Photosynthesis and Respiration in Mesophyll Protoplasts of Pea *(Pisum sativum)* in Response to Osmotic Stress and Photoinhibition

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

The candidate declares that this work has been carried out by her under the supervision of ***Dr. AS. Raghavendra,*** Associate Professor, Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad - 500 046, INDIA and that this work has not been submitted for any degree or diploma of any other University.

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CERTIFICATE

This is to certify that the thesis entitled **"Photosynthesis and Respiration in Mesophyll Protoplasts of Pea *(Pisum sativum)* in Response to Osmotic Stress and Photoinhibition"** is based on the results of the work done by **Ms. K. Sarada Devi** for the degree of **Doctor of Philosophy** under my supervision. This work has not been submitted for any degree or diploma of any other University.

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K. Sarada Devi

ABBREVIATIONS

Ap	=	stress-adaptation index
APX	=	ascorbate peroxidase
AsA	=	ascorbate
BCIP	=	5-bromo-4-chloro-3-indolylphosphate
CF_1	=	ATP synthesising component of coupling factor
CF_o	=	proton channel of coupling factor
$^3Chl^*$	=	triplet chlorophyll
D1*	=	phosphorylated form of Dl protein
DGDG	=	digalactosyldiacyglycerol
DHAR	=	dehydroascorbate reductase
DPH	=	1,6-diphenyl-1,3,5-hexatriene
DTNB	=	5,5'-dithiobis-(2-nitrobenzoic acid)
ESR	=	electron spin resonance
FBPase	=	fructose-1,7-bisphosphatase
F_m	=	maximal fluorescence
F_o	=	initial fluorescence
F_v	=	variable fluorescence
GPX	=	guaiacol peroxidase
GR	=	glutathione reductase
GSH	=	reduced glutathione
GSSG	=	oxidised glutathione
LED	=	light emitting diode
LEDR	=	light-enhanced dark respiration
MDA	=	malondialdehyde
MDHAR	=	monodehydroascrbate reductase
MGDG	=	monogalactosyldiacylglycerol
Ψ_s	=	solute potential

ψ_w	=	water potential
NBT	=	*p*-nitro-blue-tetrazolium chloride
$O_2^{\cdot-}$	=	superoxide radical
$^{\cdot}OH$	=	hydroxyl radical
$^1O_2$	=	singlet oxygen
PFD	=	photon flux density
PQ	=	plastoquinone
PVDF	=	polyvinylidene difluoride
r^2	=	correlation coefficient
Rfd	=	fluorescence decrease ratio
RuBP	=	ribulose-l,5-bisphosphate
SBPase	=	sedoheptulose-1,7-bisphosphatase
SOD	=	superoxide dismutase
SPS	=	sucrose phosphate synthase
SQDG	=	sulfoquinovosyldiacylglycerol
TBA	=	thiobarbituric acid
TBA-MDA	=	2-thiobarbituric acid-malondialdehyde adduct
TCA	=	tricarboxylic acid
TMA-DPH	=	1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5 *p*-toulenesulfonate

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Chapter 1

Introduction and Review of Literature

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Introduction and Review of Literature

Stress can be defined as any biotic or abiotic factor unfavourable for growth and reproduction of the living organisms, due to either sub-optimal or supra-optimal levels of the factor (Levitt, 1980). Water stress is one of the most important stresses experienced by plants, which limits plant growth and productivity, especially in unirrigated areas with Mediterranean type climates (Wendler et al., 1990). A better understanding of the mechanisms, which enable plants to adapt to water deficit and maintain growth and productivity during drought periods, will ultimately help in early identification and selection of drought tolerant varieties.

Water stress elicits several responses in plants, beginning with stress perception (initiated by a signal transduction pathway). These responses are manifested in changes at cellular, physiological and developmental levels (Bray, 1993). One of the common effects of water stress in plants is marked changes in levels of hormones. A well known response to osmotic stress is the increase in the levels of abscisic acid, ABA (Kahn et al., 1993). ABA prevents loss of turgor in the plant by restricting of stomatal opening and thus preventing the wilting of the plant under water deficit conditions. The accumulated ABA, in response to water stress, is also responsible for the induction and expression of several genes (Bray, 1993). Ethylene also increases under water stress which in turn promotes the abscission of leaves and young fruits. Reduced levels of cytokinin, may be responsible for early leaf senescence and inhibition of protein synthesis under drought (Aspinall, 1980).

Water stress affects nitrogen assimilation of the plant, either directly by inhibiting the activity of nitrate reductase or indirectly by reducing carbon assimilation. The activity of nitrite reductase, the second enzyme of the nitrate assimilatory pathway, appears to be relatively insensitive to water stress

(Hanson and Hitz, 1982). Plants grown **under dryland conditions exhibit a** decrease in leaf **number,** leaf area index, leaf size, **plant height and total** weight per plant **(Burke** et al., 1985). Since net CO_2 fixation is a major **component** of plant productivity, the effects of water stress on leaf photosynthesis have been studied extensively (e.g. Boyer, 1976; Bradford and Hsiao, 1982).

