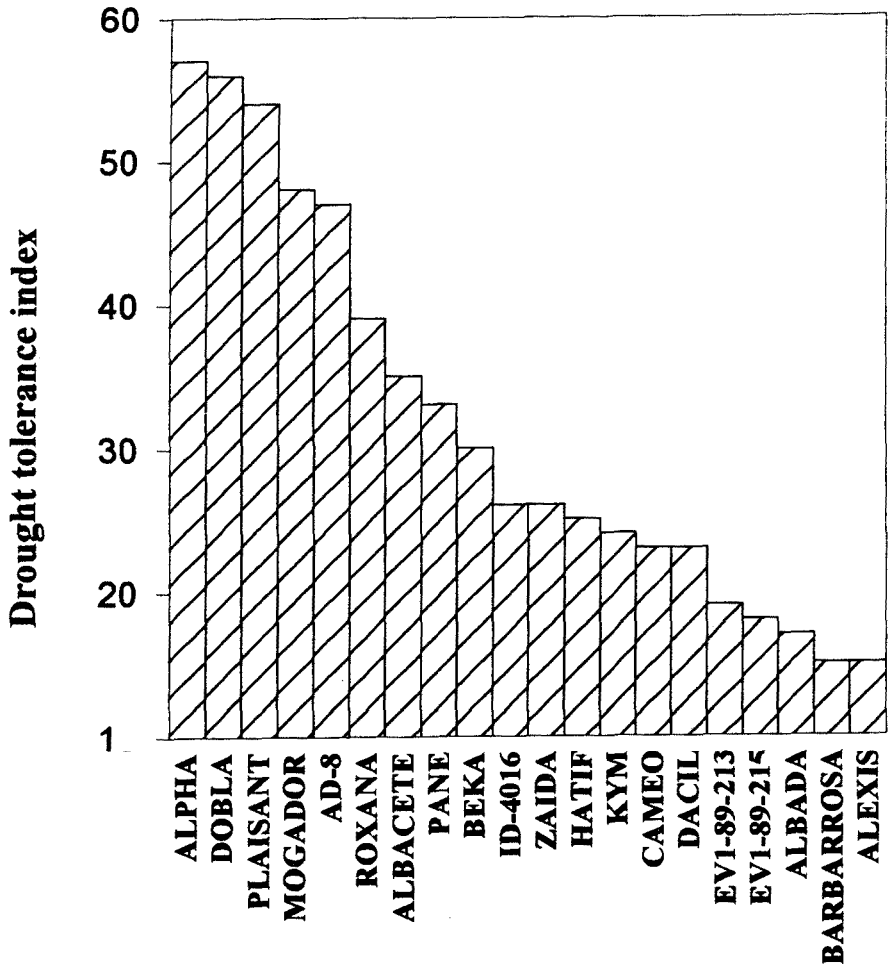


Figure 2. Screening of twenty barley genotypes according to their drought tolerance calculated combining the scores given in Table 2 separately for modulated fluorescence, net photosynthesis and water potential, and then judge the drought tolerance index from the total score.

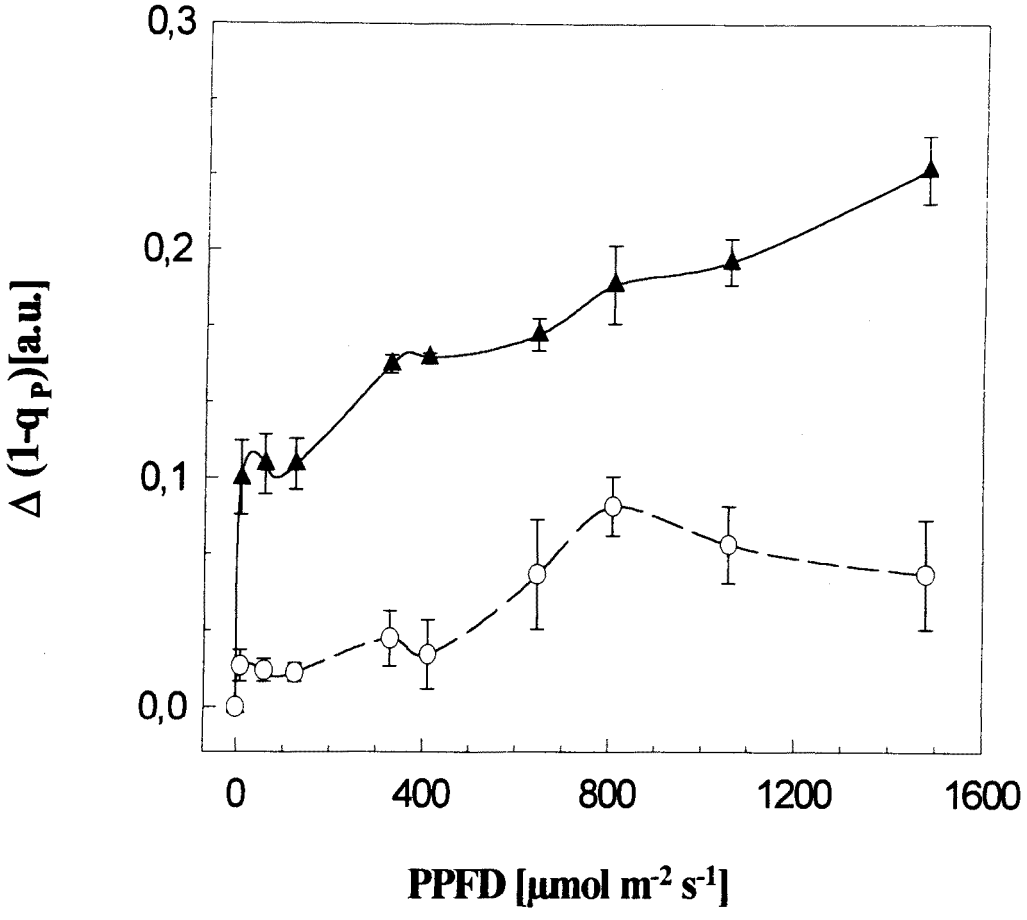


Figure 3. Irradiance response curve for the difference of the photochemical component between control and stressed plants Δ (1-qp) in barley leaves of two genotypes Dobla (circles) and EV1-89-213 (triangles) subjected to water stress. Dobla $\psi_{w\ control}$ = -0.45±0.1 MPa and $\psi_{w\ stress}$ = -1.25±0.2 MPa, EV1-89-213 $\psi_{w\ control}$ = -0.35±0.1 MPa and $\psi_{w\ stress}$ = -1.30±0.1 MPa. Values are means (n=4)±SE.

Figure 4. The response of net photosynthesis (P_N), water use efficiency (WUE) and difference of the leaf temperature between control and stressed plants (ΔT_l) at different leaf ψ_w to photon flux in two barley genotypes: Dobla (circles) and EV1-89-213 (triangles). Open symbols are control. Closed symbols are water stressed plants. The standard errors of the means (n=4) are shown by the vertical bars when larger than the symbols.

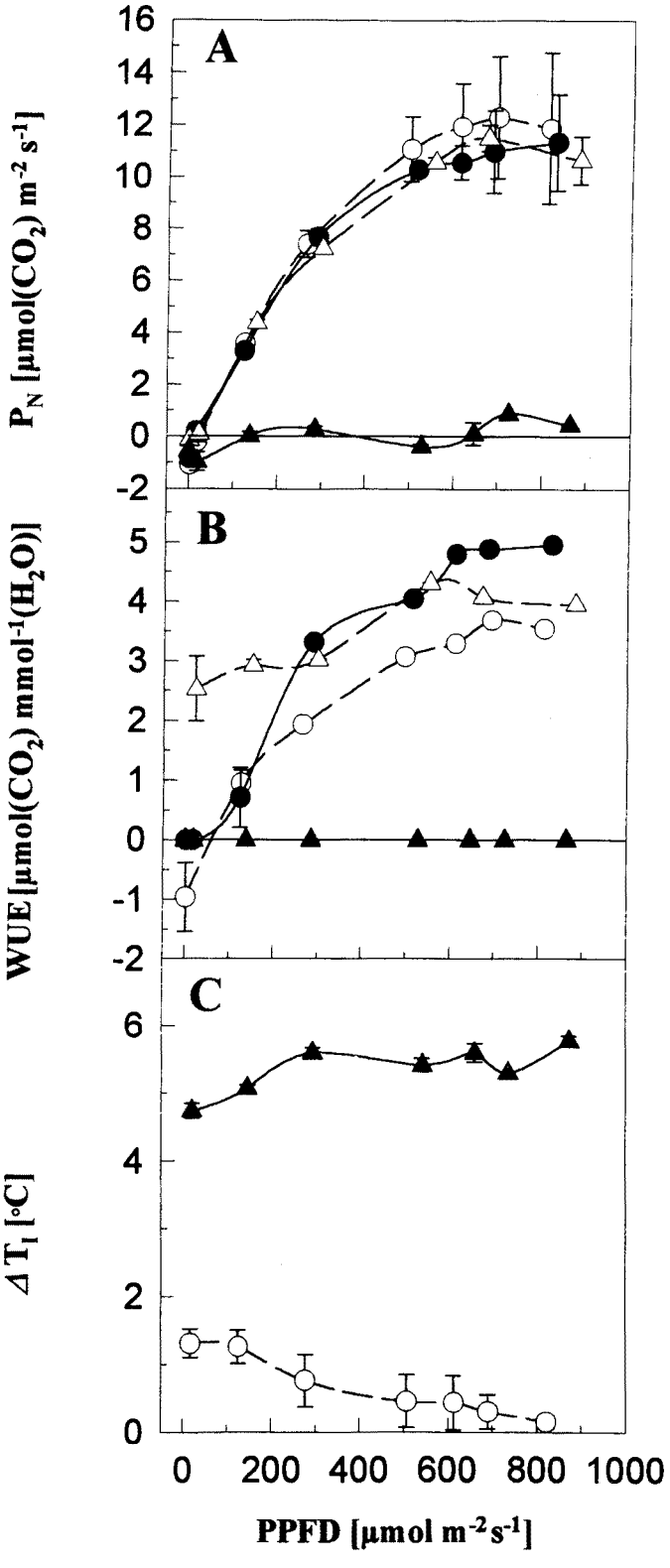

A
B
C
P_N [µmol(CO_2) m^{-2} s^{-1}]
WUE[µmol(CO_2) mmol^{-1}(H_2O)]
ΔT_l [°C]
PPFD [µmol $m^{-2}s^{-1}$]
16
14
12
10
8
6
4
2
0
-2
5
4
3
2
1
0
-1
-2
6
4
2
0
0
200
400
600
800
1000

Figure 5. Changes in the net photosynthesis (P_N), water use efficiency (WUE) and difference of the leaf temperature between control and stressed plants (ΔT_l) as a function of time in two barley genotypes: Dobla (circles) and EV1-89-213 (triangles). Open symbols are control. Closed symbols are water stressed plants. Stressed plants were re-watered (arrows) after 13 (Dobla) and 9 (EV1-89-213) days of started water stress. The standard errors of the means (n=4) are shown by the vertical bars when larger than the symbols.

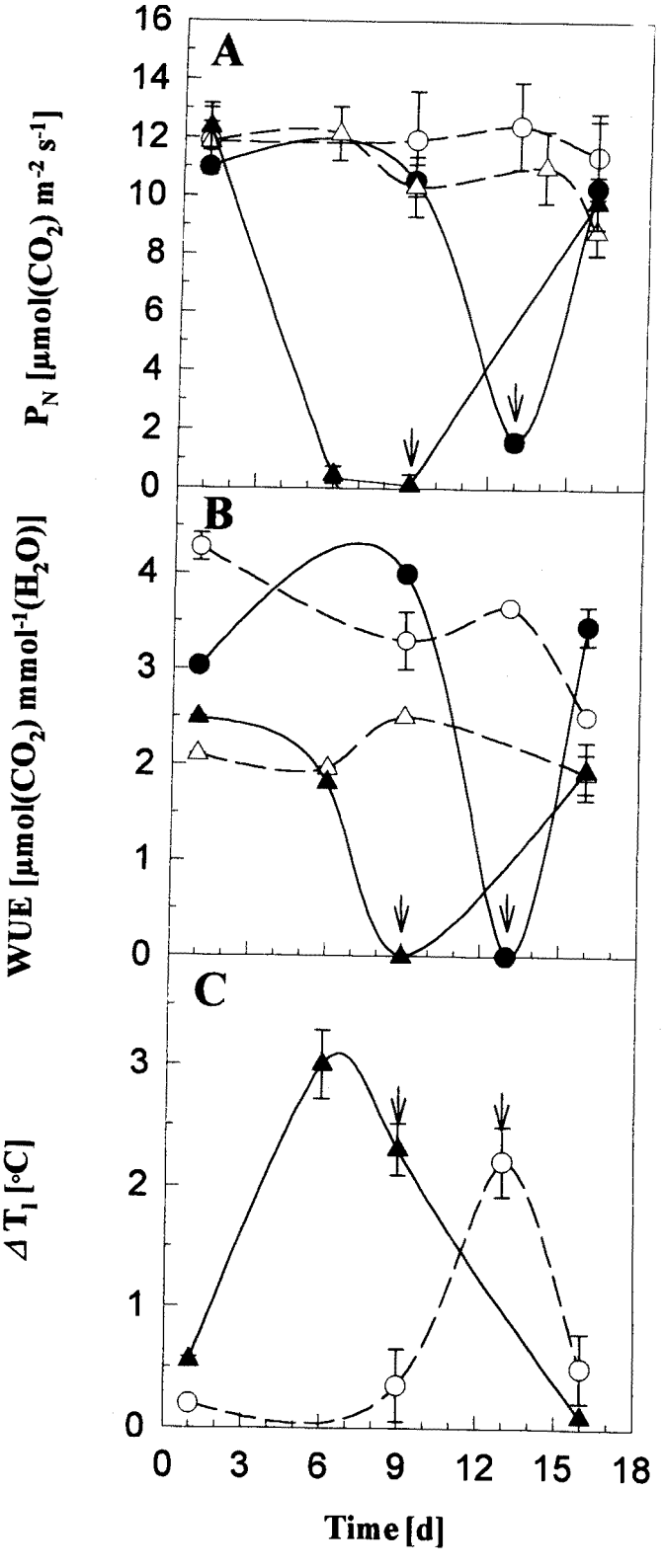
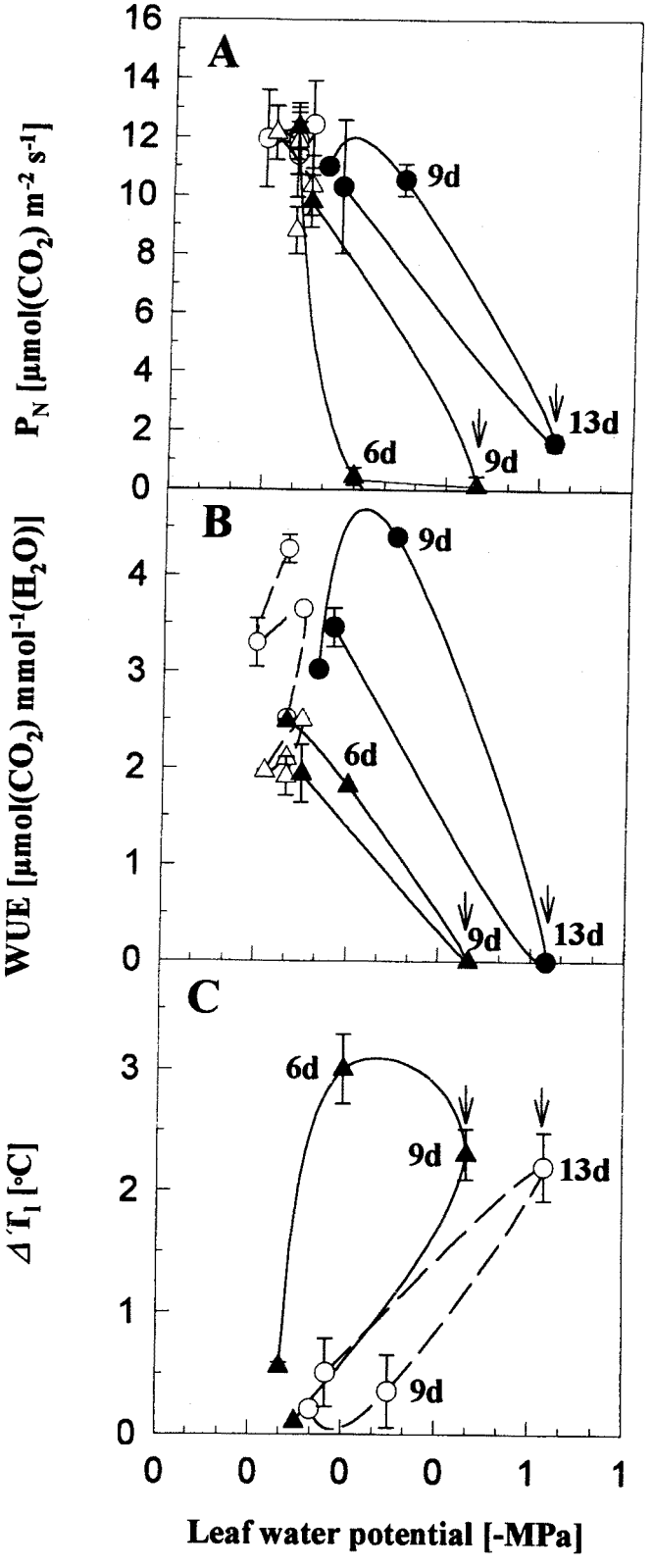

A
B
C
P_N [µmol(CO_2) m^{-2} s^{-1}]
WUE [µmol(CO_2) mmol^{-1}(H_2O)]
ΔT_l [°C]
Time [d]
0
3
6
9
12
15
18

Figure 6. The response of net photosynthesis (P_N), water use efficiency (WUE) and difference of the leaf temperature between control and stress plants (ΔT_l) to decreasing leaf water potential in two barley genotypes: Dobla (circles) and EV1-89-213 (triangles). Open symbols are control. Closed symbols are water stressed plants. Stressed plants were re-watered (arrows) after 13 (Dobla) and 9 (EV1-89-213) days of started water stress. The standard errors of the means (n=4) are shown by the vertical bars when larger than the symbols.


A
B
C
9d
6d
13d
P_N [µmol(CO_2) m^{-2} s^{-1}]
WUE [µmol(CO_2) mmol^{-1}(H_2O)]
ΔT_l [°C]
Leaf water potential [-MPa]

genotype EV1-89-213 showed a CO_2 uptake around the light compensation point. Water use efficiency (WUE), the ratio between CO_2 assimilation and transpiration (Jones, 1993), and the difference in leaf temperature between control and stressed plants were also analysed (Fig. 4). The sensitive genotype showed a much lower WUE and higher leaf temperature difference between control and stressed plants than the resistant genotype, which showed higher WUE in stressed than in unstressed plants.

The response of these variables to progressive water stress was also studied (Fig. 5). While in sensitive genotypes clear differences are observed in the values of net photosynthesis at the sixth day of water stress treatment respect to control plants and in WUE at the ninth day, the decrease is not observed in tolerant plants until thirteen days of water withholding.

No difference in the rate of CO_2 assimilation, WUE and leaf temperature as a function of ψ_W was apparent between the two genotypes in well-watered conditions (Fig. 6). In both genotypes, the P_N decreased linearly with decreasing ψ_W. However, water stress led to a much faster decrease in the CO_2 uptake rate and WUE in the sensitive genotype. Leaf temperature difference (between control and stress plants) was higher in the sensitive that in the tolerant genotype. Figures 5 and 6 also show a complete recovery of photosynthetic parameters after a moderate water stress treatment.

2.6. DISCUSSION

All the parameters used in this work (q_P, P_N and ψ_W) were influenced by water stress and could be used as rapid screening tests for drought tolerance in barley (Table 2). The homogeneity of these screenings for each parameter, as shown by Tukey's method, suggests that the techniques of modulated chlorophyll fluorescence, net photosynthesis and leaf water potential are more appropriate as indicators for severe water stress rather than mild water stress. It is thus difficult to choose only one technique for screening genotypes according to their tolerance to drought and it is much better to use several parameters simultaneously.

Havaux *et al.* (1988b) showed that leaf desiccation caused drastic changes in the different modulated fluorescence levels, resulting in a strong reduction in q_P, and the

percentage decrease in this value could be used as an index of the level of tolerance. This is further confirmed by the present study. The rapidity and sensitivity of this chlorophyll fluorescence method *in vivo* allowed us to compare twenty genotypes of barley. This comparative study gave a measure of the range of variability for drought tolerance in cereals. Drought caused a decrease in q_P (or an increase in 1-q_P) in all the genotypes tested, but the percentage of inhibition was highly dependent on the degree of drought tolerance of the genotype (Table 2). This reduction of q_P occurs because water stress restricts the availability of the sinks for the products of electron transport, *i.e.* NADPH and ATP (see section 2.2.3.). For similar values of $\psi_W \approx -1.2$ MPa, in drought-tolerant genotypes, the relative decrease in q_P, was 87% (for Dobla), whereas in drought sensitive genotypes, q_P was 57% (for EV1-89-213). Between the two extremes there was a gradation of tolerance to drought and it is difficult to establish the limits between tolerant, moderate or sensitive. The value of q_P can then be used as a rapid indicator of the degree of drought tolerance in barley genotypes, however although q_P reduction is a good method by which to distinguish between tolerant and sensitive genotypes, it fails for moderate genotypes.

The difference 1-q_P between control and stressed plants was also much greater in the sensitive than in tolerant genotypes (Fig. 3). These results are in agreement with those obtained by Jovanovic *et al.* (1990), who show, in different maize lines, that under given drought conditions the fluorescence may be a sensitive parameter of water stress and thus be used successfully for the determination of plant drought susceptibility. The increase in 1-q_P difference between control and stressed plants at high irradiance could be related with processes of photoinhibition.

Water stress led to a decrease in net photosynthesis. In all genotypes investigated, net photosynthesis declined when ψ_W decreased. In sensitive non-watered plants, the decline was larger and occurred earlier. At $\psi_W = -0.75$ MPa the P_N of the sensitive genotype (0.45 μmol m^{-2} s^{-1}) was significantly lower than P_N of the tolerant genotype (10.54 μmol m^{-2} s^{-1}). No permanent damage occurred to photosynthesis at $\psi_W = -1.2$ MPa. After rewatering, photosynthesis recovered by about 90% and the same pattern was observed in WUE and leaf temperature. Water stress has been shown to influence net photosynthesis in many ways

(Schulze 1986b, Turner 1986, Nilsen 1992). In early stages of water stress, net photosynthesis is inhibited by stomatal closure. Studies of the decrease in fluorescence *in vivo* during the onset of drought and as a function of light intensity provided information on the apparent mesophyll limitation of net CO_2 assimilation.

Under control conditions (without water stress), fully expanded leaves of both tolerant and sensitive genotypes had similar rates of net photosynthesis at saturating irradiance (Fig. 4A). Under water stress, P_N of tolerant genotypes was much greater than that of sensitive genotypes, although both genotypes showed quite similar ψ_W (about -1.0 MPa). There is much greater difference in the values of WUE in the sensitive genotypes than in the tolerant ones. Stressed plants show higher leaf temperatures than irrigated plants, the differences between control and stressed plants were higher in sensitive genotypes than in tolerant ones. The present study shows, in accordance with Sharp and Boyer (1986), that inhibition occurs even when leaves are exposed to low photosynthetic photon flux density (PPFD) during dehydration (Fig. 5A).

This study provide further support for the proposal that screening of cereal genotypes for tolerance to water stress may be achieved by combining gas exchange analysis with fluorescence quenching measurements.

2.7. CONCLUSIONS

Modulated chlorophyll fluorescence could be used as rapid screening tests for drought tolerance in barley, although applied alone, it is more appropriate as indicator for severe water stress rather than mild stress.

Screening of cereal genotypes for tolerance to any water stress treatment could be achieved by combining fluorescence quenching measurements with gas exchange analysis.

Drought tolerant genotypes show smaller variations of photochemical quenching, net photosynthesis, water use efficiency and leaf temperature than drought sensitive ones.

Tolerant barley genotypes can be distinguished from sensitive types by their ability to maintain comparable photosynthetic efficiency and light-saturated rates of CO_2 exchange under water stress conditions.

EFFECTS OF UV-B RADIATION ON PHOTOSYNTHETIC PERFORMANCE UNDER CONTROLLED ENVIRONMENT CONDITIONS USING MODULATED CHLOROPHYLL FLUORESCENCE

CHAPTER 3:

EFFECTS OF UV-B RADIATION ON PHOTOSYNTHETIC PERFORMANCE UNDER CONTROLLED ENVIRONMENT CONDITIONS USING MODULATED CHLOROPHYLL FLUORESCENCE

3.1. ABSTRACT

Mature pea leaves were exposed to two levels of UV-B radiation, with and without supplementary UV-C radiation, during 15 hours photoperiods. Simultaneous measurements of CO_2 assimilation and modulated chlorophyll fluorescence parameters demonstrated that irradiation with low level of UV-B resulted in decreases in CO_2 assimilation that are not accompanied by decreases in the maximum quantum efficiency of PSII primary photochemistry. Increased exposure to UV-B resulted in a further loss of CO_2 assimilation and decreases in the maximum quantum efficiency of PSII primary photochemistry, demonstrating that photodamage to PSII reactions centres had occurred. Addition of UV-C to UV-B treatments increased markedly the rate of inhibition of photosynthesis, however the relationships between CO_2 assimilation and PSII characteristics remained the same, indicating that UV-B and UV-C inhibit leaf photosynthesis by a similar mechanism. Stomatal conductance does not change significantly during UV-B irradiation and cannot account for the decreases in CO_2 assimilation. Further, absorbance, reflectance and transmittance of mature pea leaves do not change significantly during UV-B irradiation.

No significant decreases in the atrazine-binding capacity of thylakoids isolated from leaves were found throughout the low UV-B treatment. Increased exposure to UV-B radiation resulted in a loss in the capacity of thylakoids to bind atrazine, also indicating that photodamage to PSII reaction centres had occurred. Complete recovery of the PSII complexes from this photodamage was found to occur after removal of the UV-B radiation. Supplementing the UV-B treatments with UV-C caused large decreases in the atrazine-binding capacity of thylakoids.

It is concluded that although UV-B irradiation of pea leaves will result in photodamage to PSII reaction centres, this is not the primary cause of the inhibition of CO_2 assimilation induced by UV-B radiation. Such photodamage will only occur at UV-B levels considerably in excess of those currently experienced in the field. The initial decreases in the ability of leaves to assimilate CO_2 on exposure to UV-B are not accompanied by any decreases in the maximum quantum yield of PSII primary photochemistry or the ability of PSII to bind quinones. Consequently, factors other than damage to the PSII reaction centre must determine the loss of ability to assimilate CO_2 during the early stages of irradiation with UV-B. With

increasing exposure to UV-B, when the rate of CO_2 assimilation falls bellow 6 μmol m^{-2} s^{-1}, a slowly reversible quenching of excitation energy in the PSII antenna occurs, which is consistent with a light-induced zeaxanthin quenching and results in a decreased quantum efficiency of PSII photochemistry. However, this quenching is almost certainly an indirect effect of UV-B inhibition of carbon assimilation mediated through a feedback inhibition. An increase of the UV-B irradiation dosages will eventually result in photodamage to the PSII reaction centres.

In this study, modulated chlorophyll fluorescence was an useful and rapid probe of UV-B radiation effects on the photosynthetic performance in pea leaves grown under controlled environment conditions.

3.2. INTRODUCTION

There is a fast growing and an extremely serious international concern regarding man's influence on the global climate. The issues of concern are the depletion of stratospheric ozone and a consequent increase in tropospheric UV-B, the increase in the ground level emissions of 'greenhouse gases' and the global warning, and which have been predicted to drastic alter in terrestrial and aquatic ecosystems (Krupa and Kickert, 1989).

Stratospheric ozone limits penetration of harmful ultraviolet radiation to the earth's surface, however, over the last decades, significant decreases in stratospheric ozone concentrations have been reported above the Antarctic. This ozone layer is being depleted by the rise in atmospheric chemical pollutants, and in particular chlorofluorocarbons (Farman *et al.*, 1985; Rowland, 1989; Frederick, 1990). This has resulted in an increase in the amount of ultraviolet radiation, primarily in the UV-B region of the spectrum, and further to the UV-C region, reaching the earth surface which is potentially damaging to biological systems (Coohill, 1991). This depletion is expected to continue for another 20-30 years (Madronich *et al.*, 1994) and may therefore result in reductions to present day levels of *ca.* 15% (NAS, 1979). In the Northern hemisphere the observed decrease in stratospheric ozone is thought to have been about 4-5% per decade since the early 1980s (Stolarski *et al.*, 1992; Herman *et al.*, 1993).

Because the problem of ozone depletion has now become acute, it is necessary to begin a commitment to understanding the basic biology and biochemistry of how increased

levels of UV-B will impact on critical biological processes needed to sustain life on this planet. Perhaps more importantly, it is necessary to understand how these changes will impact ultimately on populations and ecosystems. It is already well-know that UV-B can impact on the DNA and on proteins which regulate biological reactions, repair and replace damaged cells and maintain cellular integrity. Alterations to these substances need to be clearly understood in the context of whole organisms and ecosystems (SCOPE, 1992).

3.2.1. THE OZONE LAYER

Ozone (O_3) concentrations vary with altitude above the earth's surface. The vertical column of ozone is distributed roughly as follows: 0-10 km, 10%; 10-35 km, 80 %; and above 35 km, 10% (Cicerone, 1987). Ozone concentrations in the troposphere also vary with the latitude (Pruchniewicz, 1973). Variations in total column ozone occur on several time scales, the most obvious being the annual cycle. Atmospheric winds transport ozone from low to middle and high latitudes as well as downward in the altitude region below 30 km during winter (London *et al.*, 1977).

In the stratosphere (10-50 km), a series of photochemical reactions involving O_3 and molecular oxygen, O_2, occur. Ozone strongly absorbs solar radiation in the region from $\approx$ 210 to 290 nm, whereas O_2 adsorbs radiation at < 200 nm. The absorption of light primarily by O_3 is a major factor causing the increase in temperature with altitude in the stratosphere. Excited O_2 and O photodissociate, initiating a series of reactions in which O_3 is both formed and destroyed leading to a steady state concentrations of O_3 (Finlayson-Pitts and Pitts, 1986). This O_3 serves as a shield against biologically harmful solar UV radiation, initiates key stratospheric chemical reactions and transforms solar radiation into heat and the mechanical energy of atmospheric winds (Krupa and Kickert, 1989).

The flux of photochemically-active UV-B photons into the troposphere is limited by the amount of stratospheric O_3 (Cicerone, 1987). In addition to this protective effect of stratospheric ozone against UV, clouds reflect a large part of the incoming solar radiation,

causing the albedo of the entire earth to be about twice what it would be in the absence of clouds (Ramanathan *et al.*, 1989).

3.2.2. OZONE DESTRUCTION IN THE STRATOSPHERE

The first concern is that human activities might interfere with the natural atmospheric ozone equilibrium and arose in the early 1970's. These were focused on the injection of nitrogen oxides into the stratosphere by high altitude aircraft (Johnston, 1971). These concerns were founded largely on atmospheric models that were designed to simulate the chemistry and transport dynamics of the atmosphere. As the models were subsequently refined and better measurements of various rate coefficients were made, the importance of nitrogen oxides as catalysts of ozone reduction was considerably diminished and the importance of halogens (as ozone catalysts) was recognised. Molina and Rowland (1974) proposed that stratospheric photodissociation of chlorofluoromethanes might constitute a significant source of chlorine which could act against atmospheric ozone.

The CFCs and HCFCs contribute to stratospheric ozone dilution by its conversion to oxygen. It has been calculated that more than 80% of the current chlorine loading of the stratosphere stems from anthropogenic CFCs (Chipperfield, 1993). These CFCs are released into atmosphere as a result of their use as aerosol sprays propellants, polymeric foam blowing agents in industry, and coolants in refrigerators and in air conditioners. They are also used in fire extinguishers and in industrial cleaning applications, especially in the electronics industry (Sauders, 1992).

It has been calculated that a single Cl atom released from CFCs or HCFCs can produce approximately 10^5 O_2 molecules from ozone before it is permanently removed from the atmosphere (Molina and Rowland, 1974). If present global release rates of CFCs are maintained, an ozone reduction of 16% will eventually result. The dynamics of the stratosphere are so slow that a century will be required for an equilibrium ozone reduction to be achieved. If release rates increase 7% per year from 1980 to 2000 and remain constant thereafter, an ozone reduction at equilibrium in excess of 30% is predicted (Caldwell, 1981).

The nitrogen oxides produced by high-flying supersonic aircraft also affect the natural balance between ozone and molecular oxygen (McKersie and Leshem, 1994).

3.2.3. THE ANTARCTIC 'OZONE HOLE'

A rapid decrease of the total column amount of ozone in late winter and early spring over the Halley Bay station in Antarctica (76°S, 27°W) has been reported by Farman *et al.* (1985). They attributed the decrease to the increase in stratospheric chlorine due to chlorofluorocarbon release, and proposed that the unique conditions of extreme cold and low sunlight in the Antarctic winter and spring enhanced the effect.

Stolarski *et al.* (1986) have provided global measurements of ozone from 1978 by the Solar Backscatter Ultraviolet instrument and the Total Ozone Mapping Spectrometer aboard the Nimbus 7 satellite which confirm the reported decline of total ozone and show the phenomenon to be regional in extent. The decrease occurs during September as the Sun rises, reaching a minimum in mid-October.

The observed ozone content was about 50% lower in the Antarctic spring seasons (October) of 1990-93 than in the springs of 1957-73 (Fig. 7).

According to McElroy and Salawitch (1989), the annual averaged column density of ozone declined between 1.7 and 3.0% in the latitude band 30° to 64° N between 1969 and 1986.

Recently, decreased levels of stratospheric ozone over heavily populated areas of the Northern hemisphere have been found (Stone, 1993; Kerr, 1993). In the Northern hemisphere the observed decrease in stratospheric ozone is thought to have been about 4-5% per decade since the early 1980s (Stolarski *et al.*, 1992; Herman *et al.*, 1993). This annual mean disguises considerable seasonal variation in depletion: rates of loss are greatest in late winter and spring and least in late summer and autumn (Herman *et al.*, 1993; Hughes *et al.*, 1994).

3.2.4. THE UV-B ACTION SPECTRA

Reduced amounts of atmospheric ozone will permit large amounts of UV-B radiation to penetrate through the atmosphere, for example, with overhead sun and typical ozone amounts, a 10% decrease in ozone was predicted to result in a 20% increase in UV-B penetration at 305 nm (Cutchis, 1974). Stratospheric ozone depletion increases UV-B intensity and shifts UV-B radiation to shorter wavelengths than normally found in global radiation (Tevini *et al.*, 1988). Thereby, at 290 nm and below (UV-C) all incoming UV radiation is virtually absorbed by ozone. At longer UV-wavelengths, the absorption of ozone falls off rapidly so that it is nearly 50% at 310 nm (UV-B) and close to zero at 330 nm (UV-A). As a result, the UV cut-off of ozone for solar radiation is at approx. 290 nm. A decrease in the stratospheric ozone content would shift this cut-off and allow shorter wavelengths of UV radiation to reach the earth (Krizek, 1981).

Because different biological processes exhibit different degrees of sensitivity to different wavelengths of UV-B, an *action spectra* must be used as a weighting factor to adjust the measured UV-B flux (Gerstl *et al.*, 1981; Rundel, 1983). Action spectra of UV damage to plants must be used as weighting functions to evaluate the relative increase of solar UV radiation that would result from a decreased atmospheric ozone layer, evaluate the existing natural gradients of solar UV irradiance on the earth, and compare UV radiation from lamp systems in experiments with solar UV radiation in nature (Caldwell *et al.*, 1986). In this study, the generalised plant action spectrum (Caldwell, 1971) was used as weighting function.

According to Frederick *et al.* (1989), the biologically effective UV-B irradiance at the earth's surface varies with the elevation of the sun, the amount of atmospheric ozone, and with the abundance of atmospheric matter generated by natural and anthropogenic processes, that have scattering and adsorbing properties. Also, cloud cover, elevation above sea level and ground reflectance greatly influence solar UV irradiance (Caldwell, 1981).

Based on Björn and Murphy's modified radiation transfer model (Björn and Murphy, 1985), the current plant weighted daily UV-B dosage for midsummer in the UK is about 4.4 KJ m^{-2} d^{-1}. According to Madronich (1993), the current maximum daily biologically effective dose is just over 5 KJ m^{-2} d^{-1}.

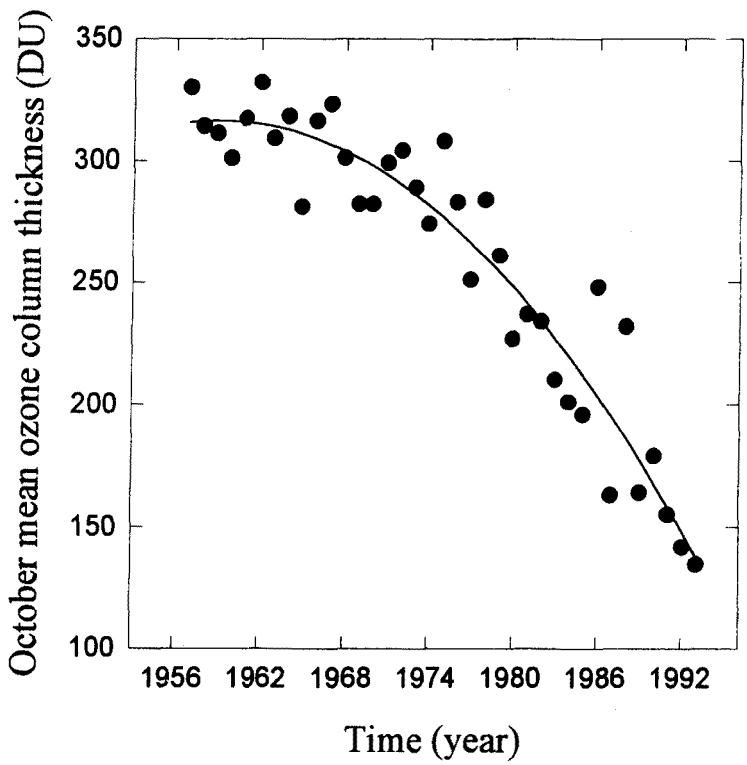


Figure 7. Monthly means of total ozone in October at Halley Bay station in Antarctica from 1957 to 1993 (Farman *et al.*, 1985; WMO, 1991; Gleason *et al.*, 1993; Walker, 1993).

3.2.5. EFFECTS OF UV-B ON BIOLOGICAL SYSTEMS

Many studies have shown that UV radiation penetrates to ecologically significant depths into natural waters (Jerlov, 1950; Smith and Baker, 1979). Pure water is quite transparent to UV, but scattering and adsorption by dissolved organic matter and other material in natural waters can alter the penetrance of solar UV considerably (Caldwell, 1981).

The negative effect on an ozone-dependent increase in UV-B radiation on primary production in an Antarctic natural marine community has been demonstrated by Smith *et al.* (1992). In addition, laboratory studies demonstrate that exposure to UV-B radiation decreases algal productivity and causes damage to various forms of aquatic organisms (Worrest and Häder, 1989; El-Sayed *et al.*, 1990).

There is convincing evidence that UV-B radiation, at levels currently incident at the surface of the ocean, may have an influence on phytoplankton productivity (Smith, 1989). Direct influence of UV-B on protein content, dry weight and pigment concentration of phytoplankton have also been observed by Dohler (1984, 1985). Also, long-term (24-48 h) exposure of phytoplankton to UV radiation under ambient visible light conditions produced drastic changes in phytoplankton pigmentation (Bidigare, 1989).

Increased UV-B causes irreversible damage and/or death to various zooplankton organisms. Research shows that enhanced UV-B can increase the mortality of some organisms and decrease the fecundity of survivors. Further, the drifting eggs and larvae of many species of marine fish may be sensitive to enhanced levels of UV-B because they occur at or near the sea surface (Smith, 1989). Chapman and Hardy (1988) have reviewed the potential impacts of enhanced UV-B on marine fisheries. They point out that fisheries will most likely be directly influenced by damage to near surface fish eggs and larvae and possibly indirectly by effects on the food chain on which the larvae depend.

Over the longer term, ecosystem productivity is strongly affected by nutrient cycling dynamics. Increasing levels of UV-B radiation may affect long-term nutrient cycles by increasing turnover of compounds otherwise resistant to biological decay (*e.g.* lignins, humic substances) (Pauli, 1964; Moorhead and Reynolds, 1989; Kieber *et al.*, 1989; Mopper *et al.*, 1991).

Enhanced solar ultraviolet radiation affects biochemical cycles in three major ways:

(i) Biological processes are directly affected through increased damage to DNA and other important molecules such as chlorophyll. Resulting effects include changes in photosynthesis and respiration by terrestrial plants (Tevini and Teramura, 1989) and marine phytoplankton alterations in microbial respiration, nitrification, sulphur cycling and metal redox

reactions (SCOPE, 1992). These effects are likely to be linked to other global changes such as warning and the CO_2 build-up (Tevini and Teramura, 1989).

(ii) Geochemical processes in the sea and on land are strongly influenced by enhanced ultraviolet radiation. Such processes at the land surface and in the sea include formation of greenhouse and chemically important trace gases (*e.g.* CO_X, NO, NMHCs) (SCOPE, 1992); conversion of organically bound nitrogen and phosphorus to biologically available inorganic N and P; conversion of refractory organic matter to lower molecular weight, biologically available organic compounds (Kieber *et al.*, 1989); deeper penetration of solar UV radiation into the sea (Kouassi *et al.*, 1990; Zepp, 1988); and changes in the redox state of the upper ocean (Zepp, 1991).

(iii) Chemical processes in the atmosphere are modified with resulting changes in the oxidising capacity of the troposphere; the build-up of greenhouse gases such as methane and ozone; aerosol concentrations and cloud condensation nuclei; and concentrations of ozone-depleting gases, including various halogenated compounds (SCOPE, 1992). These effects are likely to interact strongly with other global phenomena such as the release of chemical active gases by biomass burning and other human activities (Andreae, 1991).

3.2.6. EFFECTS OF UV-B ON TERRESTRIAL PLANTS

The biological effects and consequences of enhanced UV-B radiation for terrestrial plants under a variety of growing conditions are numerous but nevertheless still insufficiently understood. Several laboratory and glasshouse based experiments have demonstrated that UV-B radiation can induce a wide range of responses in plants, including increased concentrations of protective UV-B absorbing pigments in leaves, reductions in the rates of CO_2 assimilation and plant growth, and changes in chlorophyll fluorescence (Tevini *et al.*, 1981; Strid *et al.*, 1990; Teramura *et al.*, 1991; Strid and Porra, 1992; Jordan *et al.*, 1994).

Table 4 lists the various effects on plants of enhanced UV-B radiation due to stratospheric ozone depletion. Photosynthesis was found to be sensitive to increased UV-B radiation in many studies. Leaf conductance was found to be not affected in a number of studies. Many studies have demonstrated a reduction in leaf area in plants growth under

enhanced UV-B. Studies of effects on dry matter production and crop yield have shown a decrease in a number of plants (Krupa and Kickert, 1989).

Table 4. Overview of the effects of enhanced UV-B radiation on plants (partially from Krupa and Kickert, 1989).

Plant parameter	Plant response
Photosynthesis	Decreases in many C3 and C4 plants
Leaf conductance	Not affected in many plants
Rubisco	Decreases in most plants
Water use efficiency	Decreases in most plants
Leaf area	Decreases in many plants
Specific leaf weight	Increases in many plants
Crop maturation rate	Not affected in most plants
Flowering	Inhibits or stimulates in some plants
Dry matter production and yield	Decreases in many plants

Growth characteristics such as plant height and leaf area are reduced in UV-B sensitive plants to various extents, depending on plant species and cultivar (Lydon *et al.*, 1986; Murali and Teramura, 1986). Both artificially and naturally supplied levels of UV-B radiation decreased stem length and leaf area in cucumber seedlings (Teramura and Sullivan, 1987).

Growth studies with soybean [*Glycine max* (L.) cv. Essex] in a greenhouse revealed that UV-B effects (simulating a 25% ozone depletion) varied with plant growth stage. The most UV-effective period occurred during the transition time between vegetative and reproductive stages. Even intermediate levels of UV-B radiation (simulating a 16% ozone

depletion) reduced plant height, leaf area and total dry weight at the end of the vegetative and reproductive phases (Teramura and Sullivan, 1987).

Differences in total biomass accumulation were also found in five cucumber cultivars in greenhouse studies. The reason for this diversity might be differential accumulation of UV-screening pigments or morphological alterations (Murali and Teramura, 1986). Many UV-B irradiated plants respond to reduced leaf area with increased leaf thickness as shown in an anatomical study of cucumber cotyledons (Tevini *et al.*, 1983) and soybean leaves (Mirecki and Teramura, 1984).

Caldwell (1981) emphasises that the disproportionate growth of plant organs, the depressed flower development, the loss of apical dominance, the abscission of leaves and the altered mineral nutrient concentrations, which were observed after UV-B treatments, still remain to be demonstrated as occurring in nature outside laboratories and growth chamber conditions.

The molecular reasons for growth reductions can be attributed to changes in DNA and/or phytohormones. Phytohormones can affect growth by altering their concentration in the growth sensitivite tissue and by changing photohormone dependent processes (Tevini, 1994).

UV-B induced growth reductions are associated with changes in cell division and/or cell elongation. A clear interaction with the growth regulator IAA was demonstrated in hypocotyls of sunflower seedlings. IAA adsorbs in the UV-B waveband and can be converted *in vitro* and *in vivo* to various photooxidation products. One of these photoproducts, 3-methylene-oxindole, inhibits hypocotyl growth when applied exogenously (Tevini and Teramura, 1989). Furthermore, the plastic epidermal cell wall extensibility, which is auxin-induced elongation growth, was also reduced. Another phytohormone, ethylene, which changes elogantion to radial growth, is produced to a greater extent in UV-B irradiated sunflower seedlings. In UV-B exposed cucumber and bean seedlings growth could be stimulated by gibberellins (Tevini, 1994).

Despite considerable research during the past two decades the UV-adsorbing photosensory system have remained elusive. There has been no definitive isolation and characterisation of a higher plant blue photoreceptor (Short and Briggs, 1994). Genes for the four major flavonoid biosynthesis enzymes [PAL, CHS, chalcone isomerase (CHL), and dihydroflavonol reductase (DFR)] in red-grown *Arabidopsis thaliana* seedlings shown

induction by both UV-B and blue acting through specific photoreceptors separate from phytochrome. This result is consistent with previous findings that CHS gene expression is co-ordinately regulated by distinct receptors of UV-B, blue and red in parsley cell cultures (Short and Briggs, 1994).

Pollen enclosed in the anthers is well-protected against UV-B radiation since the anther walls filter out over 98% of incident UV-B (Flint and Caldwell, 1983). The pollen wall itself contains UV-adsorbing compounds and is therefore also well-protected during pollination. However, after transfer to the stigma the pollen tube may be susceptible especially for binucleate types with longer time courses for germination and penetration than trinucleate types (Flint and Caldwell, 1986). Even low irradiance of 50-70 mW m^{-2} UV-B inhibited germination of two species by 65% within 1 hour and that other four species by 33% within 3 hours (Campbell *et al.*, 1975). It was further shown in three of four species, that the UV-B irradiation of high elevations or low latitudes are effective in partially inhibiting germination *in vitro* (Flint and Caldwell, 1984). Ovules are well hidden in the ovaries and may therefore be sufficiently protected against solar UV-B radiation (Tevini and Teramura, 1989).

Generally flowering was increased when UV-B was excluded by Mylar plastic films or glass. Even 100 mW m^{-2} UV-B led to a 20% decrease in flowering compared to plants irradiated only with white light, whereas 300 mW m^{-2} produced a 50% reduction in flowering. This inhibition is correlated with the suppression of gibberellic acid content (Tevini and Teramura, 1989). In addition, a clear UV-B fluency rate and fluency dependent inhibition of photoperiodic flower induction was observed in the long day plant *Hyoscyamus niger* (Rau *et al.*, 1988).

3.2.6.1. EFFECTS OF UV-B ON PHOTOSYNTHESIS

Photosynthesis and photosynthetic productivity in many plant species can be inhibited by increased exposure to UV-B radiation (Caldwell *et al.*, 1989; Tevini and Teramura, 1989; Teramura, 1990). To date ,however, there is no consensus for the mechanistic basis of UV-B-induced inhibition of CO_2 assimilation in mature leaves. Photosynthesis of phytoplankton is

also especially liable to photoinhibition by UV-B radiation (Caldwell, 1981). Damaging effects of UV radiation, mainly UV-C, on photosynthesis of algae and isolated chloroplasts are also well documented (Renger *et al.*, 1986).

Jones and Kok (1966a,b) suggested that UV photoinhibition is independent of oxygen, which clearly distinguishes it from photobleaching by intense visible radiation of chlorophyll which is quite oxygen-dependent.

Numerous studies have shown that PSII is affected by increased exposure to UV-B (Noorudeen and Kulandaivelu, 1982; Iwanzik *et al.*, 1983; Renger *et al.*, 1989; Strid *et al.*, 1990; Melis *et al.*, 1992) and have implicated PSII damage as a potential limitation to photosynthesis in UV-B treated leaves (Teramura and Ziska, 1995), as is the case in the photoinhibition of photosynthesis by photosynthetically-active radiation (Baker and Bowyer, 1994). Damage is assessed, for example, by measuring the increase in variable chlorophyll fluorescence; an increase in fluorescence can be observed after doses of UV-B radiation in the physiological relevant range (Tevini *et al.*, 1991).

From previous experiments it has been assumed that UV-C acts primarily on the water-splitting system or on the oxidising site of PSII (Kulandaivelu and Noorudeen, 1983; Renger *et al.*, 1989). However, UV-B acts on either the reaction centre itself, producing dissipative sinks which quench the variable fluorescence (Iwanzik *et al.*, 1983), and/or on the reducing site of PSII (Melis *et al.*, 1992). Evidence for the first action comes from the additional appearances of polypeptide fragments from the PSII reaction centre under UV-B irradiation. However, it was also shown that modification of Q_A by UV-B can prevent electron transport from reduced pheophytin to Q_A, thus allowing charge recombination reactions between P-680$^+$ and Pheo$^-$ and quenching of the variable fluorescence (Melis *et al.*, 1992). Further targets seem to be the quinone Q_B and/or the quinone pool inducing the degradation of the D1 protein as seen in photoinhibition, but UV-B damage is different to damage by visible radiation because the D1-degradation rate is much slower (5-7h) than that during photoinhibition (Tevini, 1994). UV-C destroys plastoquinone and plastohydroquinone, whereas UV-B does not, except when photosynthetic electron flow is already low as the result of prolonged irradiation periods with UV-B irradiances, as demonstrated in radish leaves (Tevini and Iwanzik, 1983). In the field, when longer UV-B irradiation periods occur,

photoinhibition and UV-B damage may be additive and thus increase impact on net photosynthesis. This was also deduced from the inhibition of violaxanthin de-epoxidase by UV-B, which is thought to have a protective role against photoinhibition (Demming *et al.*, 1987). This enzyme synthesises zeaxanthin (via antherazanthin) which dissipates some of the adsorbed excessive light energy (Pfündel *et al.*, 1992). PSI seems much more resistant to UV-B radiation (Iwanzik *et al.*, 1983; Renger *et al.*, 1989; Strid *et al.*, 1990).

Elucidation of the role of the xanthophyll cycle in the non-photochemical quenching is an example of the way correlative biological evidence drives new biophysical research (Osmond, 1994). Demming *et al.* (1987) recognised that the electron transport dependence of the interconversion of the xanthophyll pigments violaxanthin, antheraxanthin and zeaxanthin in chloroplasts may be relevant to photoinhibition. They found that, in leaves under conditions of photon excess, violaxanthin conversion to zeaxanthin was highly correlated with non-photochemical quenching, a relationship now confirmed in a wide range of situations and organisms (Osmond, 1994).

The role of violaxanthin-zeaxanthin interconversion in the molecular mechanism of the non-photoquemical quenching has been well summarised by Owens (1994). Violaxanthin transfers energy to chl *a* with high efficiency in some algal light-harvesting systems. Conversion of violaxanthin to zeaxanthin lowers the carotenoid S_1 state energy close to that of chl *a* and introduces a potential new pathway for reversible energy transfer among adjacent chl *a*-zeaxanthin pairs. Because the total rate of radiative and non-radiative transitions from S_1 to the ground state is more than 2 orders of magnitude larger for carotenoids than for chl *a*, the occurrence of reversible energy transfer between antenna chl *a* and zeaxanthin would result in the formation of weak quenching centres in the antenna. The quenching efficiency at each zeaxanthin site will be a complex function of energy transfer and internal conversion rates on all coupled antenna pigments and, in general, will be determined by the dynamics of excited state motion and trapping in PSII.

Chlorophyll reduction was only evident in plant leaves which were exposed to particularly large doses of UV-B (Sisson and Caldwell, 1976; Brandle *et al.*, 1977; Teramura *et al.*, 1980) or when plants were exposed to UV-B while in an environment of very low visible irradiance (Vu *et al.*, 1982).

Chlorophyll destruction was a function of UV-B fluence rate and was only found in UV-sensitive plants such as bean and cucumber in growth chamber studies (Tevini *et al.*, 1983). Solar UV-B simulating a 12% ozone depletion reduced chlorophyll content when expressed on a per plant basis, but increase it when expressed on leaf area, despite reduction in overall area (Tevini and Teramura, 1989).

The fresh weight, the leaf area, the amounts of chlorophylls, carotenoids and the galactolipids of the chloroplasts were reduced in barley (*Hordeum vulgare*), corn (*Zea mays*), bean (*Phaseolus vulgaris*), and radish (*Raphanus sativus*) seedlings irradiated under a lighting device for 5-10 days at an increased UV-B fluency rate (Tevini *et al.*, 1981).

Decreases in Rubisco activity and stomatal conductance may also be implicated as factors limiting CO_2 assimilation in leaves exposed to elevated levels of UV-B. Rubisco has been shown, at least in pea leaves, to be very UV-sensitive (Strid *et al.*, 1990; Jordan *et al.*, 1992). Prolonged exposure to elevated levels of UV-B has been demonstrated to result in decreases in both Rubisco activity and content (Vu *et al.*, 1984; Strid *et al.*, 1990; Jordan *et al.*, 1992), and is accompanied by large decreases in the mRNA transcripts of both the large and small subunits of Rubisco (Jordan *et al.*, 1992). Such decreases in Rubisco are consistent with the observed decrease in the leaf carboxylation efficiency, determined from the initial slope of the response of CO_2 assimilation to increasing CO_2 concentration, when leaves are given supplemental UV-B radiation (Ziska and Teramura, 1992).

Phosphoenolpyruvate-carboxylase activity in the C4 plant sweet corn was found to be lowered by UV-B irradiation (Vu *et al.*, 1982).

Exposure to UV-B has been found to modify the rates of stomatal closing, and consequently the rate of leaf transpiration (Wright and Murphy, 1982; Murali and Saxe, 1984). Guard cell activity is very sensitive to ultraviolet radiation. In short-term experiments the stomata respond to UV-B by closing, and the guard cells continuously lose ions. This indicates that both the plasmalemma and the tonoplast are affected. UV-B radiation could also indirectly affect the ATP supply needed for ion transport into guard cells (Bakken, 1989).

The action spectrum showed that UV of 285 nm or shorter wavelengths was the most efficient in causing stomatal closure. UV radiation of 313 nm or longer wavelengths was practically without effect. Since stomatal closure is caused by K^+ efflux from the guard cells (Wright and Murphy, 1982), UV-induced potassium efflux is a possible mechanism for UV-

induced stomatal closure. Furthermore, when higher white light (450 μmol m^{-2} s^{-1}) was administered, stomates re-opened rapidly irrespective of whether the UV was on or off, although a subsequent gradual closing tendency was observed when the UV was on (Negash and Björn, 1986). Further, no effects on leaf gas exchange were found in grasses like wheat and wild oat even when high supplemental UV-B (simulating a 40% ozone reduction) was supplied in the field (Beyschlag *et al.*, 1988). Transpiration was reduced in some UV-sensitive seedlings such as cucumber grown under artificial UV-B in growth chambers or in sunflower seedlings grown under ambient solar UV radiation in Portugal compared with those receiving a reduced fluence. The time course for stomatal closure was rapid at low UV-B fluences and stomatal opening was slow at higher UV-B fluences (Tevini and Teramura, 1989).

Most higher plants accumulate UV-adsorbing pigments in their leaves particularly phenylpropanoids, such as cinnamic acid esters, flavones, flavonols and anthocyanins, after irradiation with UV-B. These compounds are commonly located in the upper epidermal layers of leaves (Tevini and Teramura, 1989). *Rumex patientia*, a relatively UV-B sensitive plant, has a higher epidermal UV transmittance than the more resistant specie *R. obtusifolius*, which indicated that the UV-B flux at the mesophyll layer for *R. patientia* would also be higher. Attenuation of UV-B radiation increased in *R. obtusifolius* by 27% after exposure to solar UV-B radiation (Robberecht and Caldwell, 1986). In addition to phenylpropanoids, other important products of the shikimic acid pathway such as furanocoumarins, and terpenoids, also accumulate under increased UV-B radiation. Since these compounds are toxic to many organisms including insects, changes in their concentration might affect their resistance to herbivores (Tevini and Teramura, 1989).

It has been clearly shown that flavonol accumulation is specifically UV-induced and linearly dependent on UV-B fluency (Wellmann, 1985). The content of flavonoids increased in barley and radish seedlings by about 50% on exposure to UV-B radiation (Tevini *et al.*, 1981). This increase in flavonoid concentration is due to a higher activity of the enzyme PAL and/or to higher rates of biosynthesis of this enzyme. Both possibilities were found in UV irradiated primary leaves of rye seedlings which accumulated two isovitexin derivatives exclusively located in the epidermal layers. In rye, PAL activity increased within minutes by decreasing the

PAL inhibitor, trans-cinnamic acid through its isomerization to the cis form. The equilibrium between the cis and trans from is shifted to the cis form by shorter wavelengths in the UV-B, thus lowering the feedback inhibition of the enzyme PAL, and possibly also of the subsequent enzymes, normally performed by trans cinnamic acids (Tevini, 1994). The UV regulation at the genetic level was investigated in cultured cells and leaves of *Petroselinum* by Chappell and Hahlbrock (1984), Kuhn *et al.* (1984) and Schmelzer *et al.* (1988), demonstrating that the increase in mRNA transcripts for PAL, 4-coumarate: CoA ligase (4CL) and CHS is caused by UV-induced changes in transcription.

In addition to flavonoids, polyamines which stabilise the membrane structure and inhibit lipid peroxidation, may also play a role in protecting plants from UV-B stress (Kramer *et al.*, 1992).

Since photosynthesis is essential for plant productivity, UV-induced damage to photosynthetic function will often manifest itself in less biomass or lower yield.

A field study with six soybean cultivars simulating a 16 and 25% ozone depletion, showed a UV-B fluency dependent reduction in relative growth rate, height growth and net assimilation rate (Lydon *et al.*, 1986). In a 6-years study, reductions in plant height and leaf blade length were found both in wheat and wild oat when grown in monocultures (Barnes *et al.*, 1988). These reductions were not associated with reduced photosynthesis which remained unchanged (Beyschlag *et al.*, 1988). Therefore, reductions in plant height or leaf area are not always correlated with total biomass reductions, especially in grasses like wheat and wild oat where leaf area is displayed vertically, rather than horizontally as in herbaceous plants (Barnes *et al.*, 1988).

In another field study using filtered lamps and simulating 10 and 25% ozone reductions, no UV-B effects were demonstrated in three cabbage cultivars, nor in lettuce and rape (Tevini and Teramura, 1989). Biggs and Kossuth (1978) found yield reductions between 5 and 90% in half of them, among them crops like wheat (-5%), potato (-21%) and squash (-90%), whereas rice, peanut and corn were unaffected. This results indicate a high degree on interspecific variability complicated further by differences in artificial light sources, climate, soil quality, day length, etc.

In addition to this interspecific variability, there is a high degree of intraspecific variability among cultivars. For example, twenty-three soybean cultivars were grown in a greenhouse and six were selected for a comparative field study. Results from the greenhouse and the field indicate that the cultivar "Forrest" was the most tolerant whereas "Shore" and "York" were the most susceptible to UV-B, based upon a combination of responses including plant height, leaf area, total dry weight and seed yield ("York" had a significant 25% reduction in yield when exposed to a simulated 16% ozone reduction) (Teramura and Murali, 1986).

3.2.6.2. UV-B SENSITIVITY OF PLANTS

Species and ecotypes differ in sensitivity, and the damaging effect depends on other environmental factors. The most important is the level of PPFD given together with the UV-B radiation (Bakken, 1989). Data for UV effects on terrestrial higher plants has been collected, mainly for important crops, and forest species.

Sullivan and Teramura (1988) point out that about two-thirds of the 200 plant species so far tested appear to be sensitive to UV-B. The sensitivity of more than 45 different crop species has been screened under a diverse range of environmental conditions (Van *et al.*, 1976; Teramura, 1983). In general, monocotyledons seem to be less affected by UV-B radiation than dicotyledons, and crops with the C3 photosynthetic pathway are more affected than those with the C4 pathway (Bakken, 1989). Among important crop species which appear to be sensitive are **pea** (*Pisum sativum* L.), collard (*Brassica oleracea* L. var. acephala), cabbage (*Brassica oleracea* L. var. capitata), soybean (*Glycine max* L.) and oat (*Avena sativa* L.) (Van *et al.*, 1976).

Several cultivars of some major crop species are sensitive to UV-B. These include soybeans, beans, wheat, **peas**, rice, potato, squash, and cassava. Some studies show other cultivars of rice and wheat to be insensitive to UV-B and other crops appear to be UV resistant, *e.g.* corn, peanuts, and cabbage (Coohill, 1991).

3.3. AIMS

The aim of this study is to use modulated chlorophyll fluorescence to evaluate the role of PSII inactivation in the decreases in CO_2 assimilation when mature pea leaves are exposed to elevated levels of UV-B radiation under normal growth conditions in a glasshouse. The maximum quantum efficiency of PSII photochemistry was determined during UV-B-induced decreases in CO_2 assimilation of mature pea leaves. Stomatal conductance, and absorbance, reflectance and transmittance of mature pea leaves were also determined during UV-B irradiation. The capacity of thylakoids to bind atrazine was also determined during UV-B irradiation of mature pea leaves.

3.4. MATERIALS AND METHODS

The general principle in the experiments to determine the effects of UV-B on plants involves the use of a UV source coupled with different types of filters to exclude bands of UV wavelength not desired in the experiment. The intensity of UV is varied by changing the height distance between the UV source and the plant canopy (Caldwell *et al.*, 1986).

Plants should be grown under the appropriate white light source with an irradiance at the plant level of not less than 400 μmol m^{-2} s^{-1}, since UV-B effects on plants are exaggerated under very low levels of PPFD (Sullivan and Teramura, 1988). The UV-B sources can be, for example, Philips TL40W/12 or Westinghouse FS-40 lamps filtered to remove wavelengths shorter than 280 nm. One such lamp suspended 40 cm above the plants will provide the equivalent of a 16% reduction in stratospheric ozone over a footprint of approximately 0.5 m^2 (Hendry, 1993).

In greenhouse or in the field cellulose acetate plastic films were used to shape the spectral distribution or radiation produced by low-pressure fluorescent sunlamps, eliminating harmful UV-C radiation (Fig. 8). Filters may be either cheap with a short life (1 mm thick cellulose acetate) or more expensive but with an indefinite life (3 mm Schott glass filters WG280). In greenhouses, control irradiances are provided by the further addition of UV-B lamps filtered to include UV-A but exclude all UV-B irradiance (filters either 0.13 mm Mylar

type D replaced weekly or 3 mm Schott glass WG320) and a second control where is no added UV-B irradiance (Hendry, 1993).

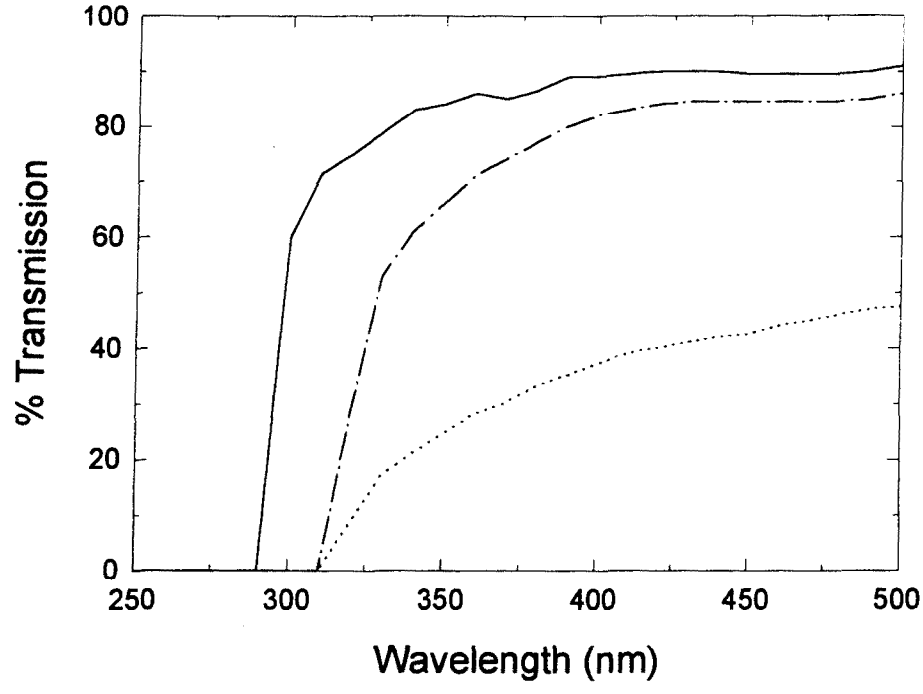


Figure 8. The transmittance of cellulose acetate (—), Mylar type D (-·-) and Mylar type A (···) filters as a function of wavelength.

3.4.1. PLANT MATERIAL AND GROWTH CONDITIONS

Seeds of vining pea (*Pisum sativum* L., cv. Meteor) were soaked in running tap water for 24 hours at room temperature. Then, they were germinated on wet filter papers at 30°C for 3 days, sown in a mixture of perlite:Levington F2 compost (1:1, v/v) in freely-draining pots and watered regularly with Hoagland's nutrient solution (Hoagland and Arnon, 1950).

Plants were grown in a glasshouse at Essex University (Colchester, UK) with a minimum day and night temperature of 25 and 20°C and under supplementary lighting to provide a minimum PPFD of 450 μmol m^{-2} s^{-1} for the 15 hours photoperiod.

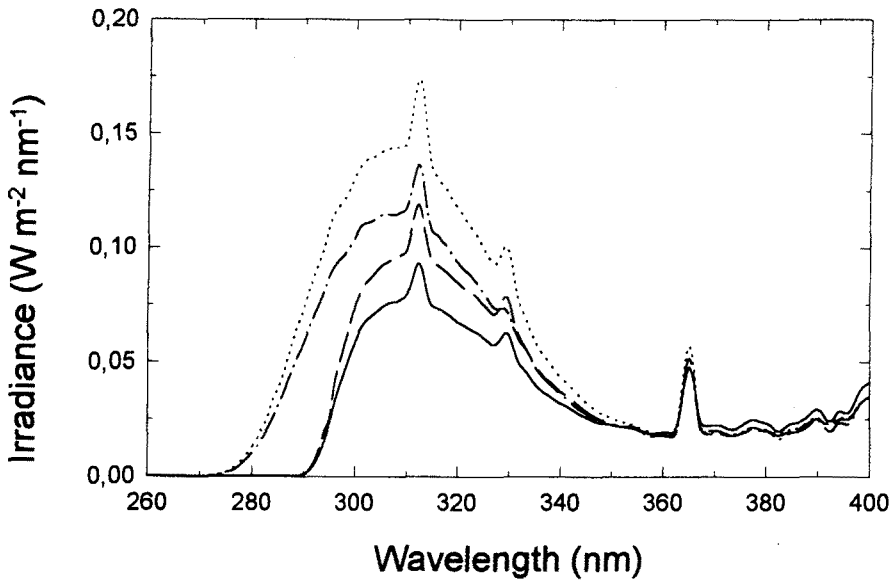


Figure 9. Spectral irradiance at the leaf surface in the four UV treatments: (—) low UV-B; (- -) high UV-B; (-·-) low UV-B + UV-C; and (···) high UV-B + UV-C.

3.4.2. UV TREATMENTS

After fourteen days from sowing the plants were transferred to a UV cabinet kept in the same glasshouse. The UV cabinet was made using 0.13 mm Mylar type D plastic films and divided into control and UV supplemented compartments. The irradiance at the top of the canopy was maintained at 450 μmol m^{-2} s^{-1}. Half of the plants were placed in the control

compartment and the other half in the UV supplemented compartment where UV radiation was provided by two or three UV-B tubes (Philips TL40W/12) mounted 40 cm above the plants. In all treatments the UV-B tubes were switched on and off with the supplementary lighting at the beginning and end of the daily photoperiod respectively, thus ensuring the plants only received UV radiation when exposed to a minimum PPFD of 450 μmol m^{-2} s^{-1}.

Table 5. Photon flux density between 260 and 400 nm and the total biologically effective UV irradiance (280-320 nm), as determined from the generalised plant response action spectrum of Caldwell (1971), of the four UV treatments.

Treatment	Photon flux density (μmol m^{-2} s^{-1})	Biologically effective irradiance (W m^{-2})
Low UV-B	11.8	0.74
High UV-B	13.3	0.93
Low UV-B + UV-C	16.4	2.92
High UV-B + UV-C	19.9	3.67

Plants were exposed to one of four UV treatments designated as (a) low UV-B, (b) high UV-B, (c) low UV-B + UV-C, and (d) high UV-B + UV-C. To exclude UV-C radiation in low and high UV-B treatments, the UV-B tubes were wrapped in cellulose diacetate sheets (Fig. 9). Since the transmission spectrum of cellulose acetate sheets changed after exposure to UV-B, the sheets were replaced daily (Strid *et al.*, 1990).

The unweighted UV spectral irradiances for each treatment, as measured with a Macam SR9910-PC spectroradiometer (Macam Photometrics, Livingston, U.K.) at the level of the sampled leaves, are shown in Figure 9. The integrated photon flux densities between 260 and 400 nm and the total biologically effective UV irradiances, as determined between 280 and

320 nm from the generalised plant response action spectrum (Caldwell, 1971), of the four UV-B treatments are given in Table 5. The low UV-B treatment provided a daily biologically effective UV-B dosage of 40 kJ m^{-2} d^{-1}, which is approximately eight times greater than the level detected in midsummer in the U.K. (see section 3.2.4.).

The youngest fully, expanded leaves were used in all experiments. Whole pea leaves were excised in the glasshouse and placed in wet Petri's dishes for transport to the laboratory. Leaves were then placed in the chamber in which the photosynthetic parameters were measured, ensuring that the cut surface was continuously irrigated with distilled water circulated by a peristaltic pump (MHRE 200, Watson-Marlow Ltd., Falmouth, U.K.).

3.4.3. LEAF GAS EXCHANGE

CO_2 ASSIMILATION

Measurements of carbon dioxide assimilation for detached leaves operating at steady state photosynthesis under a PPFD of 450 μmol m^{-2} s^{-1}, which was similar to the mean growth PPFD, were made at 25°C in an open gas-exchange system described by Stirling *et al.* (1991). Air was draw from the roof of the laboratory and passed through a vessel contained distilled water to minimise short-term fluctuations in the atmospheric CO_2 concentration. A leaf chamber constructed from stainless aluminium with provision of coolant water channels to allow temperature control, was used to determine the rate of CO_2 uptake by leaves.

The CO_2 gradient across the chamber was determined with an infrared CO_2 analyser (Type 225-Mk3, Analytical Development Co., Hoddesdon, Herts, UK). Differential calibration followed the method of Parkinson and Legg (1971) using a known mixture of CO_2 in air. Leaf temperature was maintained at 25°C by circulating water from a heater/circulator (C-400, Techne Ltd., Duxford, UK) and flow-through cooler (EN-350 Neslab, Portsmouth, NH,

U.S.A.). Leaf temperature was monitored by an electronic thermometer (1625 Cu/Co, Comark Ltd., Littlehampton, U. K.).

The chamber was illuminated with a Walz lamp (KL1500, Effeltrich, Germany) and a 250-W quartz-iodide source (Scholly Fiberoptik, GmbH, Germany) for the saturated light points. PPFD incident at the leaf surface was determined using a quantum sensor (Li-185B, Li-Cor inc., Nebraska, U.S.A.), placed below the chamber window in the position normally occupied by the leaf.

The possibility that UV-B-induced decreases in CO_2 assimilation may be related to differential effects on photorespiratory and carbon assimilation metabolism was examined by reducing the O_2 concentration of the leaf chamber atmosphere in which the photosynthetic parameters were measured from 21 to 2%, in order to inhibit photorespiration. N_2, O_2 and CO_2 were mixed at controlled rates using thermal mass flow controllers (FC-260, Tylan Inc.) and humidified using a water vapour generator (WG600, ADC Ltd.).

The A_{sat} of the leaves was determined at PPFD of 1500 μmol m^{-2} s^{-1} using the standard equations of von Caemmerer and Farquhar (1981). The ϕ_{CO2} of leaves was calculated by dividing the rate of CO_2 assimilation of the irradiated leaf area by the rate at which quanta were adsorbed by this area (see section 3.4.4.).

STOMATAL CONDUCTANCE

Measurements of stomatal conductance for detached leaves operating under a PPFD of *ca.* 1200 μmol m-2 s-1, ensuring that light intensity was not limited during the measurements, were made at 25°C using a temperature-controlled leaf section chamber (LSC, ADC Ltd., Hoddesdon, UK) described by Farage *et al.* (1991). Leaf temperature was measured using a thermocouple, connected to an electronic thermometer displays (1625 Cu/Co, Comark Ltd., Littlehampton, UK). The leaf was illuminated by a 250-W quart iodide source (Schölly Fiberoptick, GmbH, FRG) used in conjunction with a heat-reflecting filter (OCLI Ltd., High Wycombe, UK). PPFD was determined by placing a quantum sensor (Li-

185B, Li-Cor inc., Nebraska, U.S.A.) below the window at the position normally occupied by the leaf.

Gas mixtures were supplied to the chamber from three cylinders, N_2, O_2 and 10% CO_2 in N_2 (BOC Special Gases, London, UK). A gas blender (853 VI-5, Signal Instrument Co. Ltd., Camberley, UK) was used to supply the leaf chamber with 80% N_2, 20% O_2, and a range of CO_2 concentrations from 0 to 1750 μmol mol^{-1}. Before reaching the chamber, the dry air was passed through a temperature-controlled column of ferrous sulphate crystals that rehumidified the air. Absolute and differential water vapour pressure and carbon dioxide concentrations were measured using an infrared gas analyser (Li-6262, Li-Cor inc., Nebraska, U.S.A.). Gas flow through the chamber was measured using precision-variable area flow meters (Fisher Controls Ltd., Croydon, UK). After stomatal conductance measurements, the leaf area was measured using an area meter (AM1, Delta T Devices Ltd., Burwell, UK).

Stomatal conductance (g_s) was calculated using the equations of von Caemmerer and Farquhar (1981).

Measurements of **modulated chlorophyll fluorescence** yield from the upper surface of leaves, operating at steady state photosynthesis under a PPFD of 450 μmol m^{-2} s^{-1}, were made using a pulse amplitude modulation fluorimeter (PAM 101-102-103, H. Walz, Effeltrich, Germany) described in the general introduction (see section 1.2.2.1.). The measuring modulated light intensity was approximately 0.1 μmol m^{-2} s^{-1} and sufficiently low not to produce any significant variable fluorescence. A 2 seconds pulse of high-intensity (PPFD < 10,000 μmol m^{-2} s^{-1}) white light was used to produce a transient closure of the PSII photochemical reaction centres. Far-red light (190 μmol m^{-2} s^{-1} at 710-730 nm) was used to produce maximal oxidation of PSII electron acceptors (Genty *et al.*, 1989). Measurements of the maximum quantum yield of PSII primary photochemistry were made from the Fv/Fm ratio of leaves that had been dark-adapted for 30 minutes. The relative quantum efficiency of PSII photochemistry for leaves at steady-state photosynthesis (ϕ_{PSII}) was estimated from (Fm'-Fs')/Fm' (see section 1.2.2.1.).

3.4.4. LIGHT ADSORPTION BY LEAVES

Measurements of leaf absorbance, reflectance and transmittance in any given light environment for each UV-B treatments were determined in a Taylor integrating sphere (SO143-000, Varian Associates, Palo Alto, CA, U.S.A.), using a method based on that of Rackham and Wilson (1968). The integrating sphere was illuminated with a 250-W quartz-iodide source (Scholly Fiberoptik, GmbH, Germany) and the photon flux value recorded. The leaf was then placed in the back-holder and the reflectance value was recorded. Finally, the leaf was placed in the front-holder and the transmittance value was again recorded.

3.4.5. THYLAKOID MEMBRANE PREPARATIONS

The experimental procedure for thylakoid isolation was essentially similar to those described previously (Jenkins and Russ, 1984; Bradbury and Baker, 1986). Pea leaves were cut, mixed with cold grinding medium (1 mM Na_2EDTA, 330 mM Sorbitol, 10 mM $MgCl_2$, 150 mM NaCl, 40 mM HEPES/NaOH pH=7.6), and homogenised in a Waring Blender for 2 bursts, each 5 seconds long. Immediately the homogenate was filtered through 2 layers of Miracloth and then centrifuged at 5000 rpm for 3 minutes. The supernatant was discarded; the pellet was gently resuspended with a soft paintbrush in the resuspension medium (1 mM Na_2EDTA, 10 mM $MgCl_2$, 150 mM NaCl, 40 mM HEPES/NaOH pH=7.6) and then centrifuged at 10000 rpm for 2 minutes. The supernatant was again discarded and the pellet resuspended in 1 ml resuspension medium to produce a final chlorophyll concentration of *ca.* 1.67 mg ml^{-1}.

3.4.6. CHLOROPHYLL CONTENT

Chlorophyll concentrations of the final resuspended thylakoids solutions were measured in 80% (v/v) acetone by the method of Arnon (1949), using the following equation:

$$[Chl] = (8.02 \times A_{645}) + (20.2 \times A_{663})$$

where A_{645} and A_{663} are the absorbances of the resuspended thylakoid solutions at 645 nm and 663 nm, respectively.

3.4.7. THYLAKOID PROTEIN CONTENT

Thylakoid protein content was determined using the bicinchonic acid (BCA) protein assay reagent (Pierce, Rockford, IL, USA) according to the manufacturer's protocol.

The BCA assay reagent is a highly sensitive reagent for the spectrophotometric determination of protein concentration (Smith *et al.*, 1985). This assay reagent eliminates the need for precisely timed reagent additions and vortexing inherent with the Lowry method (Lowry *et al.*, 1951) and combines the well-known biuret reaction (*i.e.* protein reacting with Cu^{2+} in an alkaline medium to produce Cu^{1+}) with the BCA. The purple reaction product, formed by the interaction of two molecules of BCA with one cuprous ion, is water soluble and exhibits a strong absorbance at 562 nm. This allows the spectrophotometric quantitation of protein in aqueous solutions.

The BCA protein assay begins with the preparation of the working reagent. Then, a set of protein standards of known concentration was prepared by diluting the stock 2mg/ml BSA standard. The set of protein standards should cover the range of concentrations suitable for the assay protocol. Afterwards, 2ml of working reagent were added to 0.1ml of each standard. All tubes were incubated at 37°C for 30 minutes (standard protocol). After incubation, tubes were cooled at room temperature for 5 minutes. Absorbance of each tube was measured at 562 nm using a spectrophotometer.

Chlorophyll protein ratios determined for thylakoid preparations were used to determine the thylakoid protein content per leaf area.

3.4.8. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis of chloroplast thylakoid polypeptides was performed using the discontinuous buffer system of Neville (1971) essentially described by Chua (1980) in 10-18% linear gradient polyacrylamide gels.

Thylakoids can be loaded in the gels on the basis of their chlorophyll or protein content. Generally, thylakoid preparations contain about five times as much protein as chlorophyll (w/w), so to solubilize a sample for gels, the final SDS:protein ratio has to be 4:1, *i.e.* SDS:chl ratio has to be greater than 20.

Gradient gels with 1 mm spaced plates (150 x 150 mm) were poured using a peristaltic pump (Pharmacia-LKB Biotechnology, Uppsala, Sweden) and linear gradient mixer with equal volumes of solutions containing 10 or 18% acrylamide, 0.27 or 0.46% N,N-methylene bisacrylamide, bisdistillated water or 9.57% sucrose respectively, and 1.5 M Tris-HCl (pH=8.8), 0.1% (w/v) SDS, 0.01% (w/v) APS and 0.01% (v/v) TEMED. The stacking gel contained 3.75% (w/v) acrylamide, 0.10% (w/v) N,N-methylene bisacrylamide, 0.5 M Tris-HCl (pH=6.8), 0.1% (w/v) SDS, 0.1% (w/v) APS and 0.26% (v/v) TEMED. The tank buffer contained 0.02 M Tris-HCl (pH=8.3), 1.44% (w/v) glycine and 0.1% (w/v) SDS.

Gel lanes were loaded with membrane protein samples based on equal amounts of chlorophyll (20 μg). Electrophoresis was carried out at a constant current of 15 mA. Following electrophoresis, the gels were removed from the plates and placed in a stain solution containing 0.13% (w/v) Coomassie brilliant blue R, 7.89% (v/v) methanol, 13.16% (v/v) glacial acetic acid for 45 minutes, and then destained in a solution containing 8.4% (v/v) methanol, 5.88% (v/v) glacial acetic acid and 1.68% (v/v) glycerol overnight. The destained gels were then vacuum-dried at 80°C for 1 hour.

3.4.9. LASER DENSITOMETRY

To measure the relative amount of proteins in thylakoids, SDS-PAGE gels were analysed using an ImageQuant (Molecular Dynamics 300A, Sunnyvale, California) computing

densitometer. Gels were laser scanned to capture the whole image. Each track was then analysed using the "Integrate area" command. The optical densities of the large polypeptide bands in each track were computed and plotted. A smoothing filter was applied in order to reduce image noise and allow for more accurate band identification. The software built-in smoothing filter was set to 3, and the slope sensitivity, upward and downward counts parameters set at 0.003, 3 and 3, respectively, which were found to be optimum for band identification.

For better image visualisation, the image processing and display package (CoMOS v. 6.03, BioRad Laboratories, Hemel Hempstead, Hertfordshire, U.K. - designed for processing confocal microscopy images) was employed. Image files were transferred from ImageQuant computing densitometer to CoMOS and particular protein concentration levels were highlighted by pseudocoloring using different look-up tables (LUT) available from the system's library. LUTs translate intensity values in the image into selected colours in the display system. The "Edit output LUTs" command was set as follows: LUT=geog, max. ramp value=250, and max. pixel value=250. Pictures were then taken using a Polaroid QuickPrint video printer connected on line to the display system.

3.4.10. CAPACITY OF THYLAKOIDS TO BIND ATRAZINE

The binding of [^{14}C]atrazine to isolated thylakoids was used to determine the number of plastoquinone binding sites, *i.e.* the concentration of PSII complexes potentially capable of plastoquinone reduction, according to the principles first described by Tischer and Strotmann (1977). The experimental procedures for assay of atrazine-binding capacity were essential similar to those described by Bradbury and Baker (1986). Thylakoid preparations were obtained as described above and diluted to a chlorophyll content of 50 μg ml^{-1} with a solution of 1 mM Na_2EDTA, 10 mM $MgCl_2$, 150 mM NaCl, 40 mM HEPES(NaOH pH=7.6). A 10 μl aliquot of [ethyl-1-^{14}C]atrazine (Amersham International, Amersham, Bucks., U.K.) having a specific activity of 25 Ci mol^{-1} in 100% methanol was added to give a range of 8 final

concentrations between 20-300 nM and incubated with the thylakoid preparations at 25°C in the dark for 10 minutes.

The samples were then centrifuged in a microcentrifuge (*ca.* 10,000g) for 4 minutes and 100 µl of supernatant was removed and added to 3.5 ml of scintillation fluid (OptiPhase Hisafe II, LKB Scintillation Products, Turku, Finland). Measurements of radioactivity were performed by liquid scintillation spectrometry (LKB Wallac, model 1219 Rackbeta, Turku, Finland) with a Packard Tricarb 460C scintillation counter. The concentration of atrazine bound to the herbicide binding site on the D1 protein of PSII was calculated from the difference between the free atrazine in a control containing no resuspended thylakoids, and the atrazine measured in the supernatant of the incubated samples. The binding constant (K_m) and the number of binding sites per chlorophyll (V_{max}) were calculated from the intercepts of the ordinate and abscissa respectively of double reciprocal plots of 1/bound atrazine (mg chl nM^{-1}) versus 1/free atrazine (mmol m^{-3}) (Tischer and Strotmann, 1977).

3.5. RESULTS

The changes in A_{sat} measured at a PPFD of 1500 µmol m^{-2} s^{-1}, $\phi CO2$ and the modulated chlorophyll fluorescence parameter $\phi PSII$ measured at a PPFD similar to that under which the plants were grown (450 µmol m^{-2} s^{-1}), and Fv/Fm during four days of exposure of pea leaves to the low UV-B treatment are shown in Figure 10. After the first 12h of exposure to UV-B decreases of *ca.* 30% were observed in A_{sat}, $\phi CO2$ and $\phi PSII$. However no change was observed in Fv/Fm. Over the next two days A_{sat}, $\phi CO2$ and $\phi PSII$ did not change significantly, whereas Fv/Fm showed a slight decrease. Further decreases in all the parameters were observed after the fourth day of exposure to UV-B. The decreases in A_{sat}, $\phi CO2$ and ϕ PSII over the four day treatment period were considerably greater than the decline observed in Fv/Fm. After four days of exposure to UV-B, the UV tubes were switched off. After 39 hours in the absence of UV-B no significant recovery was observed in A_{sat}, $\phi CO2$ and $\phi PSII$ although Fv/Fm did show some recovery (Fig. 10).

When leaves were exposed to an increased UV-B dosage (high UV-B treatment) a similar pattern of change in A_{sat}, ϕ_{CO2} and ϕ_{PSII} was observed over 4 days, but the magnitude of the decreases in these parameters was considerably increased (Fig. 11). After 12 hours exposure to the high UV-B treatment A_{sat} and ϕ_{CO2} had both decreased by *ca.* 50%. In this first period of UV-B exposure ϕ_{PSII} had a significant decrease. There was also a significant decrease observed in Fv/Fm during this period of UV-B exposure. The high UV-B treatment was terminated during the fourth photoperiod by switching the UV tubes off, and after 39 hours Fv/Fm showed a considerable recovery, however no significant recovery of A_{sat}, ϕ_{CO2} and ϕ_{PSII} was observed (Fig. 11).

The consequences of supplementing the two UV-B treatments with UV-C (low UV-B + UV-C and high UV-B + UV-C treatments) on the photosynthetic performance of the leaves are shown in Figures 12 and 13. In both treatments large decreases in all of the photosynthetic parameters were observed during the first 12 hours of UV irradiation. However, in both treatments only very small decreases were observed in Fv/Fm during the first 6 hours of irradiation, whereas decreases in A_{sat}, ϕ_{CO2} and ϕ_{PSII} were considerably greater.

Oxygen is a substrate for rubisco. The oxygenation of rubisco is the primary step in photorespiration, which consumes both the products of photosynthetic electron transport (*i.e.* ATP and NADPH) and carbohydrate. At ambient atmospheric CO_2 concentrations reduction in the oxygen concentration of the atmosphere, in which the photosynthetic parameters were measured, from 21 to 2% produces an almost complete suppression of photorespiratory activity in leaves, and consequently an increase in the rate of CO_2 assimilation occurs (see Genty *et al.*, 1990). In control leaves inhibition of photorespiration resulted in significant increases in A_{sat} and ϕ_{CO2} but not in ϕ_{PSII} (Figs. 10 and 11), thus demonstrating that the removal of photorespiratory metabolism as a sink for the products of linear electron transport, although increasing the rate of CO_2 assimilation, has no effect on the flux of electrons through PSII at the growth PPFD (450 $\mu mol\ m^{-2}\ s^{-1}$). The stimulation of CO_2 assimilation by inhibition of photorespiration appeared to be maintained throughout the UV-B treatments (Figs. 10 and 11). Similar results were observed for two UV-B + UV-C treatments (Figs. 12 and 13). These data indicate that effects on photorespiration cannot account for the UV-induced decreases in CO_2 assimilation.

The changes on stomatal conductance measured at a PPFD of *ca.* 1200 μmol m^{-2} s^{-1} through the low UV-B treatment is shown in Table 6. For all periods of leaves exposed to UV-B radiation there was no significant change in the stomatal conductance.

The responses of the leaf absorbance, transmittance and reflectance measured at a PPFD of 450 μmol m^{-2} s^{-1} during four days of exposure of pea leaves to the low UV-B treatment are shown in Figure 14. No significant changes on the parameters studied were observed during four days of exposure to the two UV-B treatments.

Table 6. Stomatal conductance of leaves measured at 380 μmol mol^{-1} CO_2 and high PPFD (*ca.* 1200 μmol m^{-2} s^{-1}) through the low UV-B treatment. Leaves were UV-B irradiated during 4, 8, 12, 18 and 24 hours respectively. Data are the means of 3 replicates and the standard errors of the means were less than 10% of the mean values in all cases.

Time of exposure (hours)	Stomatal conductance [mol(H_2O) m^{-2} s^{-1}]
Control	0.025
4	0.022
8	0.021
12	0.018
18	0.019
24	0.020

The consequences of supplementing the two UV-B treatments with UV-C (low UV-B + UV-C and high UV-B + UV-C treatments) on the absorbance, transmittance and reflectance of the leaves are shown in Figure 14. In both treatments no changes in the parameters studies were observed during two days of exposure of leaves to the UV-B + UV-C treatments.

The changes in the chlorophyll protein ratios of thylakoids isolated from leaves exposed to the four UV-B treatments are shown in Figure 15. There were no significant changes on the chlorophyll protein ratios throughout the UV-B treatments. These ratios were

used to determined the thylakoid protein content per leaf area to be loaded in each track of the SDS-PAGE gels.

SDS-PAGE gels of the thylakoid polypeptides from mature pea leaves were performed for the low UV-B + UV-C (Fig. 16) and high UV-B + UV-C (Fig. 17) treatments. The densitrometric scans of these gels using a computing densitometer allowed the accurately quantification of the polypeptide bands. These bands of thylakoid membrane from fully expanded leaves have been already identified by several authors and the molecular mass values for these bands are commonly found in the literature (Griffith *et al.*, 1986; Bredenkamp, 1987; Glazer and Melis, 1987; Chitnis and Thornber, 1988; Reilly and Nelson, 1988; Ghanotakis and Yocum, 1990; Nie and Baker, 1991). According with these authors and in order of increasing electrophoretic mobility, a 65-70 kDa band is the Chl-protein I apoprotein, a 57-58 kDa is the α and β- subunits of the coupling factor (CF_1), a 33 kDa is the extrinsic water splitting protein of PSII or the cytocrom *f*, a 26-28 kDa is the apoprotein of LHCII, a 20-26 kDa is apoproteins of LHCI, a 22 kDa is the subunit II of PSI and a 17 kDa is subunit IV of the cytocrom b_6/f complex. The D1 protein of PSII reaction centre does not stain well in a solution containing Coomassie brilliant blue dye.

For control (non-irradiated) leaves, four major bands of apparent molecular weights 57-58, 38, 33 and 26-28 kDas were clearly shown. The 57-58 kDa is probably the α and β-subunits of the coupling factor and the 26-28 kDa is the apoprotein of LHCII. Some minor polypeptide bands were also found (Figs. 16 and 17).

To improve the visualisation of the changes in the polypeptide bands, laser-scanned pseudocolored images of the gels showed in Figures 16 and 17 were performed using an image processing and display package (Figs. 18 and 19). A new 22-24 kDa band was more clearly shown. This polypeptide band is probably apoproteins of LHCI.

All major polypeptide bands decrease during UV irradiation, although the 26-28 kDa and the 57-58 bands are still visible after 36h of low UV-B + UV-C irradiation. When leaves were exposed to an increased UV-B dosage (high UV-B + UV-C treatment) a similar pattern of change in the major polypeptide bands was observed, but the magnitude of the decreases in these bands was considerably increased. A significant decrease in the polypeptides bands was observed during the first period of UV exposure.

Figure 10. Changes in the light saturated rate of CO_2 assimilation (A_{sat}) measured at a PPFD of 1500 μmol m^{-2} s^{-1}, the quantum yields of CO_2 assimilation (ϕ_{CO2}) and PSII electron transport (ϕ_{PSII}) measured at a PPFD of 450 μmol m^{-2} s^{-1}, and Fv/Fm throughout the low UV-B treatment. Solid (●) symbols indicate that measurements were made on leaves maintained in a normal gas atmosphere (21% O_2, 380 μmol mol^{-1} CO_2); open (○) symbols are for measurements made on leaves maintained in an atmosphere containing 2% O_2 and 380 μmol mol^{-1} CO_2 in *ca.* 98% N_2. The open and solid boxes immediately under the abscissa indicate the photoperiods and dark periods, respectively, to which the leaves were exposed. Leaves were only exposed to UV during the photoperiods. The vertical dashed line indicates the time at which UV tubes were switched off. Data are the means of 4 replicates and the standard errors are shown when larger than the symbols.

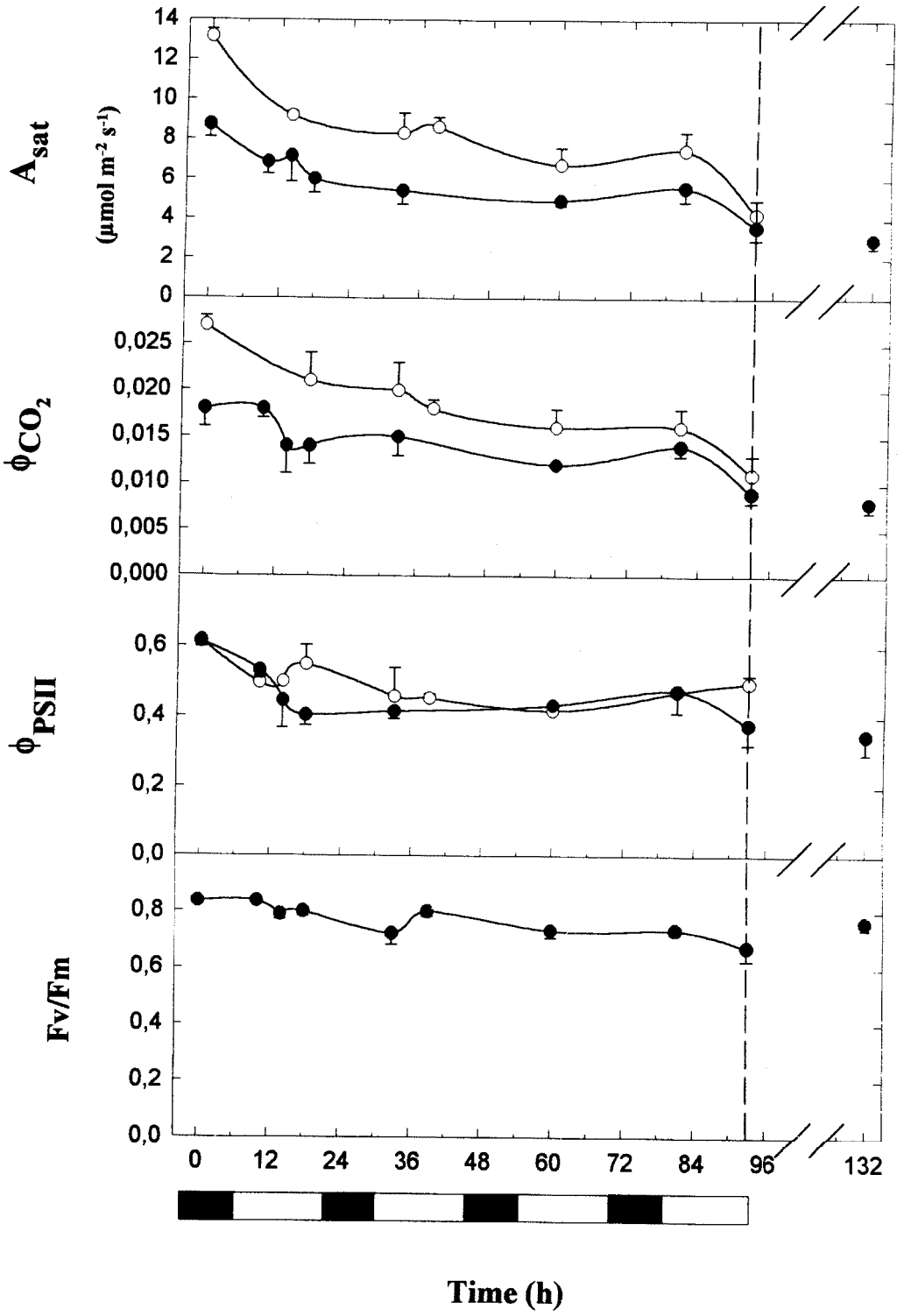

A_sat
(µmol m-2 s-1)
14
12
10
8
6
4
2
0
φCO2
0,025
0,020
0,015
0,010
0,005
0,000
φPSII
0,6
0,4
0,2
0,0
Fv/Fm
0,8
0,6
0,4
0,2
0,0
0
12
24
36
48
60
72
84
96
132
Time (h)

Figure 11. Changes in the light saturated rate of CO_2 assimilation (A_{sat}) measured at a PPFD of 1500 μmol m^{-2} s^{-1}, the quantum yields of CO_2 assimilation (ϕ_{CO2}) and PSII electron transport (ϕ_{PSII}) measured at a PPFD of 450 μmol m^{-2} s^{-1}, and Fv/Fm throughout the high UV-B treatment. All others details are as given in the legend of Fig. 10.

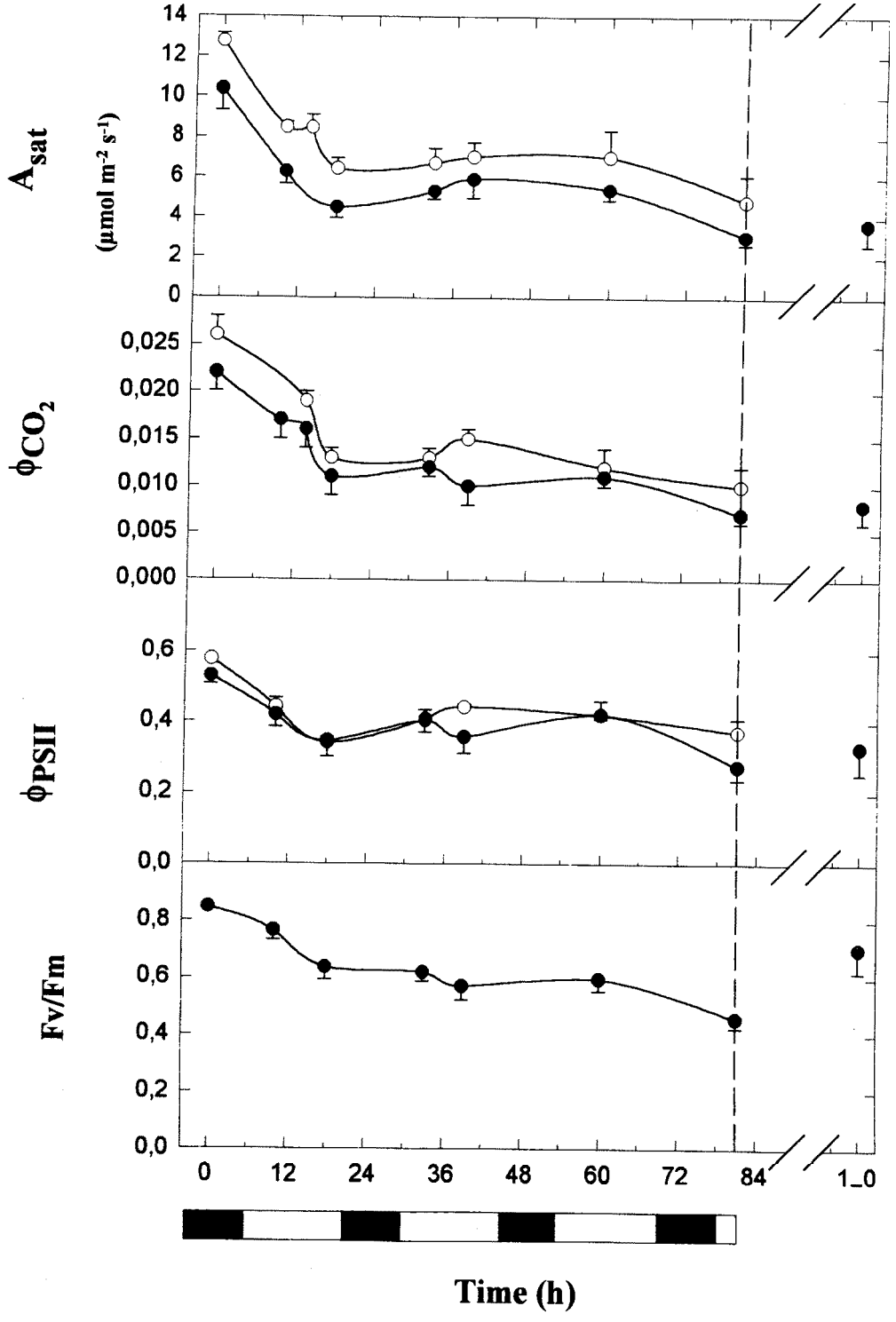

A_{sat}
(µmol m^{-2} s^{-1})
14
12
10
8
6
4
2
0
ϕCO_2
0,025
0,020
0,015
0,010
0,005
0,000
$\phi PSII$
0,6
0,4
0,2
0,0
Fv/Fm
0,8
0,6
0,4
0,2
0,0
0
12
24
36
48
60
72
84
1_0
Time (h)

Figure 12. Changes in the light saturated rate of CO_2 assimilation (A_{sat}) measured at a PPFD of 1500 μmol m^{-2} s^{-1}, the quantum yields of CO_2 assimilation (ϕ_{CO2}) and PSII electron transport (ϕ_{PSII}) measured at a PPFD of 450 μmol m^{-2} s^{-1}, and Fv/Fm throughout the low UV-B + UV-C treatment. Solid (●) symbols indicate that measurements were made on leaves maintained in a normal gas atmosphere (21% O_2, 380 μmol mol^{-1} CO_2); open (○) symbols are for measurements made on leaves maintained in an atmosphere containing 2% O_2 and 380 μmol mol^{-1} CO_2 in *ca.* 98% N_2. The open and solid boxes immediately under the abscissa indicate the photoperiods and dark periods, respectively, to which the leaves were exposed. Leaves were only exposed to UV during the photoperiods. Data are the means of 4 replicates and the standard errors are shown when larger than the symbols.

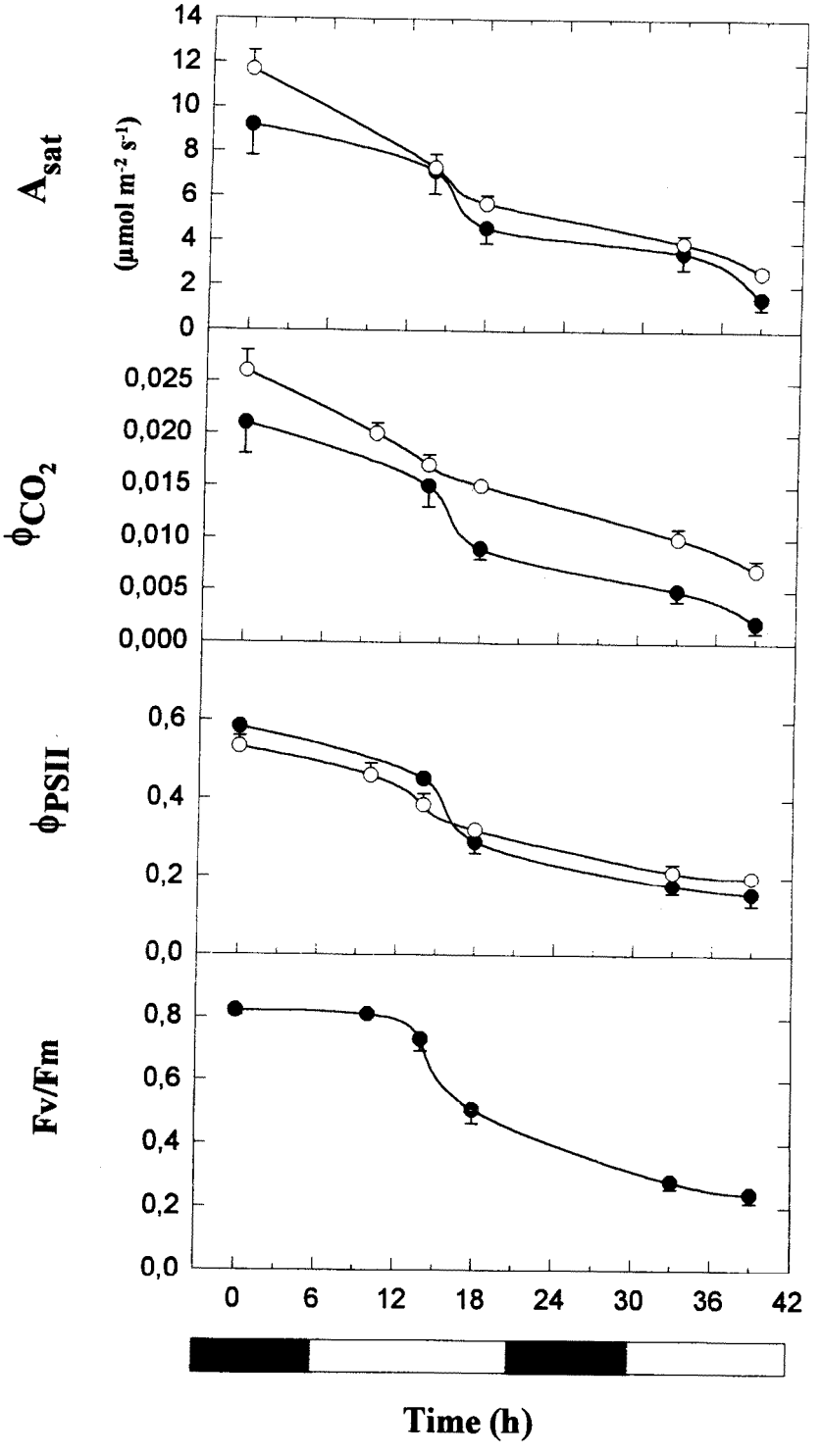

A_{sat}
(µmol m^{-2} s^{-1})
14
12
10
8
6
4
2
0
ϕCO_2
0,025
0,020
0,015
0,010
0,005
0,000
$\phi PSII$
0,6
0,4
0,2
0,0
Fv/Fm
0,8
0,6
0,4
0,2
0,0
0
6
12
18
24
30
36
42
Time (h)

Figure 13. Changes in the light saturated rate of CO_2 assimilation (A_{sat}) measured at a PPFD of 1500 μmol m^{-2} s^{-1}, the quantum yields of CO_2 assimilation (ϕ_{CO2}) and PSII electron transport (ϕ_{PSII}) measured at a PPFD of 450 μmol m^{-2} s^{-1}, and Fv/Fm throughout the high UV-B + UV-C treatment. All others details are as given in the legend of Fig. 12.

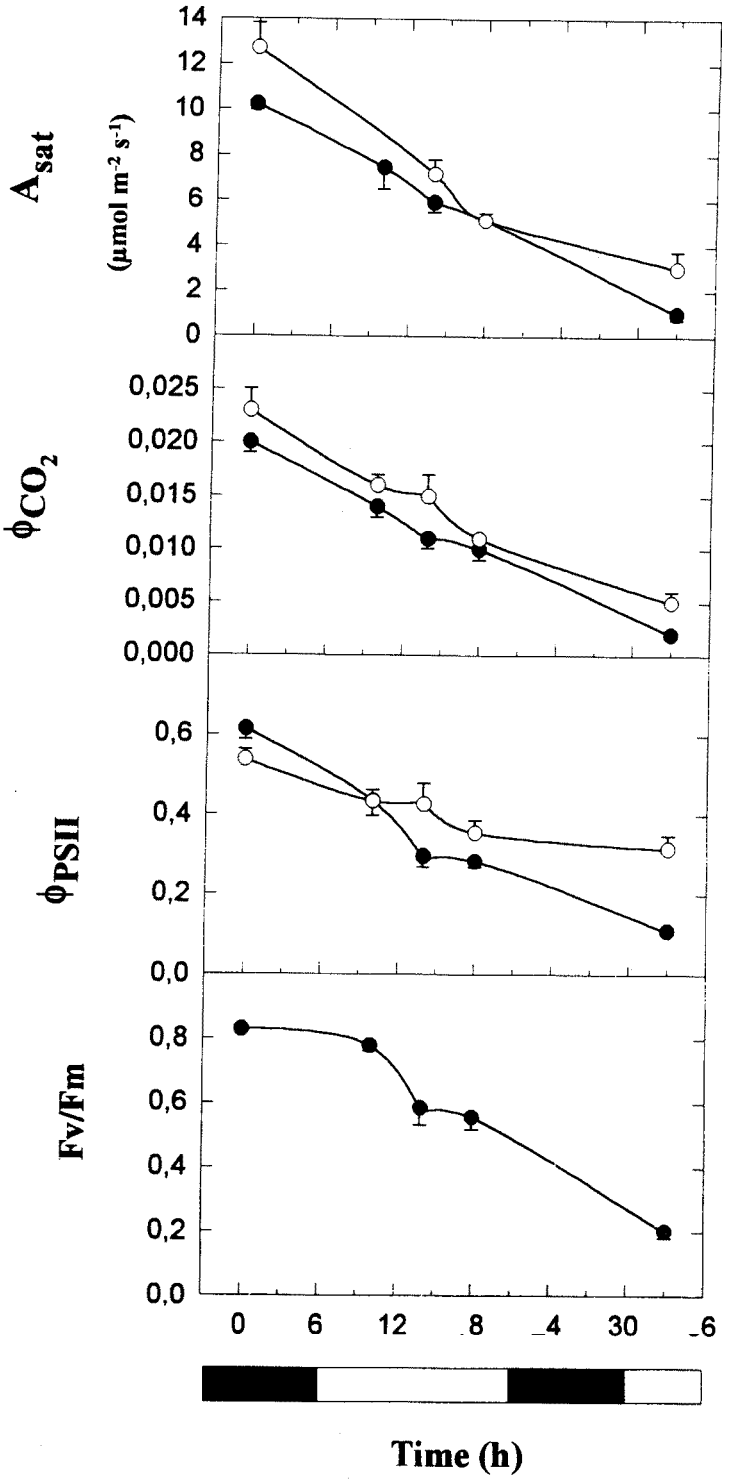
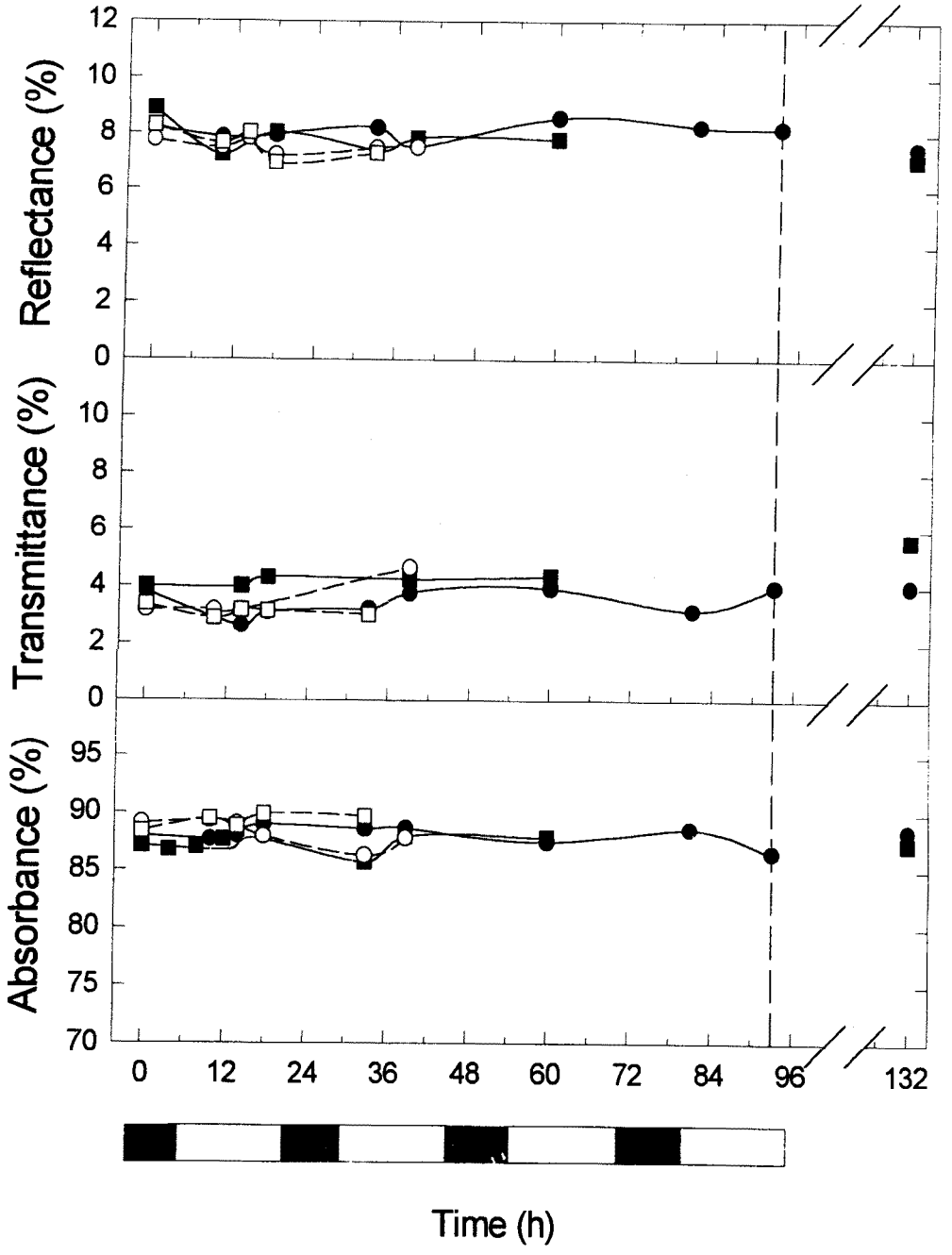

A_{sat}
(µmol m^{-2} s^{-1})
14
12
10
8
6
4
2
0
ϕCO_2
0,025
0,020
0,015
0,010
0,005
0,000
$\phi PSII$
0,6
0,4
0,2
0,0
Fv/Fm
0,8
0,6
0,4
0,2
0,0
0
6
12
8
4
30
6
Time (h)

Figure 14. Responses in the leaf absorbance, transmittance and reflectance measured at a PPFD of 450 μmol m^{-2} s^{-1} throughout the UV-B treatment. The open and solid boxes immediately under the abscissa indicate the photoperiods and dark periods, respectively, to which the leaves were exposed. Leaves were only exposed to UV during the photoperiods. The vertical dashed line indicates the time at which UV tubes were switched off. Data are the means of 4 replicates and the standard errors of the means were less than 10% of the mean values in all cases.


Reflectance (%)
Transmittance (%)
Absorbance (%)
12
10
8
6
4
2
0
95
90
85
80
75
70
0
12
24
36
48
60
72
84
96
132
Time (h)

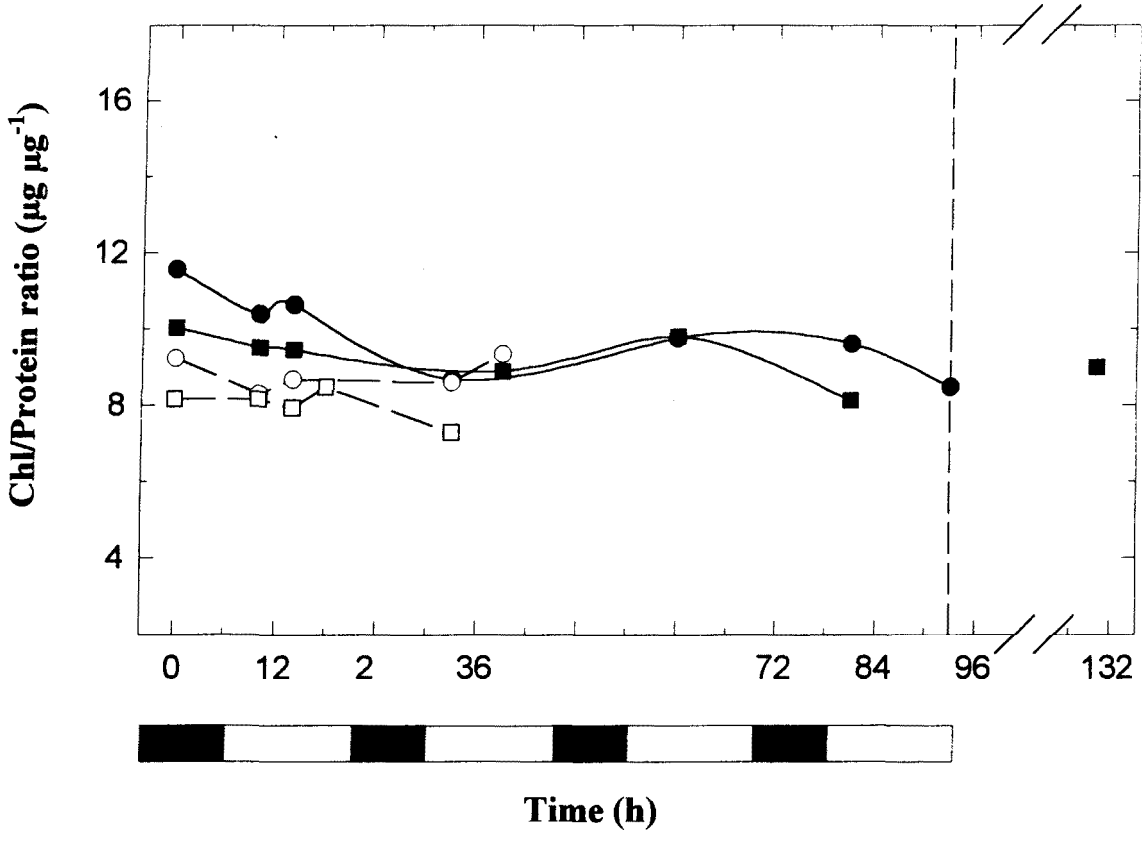
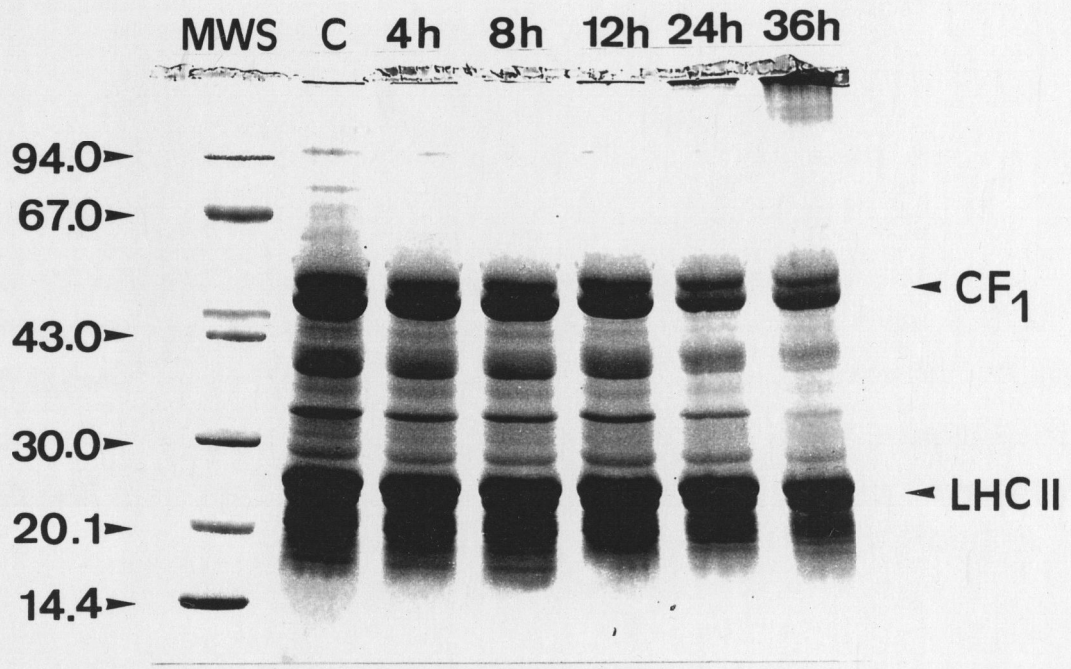


Figure 15. Changes in the chlorophyll/protein ratio of thylakoids isolated from leaves throughout the low UV-B (●), high UV-B (■), low UV-B + UV-C (○) and high UV-B + UV-C (□) treatments. The open and solid boxes immediately under the abscissa indicate the photoperiods and dark periods, respectively, to which the leaves were exposed. Leaves were only exposed to UV during the photoperiods. The vertical dashed line indicates the time at which UV tubes were switched off. Data are the means of 4 replicates and the standard errors of the means were less than 10% of the mean values in all cases.

Figure 16. Separation of thylakoid polypeptides from mature pea leaves exposed to the low UV-B + UV-C treatment by SDS-PAGE gel. Molecular weight standards are given in kDa units and the time of UV exposure are indicated. Both the 26-28 kDa (apoprotein of LHC2) and 57-58 kDa (α,β-subunits of CF_1) polypeptides are also indicated. Polypeptides were stained with Coomassie brilliant blue dye. All lanes were loaded with equal amounts of chl.

Figure 17. Separation of thylakoid polypeptides from mature pea leaves exposed to the high UV-B + UV-C treatment by SDS-PAGE gel. Molecular weight standards are given in kDa units and the time of UV exposure are indicated. All others details are as given in the legend of Fig. 16.


MWS
C
4h
8h
12h
24h
36h
94.0
67.0
43.0
30.0
20.1
14.4
CF1
LHC II

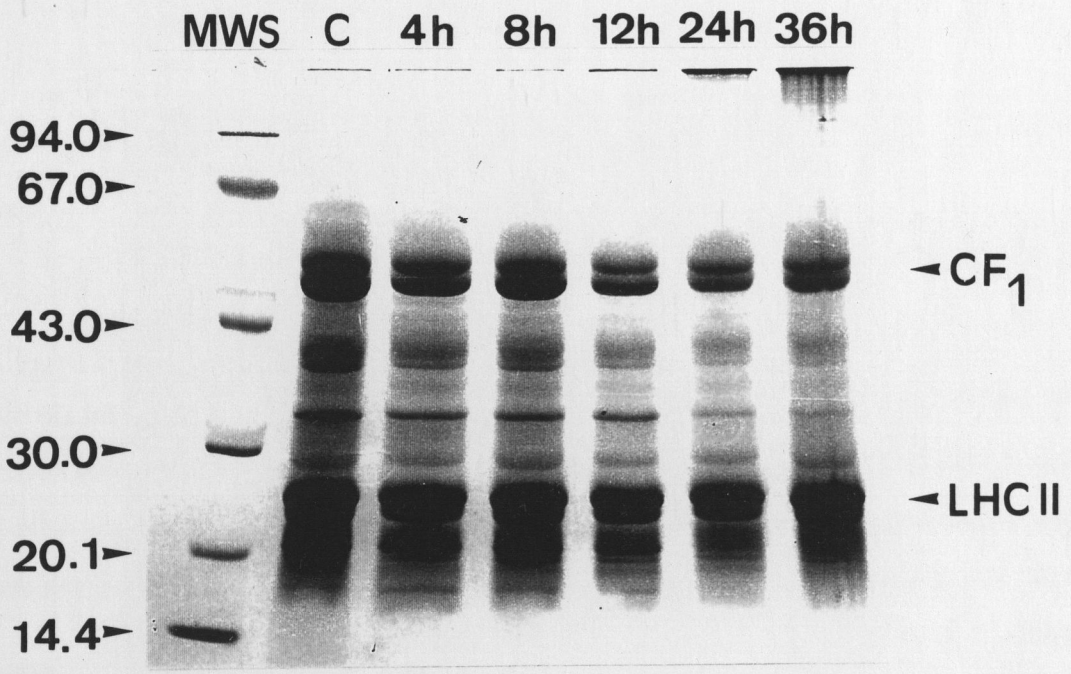
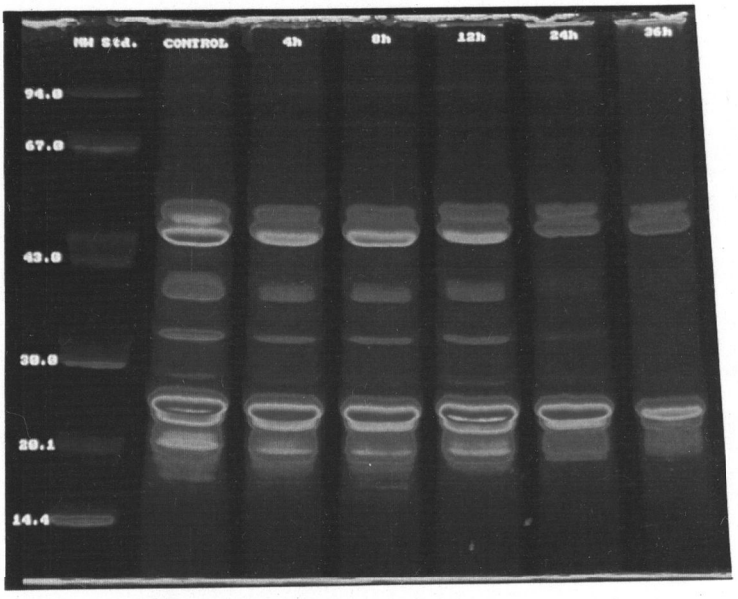
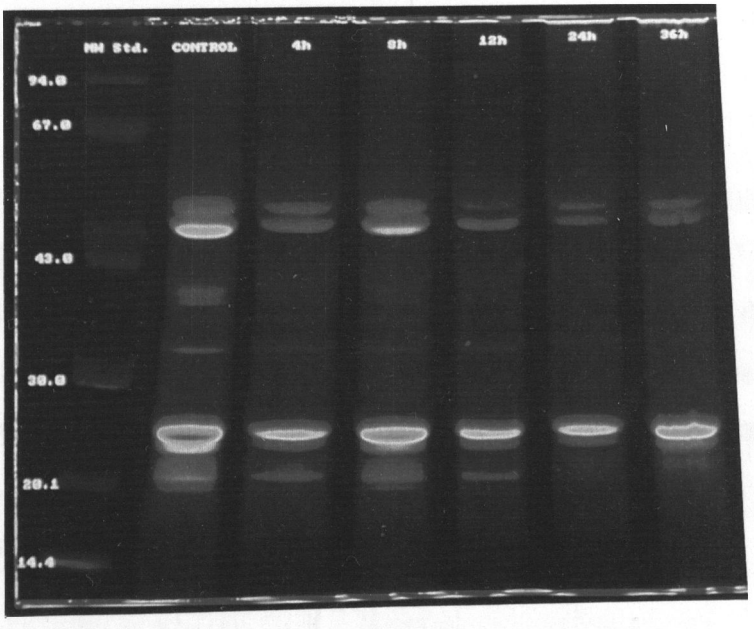

MWS
C
4h
8h
12h
24h
36h
94.0
67.0
43.0
30.0
20.1
14.4
CF1
LHC II

Figure 18. Laser-scanned pseudocolored image of the coomassie blue-stained gel shown in Figure 16. Picture was taken using a Polaroid QuickPrint video printer connected on line to the image processing and display system. Molecular weight standards are given in kDa units and the time of UV exposure to the low UV-B + UV-C treatment are indicated. Both the 57-58 kDa and 26-28 kDa polypeptide bands are more clearly shown.

Figure 19. Laser-scanned pseudocolored image of the coomassie blue-stained gel shown in Figure 17. Picture was taken using a Polaroid QuickPrint video printer connected on line to the image processing and display system. Molecular weight standards are given in kDa units and the time of UV exposure to the high UV-B + UV-C treatment are indicated. Both the 57-58 kDa and 26-28 kDa polypeptide bands are more clearly shown.


MM Std.
CONTROL
4h
8h
12h
24h
36h
94.0
67.0
43.0
30.0
20.1
14.4


MM Std.
CONTROL
4h
8h
12h
24h
36h
94.0
67.0
43.0
30.0
20.1
14.4

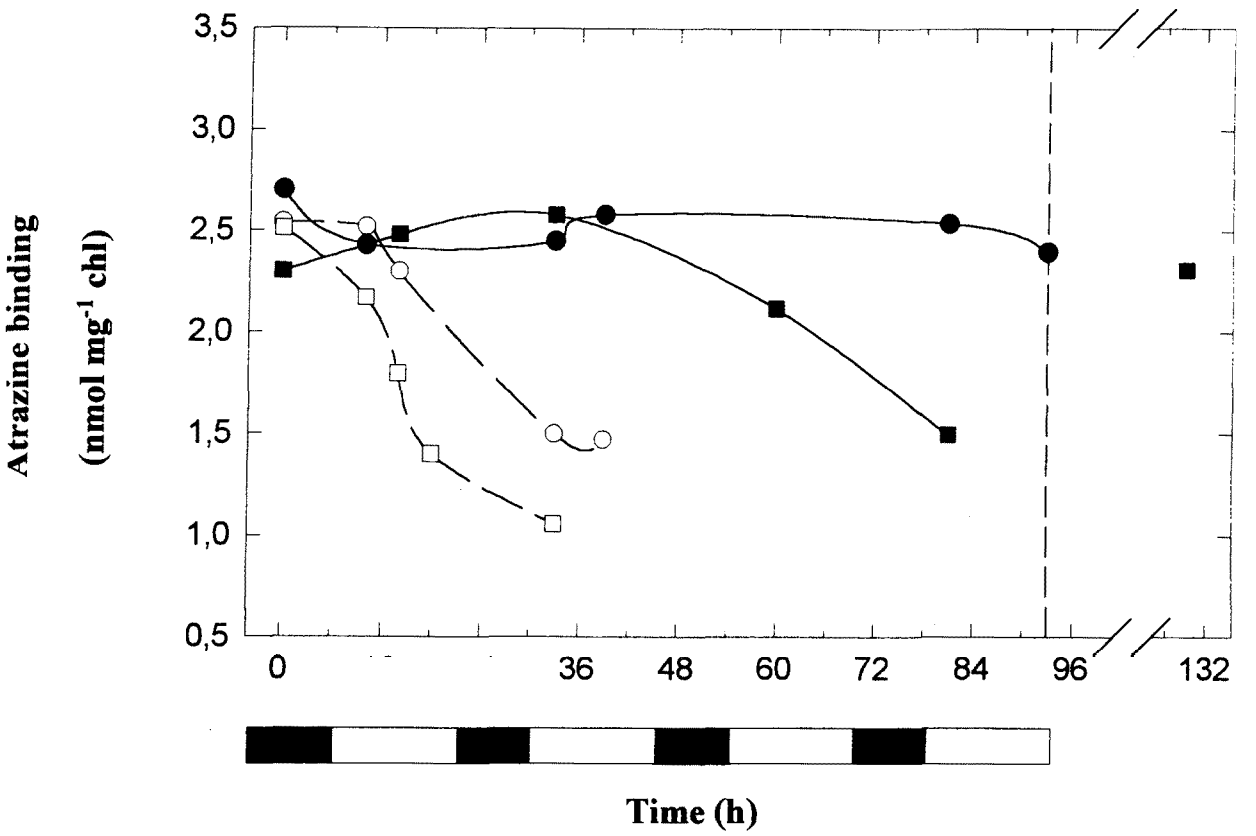


Figure 20. Changes in the binding of [^{14}C]atrazine to thylakoids isolated from leaves throughout the low UV-B (●), high UV-B (■), low UV-B + UV-C (○) and high UV-B + UV-C (□) treatments. The open and solid boxes immediately under the abscissa indicate the photoperiods and dark periods, respectively, to which the leaves were exposed. Leaves were only exposed to UV during the photoperiods. The vertical dashed line indicates the time at which UV tubes were switched off. Data are the means of 4 replicates and the standard errors of the means were less than 10% of the mean values in all cases.

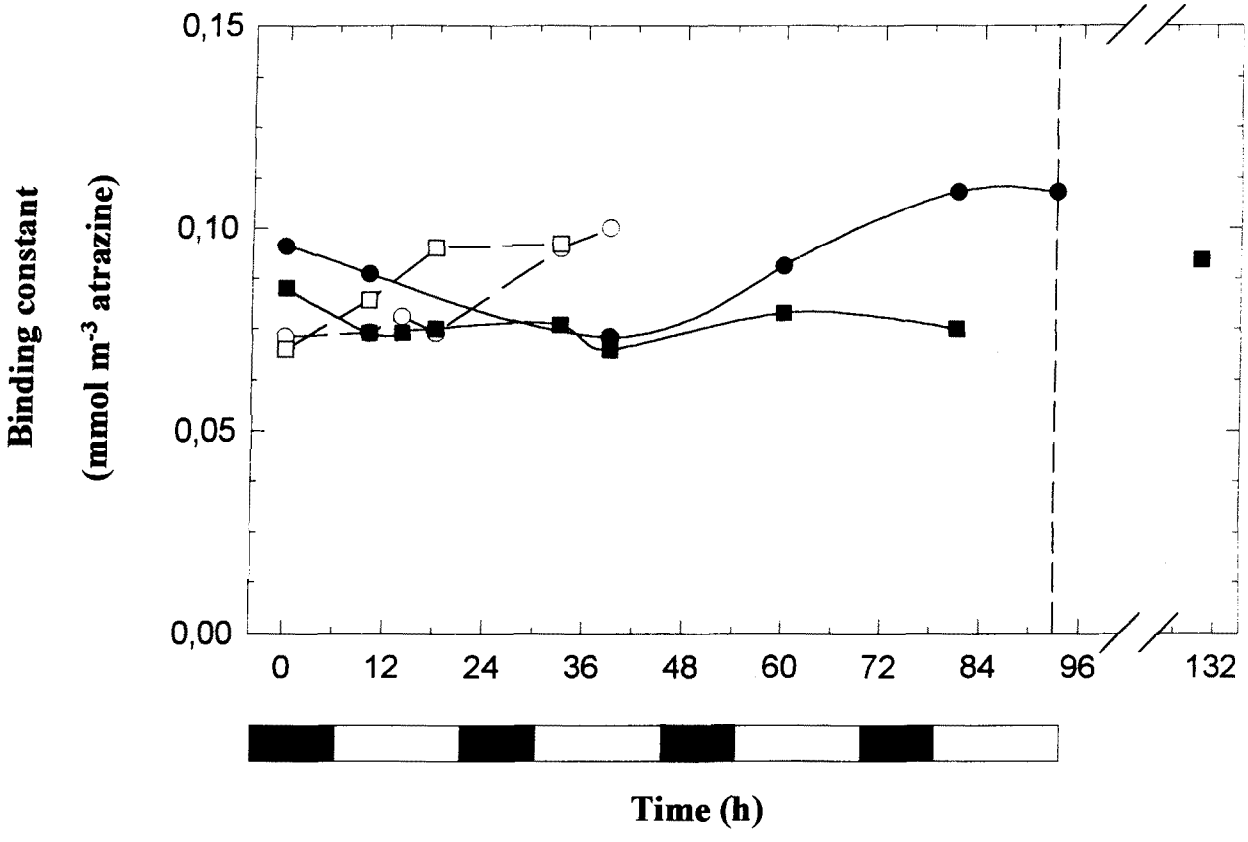


Figure 21. Changes in the binding constant of [^{14}C]atrazine to thylakoids isolated from leaves throughout the low UV-B (●), high UV-B (■), low UV-B + UV-C (○) and high UV-B + UV-C (□) treatments. Data are the means of 4 replicates and the standard errors of the means were less than 10% of the mean values in all cases. All others details are as given in the legend of Fig. 20.

The atrazine-binding capacity of isolated thylakoids is considered to be a quantitative measure of photoinhibitory damage to the plastoquinone-reductase site of PSII (Kyle *et al.*, 1984). The changes in atrazine-binding capacity of thylakoids isolated from leaves throughout the two UV-B and the two UV-B + UV-C treatments are shown in Figure 20. There were no significant decreases in the atrazine-binding capacity of thylakoids isolated from leaves throughout the four day low UV-B treatment. In the high UV-B treatment the atrazine-binding capacity decreased during the third and fourth photoperiods, indicating that photodamage to PSII complexes had occurred. Complete recovery of the PSII complexes from this photodamage was found to occur within 39 hours after removal of the UV-B irradiation (Fig. 20). Supplementing the UV-B treatments with UV-C was found to increase the photodamage to PSII, *i.e.* very large decrease in the atrazine-binding capacity of thylakoids occurred during these treatments.

The binding constant for atrazine was taken as the concentration of atrazine required to produce 50% saturating of the binding sites on the thylakoids. As we expected, the binding constant did not significantly change throughout the two UV-B and the two UV-B + UV-C treatments (Fig. 21), indicating that the quinone binding site on the D1 proteins of the PSII complexes was no affected during UV-B irradiation.

3.6. DISCUSSION

Modulated chlorophyll fluorescence was found to be an useful and rapid probe of UV-B effects on photosynthesis in pea leaves.

Throughout the low UV-B treatment only very small decreases in Fv/Fm were observed, however, these were accompanied by large decreases, *i.e.* more than 30%, in both the light-saturated rate and quantum efficiency of CO_2 assimilation (Fig. 10). During the first 15 hours photoperiod of exposure to the high UV-B treatment significant decreases in Fv/Fm occurred (Fig. 11), which are indicative of decreases in the maximum quantum efficiency of PSII photochemistry. This decrease in Fv/Fm was accompanied by large decreases in A_{sat}, ϕ CO_2, and in the modulated chlorophyll fluorescence parameter ϕ_{PSII}. Additional exposure to

UV-C radiation (Figs. 12-13) increases the rates of decreases of A_{sat}, $\phi CO2$, $\phi PSII$ and Fv/Fm.

The data in Figure 20 demonstrate that UV-B irradiation of pea leaves can result in photodamage to the plastoquinone-binding site of PSII complexes. However, such photodamage occurred only after two 15 hours photoperiods of exposure to the high UV-B treatment. When leaves were exposed to the low UV-B treatment for four photoperiods, no loss of atrazine binding capacity was observed. Further, additional exposure to UV-C increased the rate of photodamage to PSII (Fig. 20). Although no decreases in the atrazine-binding capacity of PSII were observed during the first photoperiod of exposure to this high UV-B treatment (Fig. 20), significant decreases in Fv/Fm occurred (Fig. 10) which are indicated of decreases in the maximum quantum yield of PSII primary photochemistry. Consequently, UV-B irradiation can result in decreases in the capacity for PSII photochemistry by means other than photodamage to the D1 protein. It is well established that irradiation of environmentally stressed leaves with ambient growth PPFDs can also produce decreases in Fv/Fm which have been attributed to slowly relaxing quenching of excitation energy in the antennae of PSII by zeaxanthin that is induced when the rate of excitation of the PSII reaction centres is in excess of the rate at which energy can be dissipated via linear electron transport through the PSII reaction centres (Demmig-Adams, 1990). Such light-dependent quenching of excitation energy has been shown in wheat leaves to increase with PPFD during the morning in the absence of any significant change in the herbicide-binding capacity of thylakoids isolated from the leaves (Groom and Baker, 1992). The data presented in this study are consistent with the UV-B-induced decreases in Fv/Fm, in the absence of any decreases in atrazine binding capacity of the thylakoids, being associated with slowly recovering light-induced increases in zeaxanthin quenching of PSII excitation energy that has resulted from a UV-B-induced decrease in the capacity of the leaf to dissipate excitation energy through linear electron transport. It can be seen that decreases in Fv/Fm do not occur in any of the four UV treatments until the capacity for light saturated CO_2 assimilation has fallen below *ca.* 6 $\mu mol\ m^{-2}\ s^{-1}$ (Figs. 10-13). Consequently, when the sink strength has fallen below the equivalent of this rate of CO_2

consumption, zeaxanthin quenching of excitation energy occurs and increases with decreasing sink strength.

The data in Figure 10 show that throughout the low UV-B treatment only very small decreases in Fv/Fm were observed, but, these were accompanied by large decreases in both A_{sat} and ϕ_{CO2}. Clearly, the UV-B treatment produced large decreases in CO_2 assimilation in the absence of any effect on PSII photochemistry. This implies that PSII is not the primary site associated with UV-B-induced inhibition of photosynthesis. This contention is also supported by the lack of recovery of A_{sat} and ϕ_{CO2} 39 hours after the termination of the low and high UV-B treatments while Fv/Fm recovers almost back to the control level (Figs. 10 and 11), which demonstrates that almost full recovery of PSII photochemistry can be achieved in the absence of no significant recovery of the ability to assimilate CO_2.

An increase of the UV-B irradiation dosages will eventually result in photodamage to the PSII reaction centres. This photodamage can be the direct result of absorption of UV-B by the reaction centre proteins or simply the consequences of the increasing UV-B-induced inhibition of CO_2 assimilation at a site other than PSII which results in photodamage to PSII by photosynthetically active radiation which cannot be dissipated though electron transport.

The data in Figures 10 to 13 also show that modifications to photorespiration cannot account for the UV-B-induced inhibition of photosynthesis. For all of the UV treatments similar decreases were found in A_{sat} and ϕ_{CO2} on inhibition of photorespiration, by reducing the atmospheric O_2 concentration from 21% to 2%. Inhibition of photorespiration had no significant effects on the rate of linear electron transport, as monitored by ϕ_{PSII} (Figs. 10-13), throughout all of the UV treatments.

Measurements of stomatal conductance on the pea leaves during the UV-B treatments indicated that this parameter does not change significantly and cannot account for the decreases in CO_2 assimilation (Table 6).

Consequently, Rubisco, the key enzyme of the Calvin cycle, remains as the major potential candidate for the primary site of inhibition of UV-B radiation on the photosynthetic apparatus, as already shown by Vu *et al.* (1984), Strid *et al.* (1990) and Jordan *et al.* (1992).

This study has shown that the relationships between A_{sat}, $\phi CO2$, $\phi PSII$ and Fv/Fm are similar throughout all of the UV treatments (Figs. 10-13). Increasing UV dosages (for both UV-B and UV-C irradiations) increases the rates of decrease of the parameters but their relationship remains similar. Consequently it follows that the mechanisms by which UV-B and UV-C produce an inhibition of photosynthesis in the pea leaves are similar.

No significant changes in the absorbance, reflectance and transmittance of the leaves were observed throughout the UV-B treatments (Fig. 14), although some morphological damage was observed in the leaves, particularly in pea plants exposed to UV-B + UV-C treatments. Morphological damage, plant stunting, leaf discoloration or reductions in vegetative biomass have also been observed in UV-B-irradiated plants by Teramura *et al.* (1990).

The SDS-PAGE gels in Figures 16 and 17 demonstrate clearly that thylakoid polypeptide bands from mature pea leaves exhibit markedly changes during UV irradiation. Four major polypeptide bands decrease during UV irradiation, although the 26-28 kDa (apoprotein of LHCII) and the 57-58 kDa (α and β-subunits of the coupling factor) bands are still visible after 36h of UV irradiation.

The binding constant for atrazine did not significantly change during UV-B irradiation (Fig. 21) and was maintained *ca.* 0.08±0.02 mmol m^{-3} atrazine in all UV treatments. Similar value was obtained by Habash and Baker (1990) on thylakoids isolated from control wheat leaves.

3.7. CONCLUSIONS

Modulated chlorophyll fluorescence was an useful and rapid probe of UV-B radiation effects on photosynthesis in pea leaves grown under controlled environment conditions.

Enhanced UV-B irradiation of pea leaves resulted in photodamage to PSII reaction centres. Additional exposure to UV-C increased the rate of photodamage to PSII reaction centres.

The mechanisms by which UV-B and UV-C produce an inhibition of photosynthesis in pea leaves are similar, since addition of UV-C to the UV-B treatments increased markedly the rate of inhibition of photosynthesis, however the relationships between CO_2 assimilation and PSII characteristics remained the same.

Photosystem II is not the primary target site involved with the onset of the inhibition of photosynthesis in pea leaves induced by irradiation with UV-B.

Stomatal conductance does not change significantly during UV-B irradiation.

Leaf absorbance, reflectance and transmittance do not change significantly during the days of exposure of pea leaves to the UV-B irradiation.

Four major polypeptide bands of apparent molecular weights 57-58, 38, 33 and 26-28 kDas decrease during UV irradiation, although the 57-58 kDa and the 26-28 kDa bands are still visible after 36h of UV irradiation.

The changes in atrazine-binding capacity of thylakoids isolated from pea leaves throughout the four UV treatments demonstrate that UV-B irradiation can result in photodamage to the plastoquinone-binding site of PSII complexes.

The binding constant for atrazine did not significantly change during UV-B irradiation.

FIELD STUDY OF THE EFFECTS OF UV-B RADIATION ON MODULATED CHLOROPHYLL FLUORESCENCE AND LEAF GAS EXCHANGE

CHAPTER 4:

FIELD STUDY OF THE EFFECTS OF UV-B RADIATION ON MODULATED CHLOROPHYLL FLUORESCENCE AND LEAF GAS EXCHANGE

4.1. ABSTRACT

In this study a closed loop feedback system, that provides a UV-B supplement in proportion to ambient UV-B, was used to simulate the effect of an enhanced level of UV-B resulting from an annual mean 15% reduction in stratospheric ozone on the photosynthetic performance of pea plants grow under field conditions.

Overall the enhanced UV-B treatment had no significant effect on Fv/Fm, the quantum efficiency of PSII electron transport, and the photochemical and non-photochemical quenching of chlorophyll fluorescence at different times of the day in the field or on the light response curves for PSII electron transport and CO_2 assimilation rate of detached leaves. These results suggest that reduction in growth parameters of plants exposed to such a 15% enhanced UV-B radiation may be due to direct effects of UV-B on plant growth rather than a decrease in photosynthetic capacity.

4.2. INTRODUCTION

The majority of studies on the responses of plants to enhanced UV-B radiation have been conducted under controlled environment conditions. Consequently, there is little information on which to base predictions of how plants will respond to increasing UV-B radiation in the field, where they are subjected to various environmental stresses. Only few field studies have been performed illustrating the degree of technical difficulty and uncertainty surrounding the estimates of UV-B effects on yield.

Numerous studies have shown that UV-B radiation affects the growth and physiology of many plant species under a variety of growing conditions. These effects include physiological damage to the photosynthetic apparatus, alteration in protein content and enzyme activity, effects on membranes and changes in leaf biochemistry. Morphological damage, plant stunting, leaf discoloration (chlorosis or bronzing) or reductions in vegetative biomass and seed yield have also been observed (Teramura *et al.*, 1990).

This decrease in plant growth have often be correlated to reductions in CO_2 assimilation and chlorophyll fluorescence (Strid *et al.*, 1990; Teramura *et al.*, 1991). However,

such experiments frequently compare plant growth under very high levels of UV-B radiation and/or are conducted under low levels of PPFD. Such conditions can be expected to increase plant sensitivity to damage by enhanced UV-B (Teramura, 1980; Teramura *et al.*, 1980; Caldwell *et al.*, 1994). Furthermore, in these experiments UV-B is generally the only significant environmental stress.

To overcome some problems of controlled environment experiments research effort has increasingly moved towards applying enhanced UV-B in the field. In some instances supplementary UV-B radiation had been applied to the plants as a fixed amount over a specified time period (Lydon *et al.*, 1986). However this approach generally takes no account of variation in cloud cover and enhancement levels have frequently been based on the predicted consequences of ozone depletion under clear sky conditions at the summer solstice (Sullivan and Teramura, 1992; Sullivan *et al.*, 1994). These problems have been overcome by the development of controlled feedback systems, which provide a constant proportion of UV-B radiation in a modulated fashion while monitoring the ambient solar input (Caldwell *et al.*, 1983).

In the third chapter we have demonstrated that UV-B radiation can result in decreases in CO_2 assimilation and chlorophyll fluorescence parameters by short term greenhouse experiments at relatively high light intensity (PPFD > 450 μmol m^{-2} s^{-1}). Although glasshouse and laboratory experiments may provide valuable information on the mechanisms of UV-B damage in plants, they do not simulate the consequences of future ozone depletion. In any case, the assessment of potential consequences of ozone layer reduction is greatly complicated since those experiments which are conducted in the field are necessarily more difficult and less well controlled that those conducted under growth chamber conditions (Caldwell, 1981).

Modulated chlorophyll fluorescence has now reached a point of sophistication and reliability to be applied for the assessment of *in vivo* photosynthesis under field conditions. Instrumentation and methods are available with which it is possible to judge various aspects of photosynthetic performance of plants in their natural environment. Furthermore, highly selective modulation fluorometers, with which fluorescence yield can be measured in full sunlight, are small and low in power consumption, such that they are well suited for field measurements (Schreiber *et al.*, 1995).

4.3. AIMS

The aim of this study is to evaluate the effect of an enhanced level of UV-B resulting from an annual mean 15% reduction in stratospheric ozone on chlorophyll fluorescence characteristics and CO_2 assimilation rate of plants grown under field conditions.

4.4. MATERIALS AND METHODS

The experiment was arranged in a randomised complete block design, consisting of 2 treatments (control and enhanced UV-B radiation) in 5 replicate blocks. Within each block there were 2 aluminium frames (4m long x 1.3m wide x 2.4m tall) with active UV-B lamp arrays and 2 identical frames with unconnected lamps to provide control conditions. The area under and immediately adjacent array was divided into 2 plots (1.8 x 1.5m), thus making a total of 20 plots per treatment.

Each lamp array consisted of 8 UV-B fluorescent tubes (Philips TL40W/12) supported on an adjustable aluminium frame which allowed lamps to be raised to maintain a constant height (*ca.* 1.5m) above the canopy as the crop developed.

Output from the 80 active lamps was varied by the use of 40 adjustables ballasts controlled by 2 thyristor dimmers. The 2 plots under each array contained a sub-plot (84 x 36cm) with *ca.* 28 plants, within which the UV-B supplement varied by less than ±5% at crop height. Between the 10 active arrays lamp output at this height varied by less than ±7% at minimum and less than ±4.5% at maximum output. Lamps could be reliably dimmed to *ca.* 10% of maximum output, at which point they were automatically switched off.

To remove radiation below 290 nm, cellulose diacetate sheets were wrapped around each active UV-B tub.

A closed loop control system delivered enhanced UV-B as a proportional addition to that under a control array. This was achieved by measuring radiation under a control and treatment array with 2 UV-B sensors (BW100 UV-B, Vital Technologies, Bolton, Canada). Lamp output was adjusted to maintain a fixed percentage difference between the sensors.

Sensors signals were monitored and lamp output was controlled by a data acquisition and a control program using a computer.

Sensors were calibrated against both daylight and lampspectra with a spectroradiometer (SR9910-PC, Macam Photometrics, Livingston, U.K.). Since daylight UV-B spectrum varies with solar angle, this calibration was performed from 8 a.m. until solar noon and was weighted to maximise accuracy at high irradiances. To compensate for seasonal variation in the daylight UV-B spectrum, this calibration was repeated every 7 to 14 days.

A complete description of the experimental design, lamp control and sensor calibration can be found in Mepsted *et al.* (1995).

4.4.1. PLANT MATERIAL AND GROWTH CONDITIONS

Seeds of combining pea (*Pisum sativum* L., cv. Guido) were sown (4cm deep and 12cm apart) in beds (1.8m x 2.3m) in the field at Horticulture Research International (Wellesbourne, U.K.) on the tenth and eleventh June 1994. Within each bed only plants in the subplot (described above) were measured (Mepsted *et al.*, 1995).

4.4.2. UV TREATMENT

The level of UV-B supplement required (*i.e.* percentage of UV-B increase over ambient) was calculated using the modified radiation transfer model of Björn and Murphy (1985) and the PAS300 action spectrum (Caldwell, 1971). The model was run to simulate an annual mean ozone depletion of 15% and was developed by Dr. Nigel Paul at Lancaster University (Lancaster, U.K.), which was adjusted for seasonal variations in ozone column.

Over the period of the experiment (from June to August) the mean irradiance for control and enhanced treatments were 1.75 and 2.17 kJ m^{-2} d^{-1} PAS300 respectively, which corresponded to an average enhanced of 23.3%. However, this was a 23% enhanced over that in control plots which, due to shading from the array structure, receive an average 12.5% less

UV-B radiation than ambient. Therefore the control and plus UV-B treatments were respectively -12.5 and +10.8% compared to true ambient (Mepsted *et al.*, 1995).

At mid July when chlorophyll fluorescence and gas exchange measurements were obtained the mean irradiance for control and enhanced treatments were 2.88 and 3.56 kJ m^{-2} d^{-1} PAS300 respectively, corresponding to an average enhanced of 23.6% (Fig. 22).

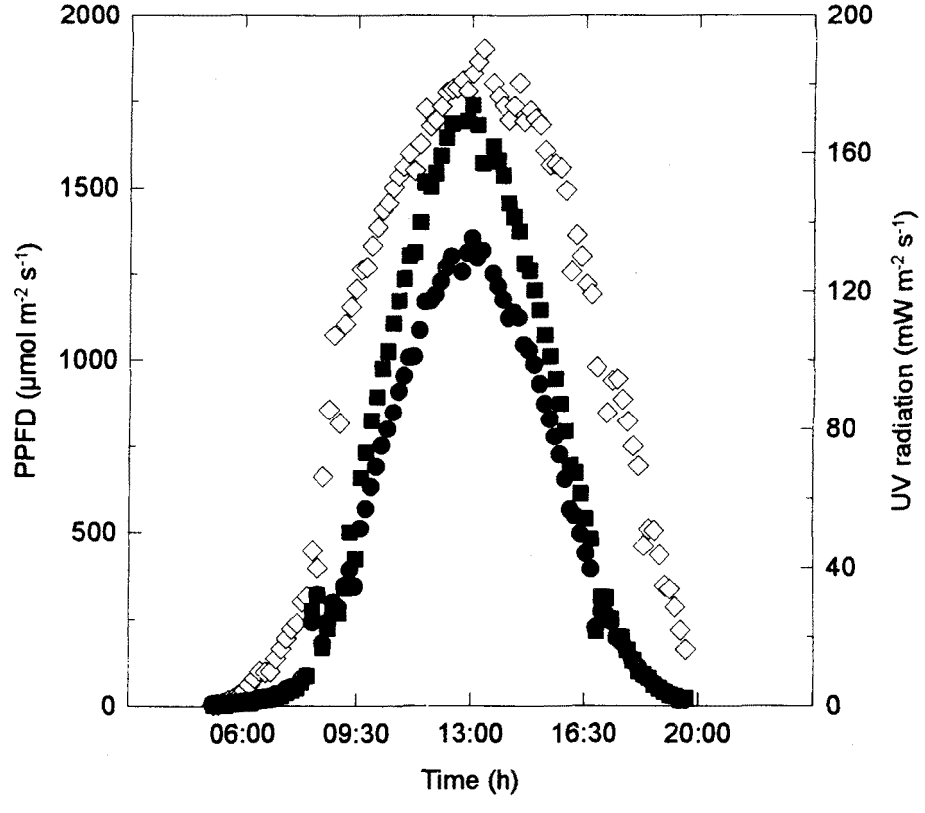


Figure 22. Ambient (●) and enhanced (■) UV radiation, and PPFD (◊) during a sunny summer day (20/7/94) in the U.K.. Mean irradiance for ambient and enhanced treatments were 53.26 and 66.04 mWatt m^{-2} d^{-1}, respectively.

4.4.3. MODULATED CHLOROPHYLL FLUORESCENCE AND LEAF GAS EXCHANGE

Between the thirty-fifth and forty-second day of the experiment, modulated chlorophyll fluorescence of the youngest fully expanded leaf was assessed during the day in the early morning (between 7:30 and 8:00), at noon (between 12:30 and 13:00) and in the late afternoon (between 17:30 and 18:00), using a PAM-2000 portable fluoremeter (Heinz Walt, Mess-und Regeltecnick, Effeltrich, Germany). As well as measuring Fv/Fm, ϕ_{PSII}, q_P, q_N and Fv'/Fm' were calculated (see section 1.2.2.1.). Changes in Fv/Fm ratio were also followed through the day from 8 a.m. to 8 p.m. on enhanced UV-B treated and control leaves.

With the PAM-2000, the measuring lamp (max. emission λ=655 nm and intensity *ca.* 0.1 μmol m^{-2} s^{-1}) was switched on and Fo was stored. Then, a saturating pulse *ca.* 10000 μmol m^{-2} s^{-1} was given to determine Fm and Fv/Fm was calculated. The actinic light source (from the red LED having a peak λ=655 nm and 300 μmol m^{-2} s^{-1}) was switched on. At steady state photosynthesis, full quenching analysis was obtained by accessing the "special analysis" mode. Once in this mode, the leaf was illuminated with a 5s pulse of far-red light (from the far-red LED having a peak λ=735nm and *ca.* 50 μmol m^{-2} s^{-1}), during which Fo' was stored. A saturating pulse automatically followed to determine Fm' and ϕ_{PSII} was calculated (see section 1.2.2.1.). From this, the quenching coefficients (q_P and q_N) were calculated. At high ambient light intensities, the far-red source of the PAM-2000 is not powerful enough to re-oxidise Q_A, and thus does not accurately determine Fo'. So, under high ambient light the leaf was covered during the far-red pulse. Leaves were dark-adapted for 3 minutes before any measurements were done. This dark adaptation period allowed dissipation of high energy state, but allows monitoring of the more slowly relaxing zeaxanthin quenching (see section 3.2.6.1.).

At the end of this measuring period, leaves were removed from the plants and taken to the laboratory where CO_2 assimilation and modulated chlorophyll fluorescence parameters, *i.e.* ϕ_{PSII}, q_P, q_N and Fv'/Fm', were measured throughout a PPFD range from dark to 1600

$\mu mol\ m^{-2}\ s^{-1}$ using an open gas exchange system (ADC LCA-3, Analytical Development Co., Hoddesdon, Herts, U.K.) and the PAM-2000 fluoremeter.

The ADC LCA-3 consists of four units: an infrared gas analyser (LCA), a leaf cuvette (PLC), an apparatus to supply air to the cuvette (ASU), and a data processor/logger (DL). With this data processor/logger, net photosynthesis was calculated from measurements of CO_2 partial pressure in air entering and leaving the cuvette as measured by LCA, mass flow of air into cuvette, atmospheric pressure, relative humidity (%) in cuvette, photon flux density incident on cuvette and cuvette air temperature (Winner *et al.*, 1989), using equations developed by von Caemmerer and Farquhar (1981).

Meteorological data (*i.e.* relative humidity, PPFD, air and soil temperatures) from the fifteenth to the twenty-second day of July in the field at the Horticulture Research International can be found in Figure 23.

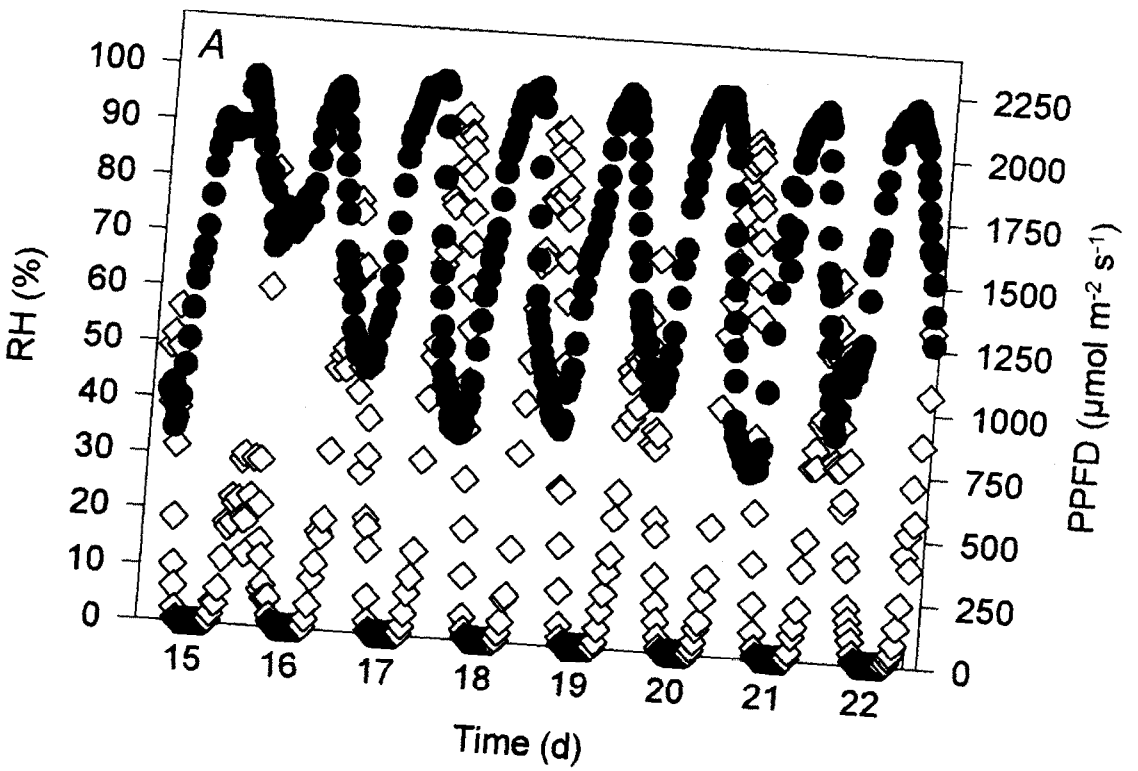
4.5. RESULTS

The effect of an enhanced UV-B radiation on the Fv/Fm, quantum efficiency of PSII electron transport, photochemical and non-photochemical quenchings, and Fv'/Fm' in the morning, at noon and late afternoon are shown in Table 7. Changes in Fv/Fm ratio were also followed through the day from 8 a.m. to 8 p.m. on enhanced UV-B treated and control leaves. Data were correlated with changes in air temperature and PPFD (Fig. 24). A decrease in Fv/Fm was found at midday, but no significant difference between enhanced UV-B treated and control leaves was found on the parameter studied.

Changes in the ϕ_{PSII}, q_P, q_N and Fv'/Fm' as a function of a PPFD range from dark to 1600 $\mu mol\ m^{-2}\ s^{-1}$ in excised pea leaves are shown in Figure 25. The light dosage response curves of CO_2 assimilation for excised pea leaves is shown in Figure 26.

Overall the enhanced UV-B treatment had no effect on Fv/Fm and the modulated chlorophyll fluorescence parameters (*i.e.* ϕ_{PSII}, q_P, q_N and Fv'/Fm') at different times of the day in the field (Table 7). This increased UV-B radiation had also no significant effect on the light response curves for the modulated chlorophyll fluorescence parameters, *i.e.* ϕ_{PSII}, q_P,q_N and Fv'/Fm', (Fig. 25) and CO_2 assimilation rate (Fig. 26) of detached leaves.

Figure 23. Meteorological data from the fifteenth to the twenty-second day of July in the field at Horticulture Research International. *A* graph: Relative humidity (●) and PPFD (◊). *B* graph: Air (■) and soil (□) temperatures.


A
RH (%)
PPFD (µmol m-2 s-1)
Time (d)
100
90
80
70
60
50
40
30
20
10
0
2250
2000
1750
1500
1250
1000
750
500
250
0
15
16
17
18
19
20
21
22

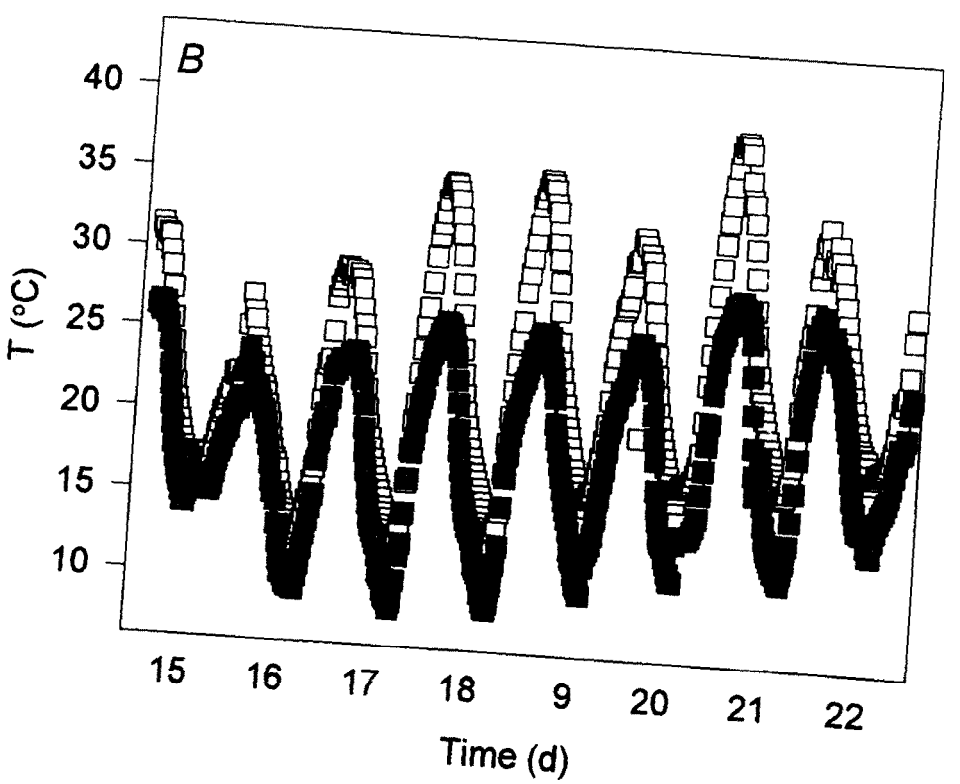

B
T (°C)
Time (d)
40
35
30
25
20
15
10
15
16
17
18
9
20
21
22

Table 7. Effect of enhanced UV-B radiation on Fv/Fm and the modulated chlorophyll fluorescence parameters (ϕ_{PSII}, q_P, q_N and Fv'/Fm') at different times of sunny summer days (from the fifteenth to the twenty-second day of July). Measurements of Fv/Fm were made after dark adaptation for 3 minutes and the modulated chlorophyll fluorescence parameters at a PPFD = 300 μmol m^{-2} s^{-1}. Values are the mean ± standard error of 8 replicate plants. Air temperature (T, °C) and photosynthetically-active photon flux density (PPFD, μmol m^{-2} s^{-1}) are also indicated.

			Fv/Fm		ϕ_{PSII}		q_P		q_N		Fv'/Fm'	
Time	T	PPFD	Control	+UVB	Control	+UVB	Control	+UVB	Control	+UVB	Control	+UVB
08:00	15.0	748	0.776±0.008	0.756±0.016	0.616±0.012	0.598±0.011	0.867±0.017	0.860±0.010	0.231±0.026	0.198±0.026	0.712±0.010	0.695±0.014
13:00	23.5	1948	0.749±0.015	0.761±0.008	0.625±0.019	0.655±0.018	0.899±0.011	0.907±0.016	0.250±0.055	0.188±0.029	0.695±0.018	0.720±0.019
18:00	25.7	1031	0.787±0.004	0.794±0.005	0.601±0.008	0.562±0.023	0.836±0.008	0.755±0.031	0.281±0.038	0.379±0.032	0.718±0.005	0.728±0.024

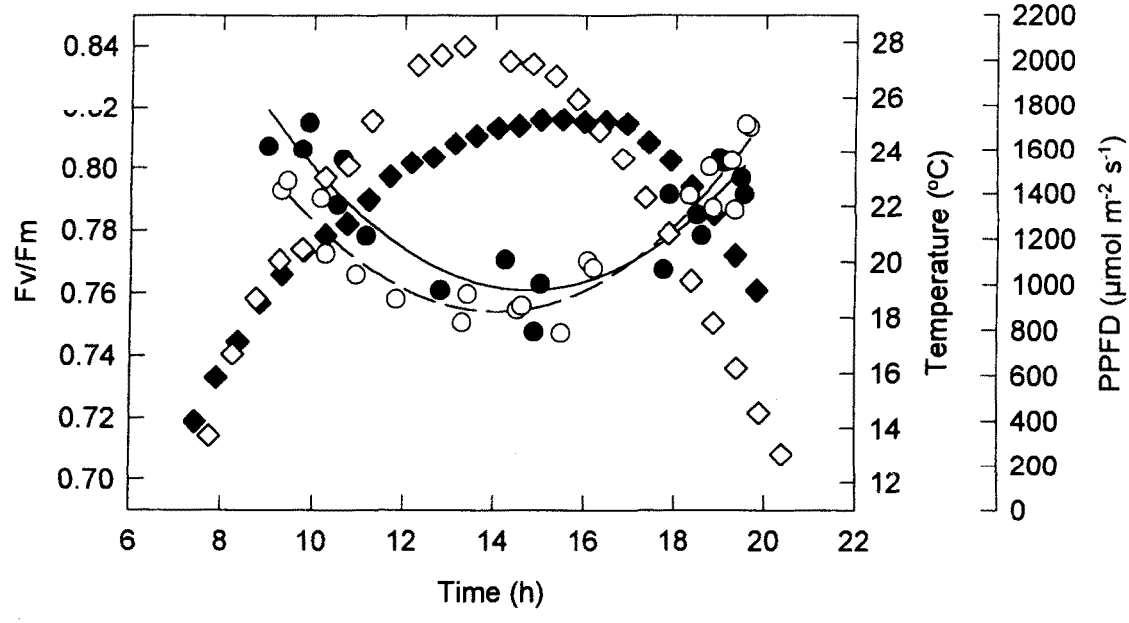
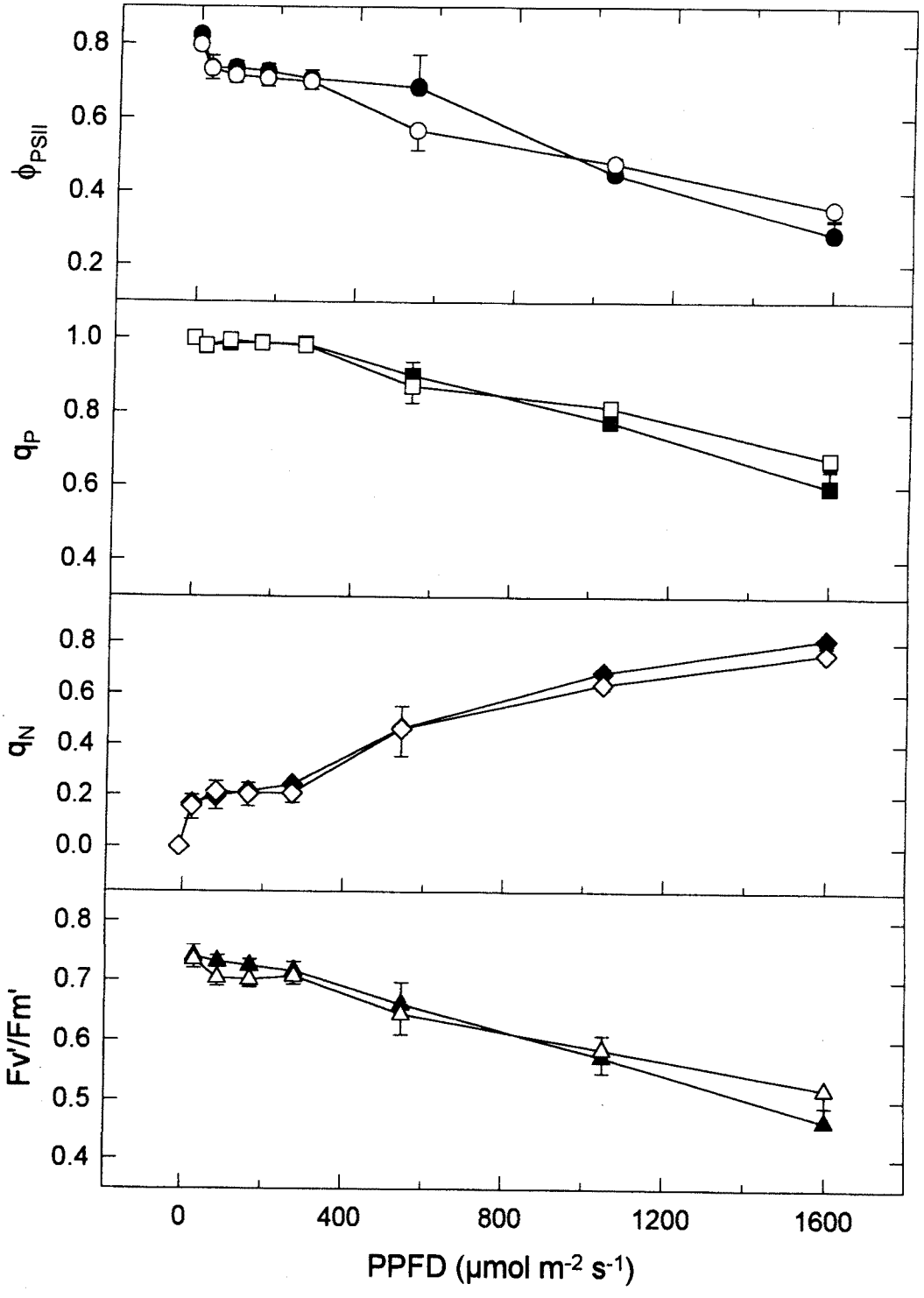


Figure 24. Changes in Fv/Fm ratio of control (●) and enhanced UV-B treated (○) leaves, air temperature (◆) and PPFD (◊) through the day. Data are the means of 4 replicates; the standard errors of the means were less than 10% of the mean values in all cases.

Figure 25. Changes in the quantum efficiency of PSII photochemistry (ϕ_{PSII}), photochemical (q_P) and non-photochemical (q_N) quenchings, and the Fv'/Fm' ratio as a function of PPFD in excised pea leaves taken from sunny summer days (from the fifteenth to the twenty-second day of July). Solid (●) and open (○) symbols are control and enhanced UV-B treated leaves, respectively. Data are the means of 5 replicates and the standard errors are shown when large than the symbols.


ϕ_{PSII}
0.8
0.6
0.4
0.2
q_P
1.0
0.8
0.6
0.4
q_N
0.8
0.6
0.4
0.2
0.0
Fv'/Fm'
0.8
0.7
0.6
0.5
0.4
0
400
800
1200
1600
PPFD (µmol m^{-2} s^{-1})

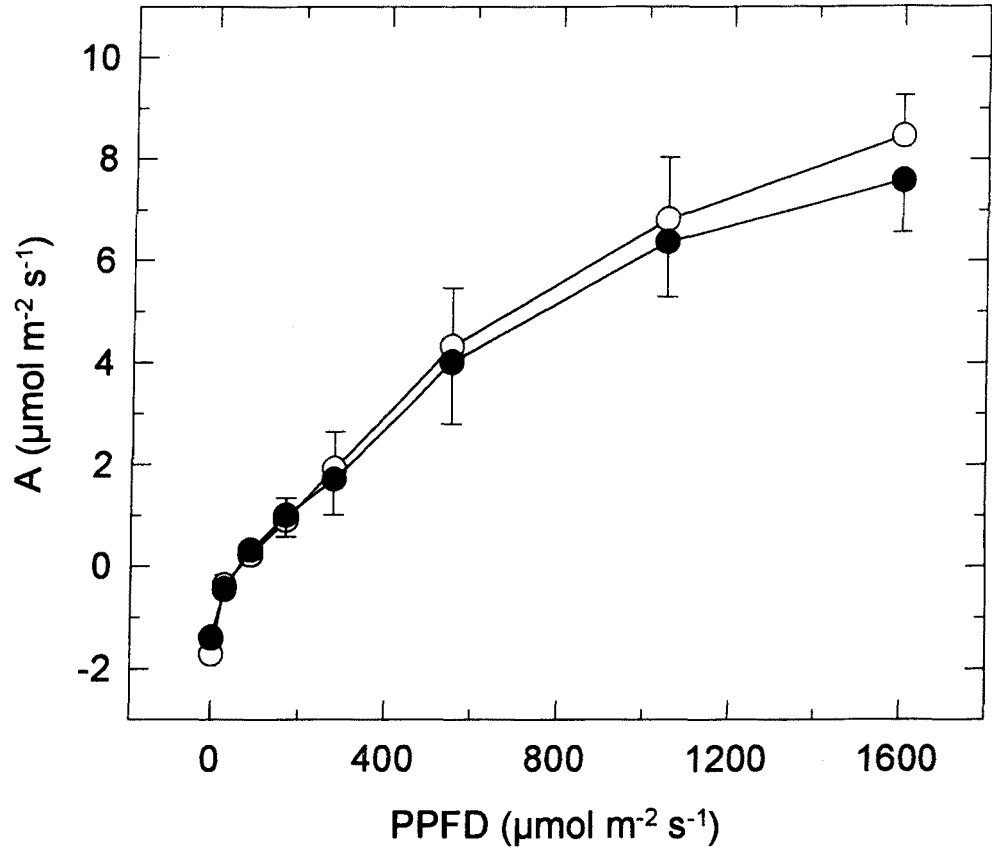


Figure 26. Light dosage response curves of CO_2 assimilation for excised pea leaves taken from sunny summer days (from the fifteenth to the twenty-second day of July). Solid (●) and open (○) symbols are control and enhanced UV-B treated leaves, respectively. Data are the means of 5 replicates and the standard errors are shown when large than the symbols.

4.6. DISCUSSION

The enhanced UV-B treatment had no effect on Fv/Fm and the modulated chlorophyll fluorescence parameters, *i.e.* ϕ_{PSII}, q_P, q_N and Fv'/Fm', at different times of the day in the field (Table 7 and Fig. 24). These results are consistent with the Fv/Fm decreases maximally around mid-day and then the increases through the afternoon as the PPFD decreased showed by Baker *et al.* (1994). Primarily light-induced reduction of the photochemical capacity appears also at noon in leaves exposed to full sunlight, but a restoration of Fv/Fm takes place until the evening (Bolhar-Nordenkampf *et al.*, 1991).

As we expected q_P decreased and q_N increased in the leaves with increasing irradiance (Fig. 25). Consequently, ϕ_{PSII} also declines with increasing irradiances since this is defined by the product of Fv'/Fm' and q_P (Genty *et al.*, 1989). Baker (1991) has shown that pretreatment of the leaves in high light induced a 26% decrease in Fv'/Fm', indicative of a large increase in non-photochemical quenching with concomitant decrease of 12% and 14% in ϕ_{PSII} and ϕ_{CO2} respectively. The decrease in ϕ_{PSII} must be attributed to an increase in non-photochemical quenching, since high light pretreatment results in a decrease in q_P, thus demonstrating that in high light-treated leaves less proportion of PSII reaction centres are oxidised than in dark-adapted leaves. Such a decrease in q_P would decrease ϕ_{PSII}. On the other hand, the decreases in ϕ_{PSII} and q_P and increase in q_N at high irradiance where not clearly found at noon in the field (Table 7), indicating that in the field other stress can account for the phenomenon. Further, the decline in Fv'/Fm' with increasing irradiance (Fig. 25) is indicative of an increase in the probability of a photon absorbed by PSII antennae being dissipated as heat, and demonstrates the occurrence of down-regulation of photosynthesis (Baker, 1993).

Measurement of most growth parameters, which has been done by others authors in the same field trial (see Mepsted *et al.*, 1995) indicated small, but often statistically significant reductions in growth as response to enhanced UV-B radiation. Mepsted *et al.* (1995) found that enhanced UV-B resulted in a 4.7% and 8.7% reductions in the number of stems and total stem length per plant, respectively. There were also found significant decreases in the dry weight of peas (10.1%) and total dry weight (8.9%) per plant. Similar small reductions in

growth parameters have been observed in field conditions with soybean (D'Surney *et al.*, 1993), loblolly pine (Sullivan and Teramura, 1992) and sweetgum (Sullivan *et al.*, 1994).

In previous studies of pea (Strid *et al.*, 1990; Teramura *et al.*, 1991) reductions in growth of plants that have been exposed to enhanced UV-B radiation have been associated with diminished chlorophyll fluorescence characteristics and CO_2 assimilation rate. In this study no such reductions in photosynthesis parameters were observed (Figs. 24-26). This discrepancy may be because in these previous studies and also in the greenhouse studies (chapter 3) very high levels of UV-B were applied to plants that were previously unexposed to UV-B radiation, *i.e.* having been exposed to low levels of UV-A and PPFD (this is not the case in the greenhouse studies - chapter 3). Such conditions may induce relatively large, and easily detected, reductions in photosynthesis.

Sullivan *et al.* (1994) found no consistent effect of UV-B on chlorophyll fluorescence characteristics and CO_2 assimilation rate of sweetgum under field conditions and suggested that the small reduction in growth (7 to 19% reduction in leaf area in the second year) may result from an even smaller reduction in photosynthetic efficiency, compounded by the reductions in the amount of photosynthetically active tissue. Such small changes in photosynthetic rate may have been undetectable, especially under field conditions.

Beyschlag *et al.* (1988) also shown that exposure to enhanced UV-B levels corresponding to a 20% reduction in the ozone column did not result in a significant depression in radiant energy-saturated net CO_2 uptake rate at ambient CO_2 partial pressure in plants of wheat and wild oat that were grown in the field or in a greenhouse. Even when plants of either species were exposed to UV-B levels corresponding to 30-45% ozone depletion levels in the greenhouse, there was no significant inhibition on net CO_2 uptake rate. They concluded that results indicated no sensitivity of photosynthesis to enhanced UV-B radiation in plants of wheat and wild oat.

These results are consistent with previous suggestions that UV-B radiation induced reductions in growth, under reasonably realistic conditions, are not primarily due to a decrease in assimilation rate per unit leaf area (Naidue *et al.*, 1993; Sullivan *et al.*, 1994; Caldwell *et al.*, 1994), although UV-B radiation induced reductions in leaf area obviously reduce total plant assimilation capacity.

In general, experiments conducted in growth chambers or greenhouses with low visible irradiance always resulted in much greater plant growth depression due to UV-B irradiation than similar experiments conducted under field conditions with comparable UV-B supplementation (Biggs and Kossuth, 1978). Whether this phenomenon stems in part from increased UV sensitivity of leaves which have developed under low light conditions and thus possess many of the morphological and physiological characteristics of shade-adapted leaves, or whether there is an immediate repair or protective process driven by visible irradiance is not clear (Caldwell, 1981).

It has been suggested that UV-B induced decreases in plant growth may be due to changes in hormone levels (Beggs *et al.*, 1985; Lingakumar and Kulandaivelu, 1993; Kato-Noguchi and Sumitomo, 1994).

4.7. CONCLUSIONS

Modulated chlorophyll fluorescence was an useful, non-invasive rapid probe of UV-B effects on photosynthesis of pea leaves under field conditions.

In pea plants, enhanced UV-B radiation had no effect on any of the chlorophyll fluorescence characteristics and CO_2 assimilation rate measured under field conditions.

Reduction in growth parameters of plants exposed to an enhanced UV-B radiation may be due to direct effects of UV-B on plant growth rather than a decrease in photosynthetic capacity.

CONCLUSIONS

CHAPTER 5:

CONCLUSIONS

5.1. MODULATED CHLOROPHYLL FLUORESCENCE

Modulated chlorophyll fluorescence is an useful, non-invasive rapid probe of the environmental stress effects on the photosynthetic performance of leaves.

This method is non-destructive and the same sample can be followed in its physiological development and changes over extended periods of time. On the basis of such information, essential conclusions on the performance of a plant in a given natural or artificial environment can be draw. In particular, this method is useful for rapid screening of plant cultivars and selecting for optical performance in environments with various forms of stress.

This study also confirmed that, using modulated chlorophyll fluorescence, relevant information which can be obtained within seconds includes the maximum quantum efficiency of PSII electron transport (Fv/Fm), the relative quantum efficiency of PSII electron transport (ϕ_{PSII}), the excitation capture efficiency (Fv'/Fm'), and photochemical and non-photochemical quenching coefficients (q_P and q_N), representative for the limitation that a sample is experiencing at a given light intensity.

5.2. WATER STRESS

Modulated chlorophyll fluorescence could be used as rapid screening tests for drought tolerance in barley, although applied alone, it is more appropriate as indicator for severe water stress rather than mild stress.

Screening of cereal genotypes for tolerance to any water stress treatment could be achieved by combining fluorescence quenching measurements with gas exchange analysis.

Drought tolerant genotypes show smaller variations of photochemical quenching, net photosynthesis, water use efficiency and leaf temperature than drought sensitive ones.

Tolerant barley genotypes can be distinguished from sensitive types by their ability to maintain comparable photosynthetic efficiency and light-saturated rates of CO_2 exchange under water stress conditions.

5.3. ENHANCED UV-B RADIATION

Controlled environment conditions

Pea leaves exposed to the low UV-B treatments resulted in decreases in CO_2 assimilation that are not accompanied by decreases in the maximum quantum efficiency of PSII primary photochemistry.

Increased exposure to UV-B resulted in a further loss of CO_2 assimilation and decreases in the maximum quantum efficiency of PSII primary photochemistry, which were accompanied by a loss in the capacity of thylakoids isolated from the leaves to bind atrazine, thus demonstrating that photodamage to PSII reaction centres had occurred.

Addition of UV-C to the UV-B treatments increased markedly the rate of inhibition of photosynthesis, however the relationships between CO_2 assimilation and PSII characteristics remained the same, indicating that UV-B and UV-C inhibit leaf photosynthesis by a similar mechanism.

Photosystem II is not the primary target site involved with the onset of the inhibition of photosynthesis in pea leaves induced by irradiation with UV-B.

Field conditions

In pea plants, enhanced UV-B radiation had no effect on chlorophyll fluorescence characteristics and CO_2 assimilation rate under field conditions.

Reduction in growth parameters of plants exposed to an enhanced UV-B radiation may be due to direct effects of UV-B on plant growth rather than a decrease in photosynthetic capacity.

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