Low leaf water potential affects photosynthesis in several ways: stomatal closure, inhibition of carbon fixation, restriction of photosynthetic electron transport and impairment of photophosphorylation (Boyer and Younis, 1984; Kaiser, 1984; Leegood et al., 1985). Disturbance of compartmentation within the cell may be an additional factor contributing to the overall inhibition of the photosynthetic apparatus under water stress (Kaiser, 1982). For e.g. leakiness of the tonoplast would result in release of vacuolar acids and decrease in pH of cytoplasm which in turn could affect photosynthesis (Kaiser et al., 1981b). Some of the other associated phenomena are the reduction in the area/duration/growth of leaves and impairment of translocation (Hanson and Hitz, 1982; Leegood et al., 1985).

The primary cause of photosynthetic inhibition during water stress is debatable (Boyer, 1976; Kaiser, 1984; Leegood et al., 1985). Water stress causes stomatal closure, sometimes 'patchy', in the leaves of several plants (Ni and Pallardy, 1992; Wise et al., 1992). Some reports considered the deleterious effects of water stress on photosynthesis to be all indirect, the primary effect being stomatal closure (Moldau, 1973; Beadle et al., 1981; Jones, 1985; Cornic et al., 1987). In contrast, other reports emphasized the importance of non-stomatal component during the inhibition of leaf photosynthesis under osmotic/water stress. Nonstomatal effect of water stress on biochemistry of photosynthesis is documented by the use of experimental systems which do not have any limitation on diffusion of CO_2, imposed by stomatal conductivity, e.g. thin leaf slices, intact cells, isolated protoplasts and chloroplasts (Jones, 1973; Plaut and Bravdo, 1973; Kaiser et al., 1981a, b; Sharkey and Badger, 1982;

Saradadevi and Raghavendra, 1994). Further, photosynthesis is extremely sensitive to water stress in leaves/leaf discs even at very high partial pressure of CO_2 (which precludes stomatal limitation), as demonstrated by the techniques of gas-exchange (Graan and Boyer, 1990), O_2 evolution (Saradadevi et al., 1995) or chlorophyll fluorescence (Ögren, 1990).

Various components of the photosynthetic process exhibit different degrees of sensitivity to water or osmotic stress (Kaiser, 1984). The two major components of photosynthesis are: the photochemical reactions (generating ATP and reduced NADP via electron transport chain) and the Calvin cycle of carbon assimilation. It has been observed that enzymatic reactions which occur in the aqueous phase of Chloroplast stroma (dark carbon fixation) or on the thylakoid stroma interface (such as Chloroplast coupling factor, CF,) are relatively more sensitive to water/osmotic stress than the reactions occurring in membrane lipid phase (photosynthetic electron transport and proton efflux) (Sharkey and Badger, 1982; Berkowitz and Gibbs, 1984; Kaiser, 1984; Martin and Ruiz-Torres, 1992; Saradadevi and Raghavendra, 1994).

The reports on changes in rates of primary photochemical reactions (involving the electron transport chain) during water/osmotic stress are quite contradictory. The effect of water stress on photochemical reactions in chloroplasts is either moderate (Keck and Boyer, 1974) or none at all (Berkowitz and Gibbs, 1982a; Kaiser et al., 1981a). The quantum yield of photosynthesis decreased under water stress in some cases (Mohanty and Boyer, 1976; Mooney et al., 1977), but not in others (Collatz, 1977).

Attempts to examine the photochemical capacity of chloroplasts using *in vivo* techniques on leaves also yielded contradictory information. Havaux et al. (1986) detected profound changes in the photochemical activity of the thylakoid membranes of rapidly desiccated leaves. Even during short desiccation stress, damage was noticed exclusively in photosystem II (PSII), presumably at the donor side. The photochemical capacity of the tissue,

monitored through chlorophyll fluorescence, was relatively unaffected in leaf discs from water stressed plants (Di Marco et al., 1988). On the contrary, measurements of delayed fluorescence, thermoluminescence and ESR signal conclude that dehydration restricts electron transfer in chloroplasts between the oxygen evolving complex and secondary electron donors of PSII and between PQ and P_{700} (Govindjee et al., 1981; Matorin et al., 1982).

Electron transport in isolated chloroplasts was decreased by 0-15% in assay media with hyper-osmotic potential (Santarius and Ernst, 1967; Potter and Boyer, 1973; Berkowitz and Gibbs 1982a) Although the observed inhibition was possibly due to decreased electron flow from PQ to P_{700} (Berkowitz and Gibbs, 1982a; Matorin et al., 1982), it could also be due to the loss of stromal adenylates (Kaiser and Heber, 1981).

According to Genty et al. (1987), PSII mediated electron flow was not affected while photosystem I (PSI) was inhibited during water stress. Keck and Boyer (1974) have shown the reduced rates of electron transport activity in chloroplasts isolated from wilted leaves *of Helianthus annus.* But it should be acknowledged that the extraction of functional chloroplasts from stressed leaves is extremely difficult and the decrease in photochemical activity may not reflect a reduction *in vivo* electron transport activities (Sharkey and Badger, 1982).

PSII mediated reactions were inhibited (but not PSI) on exposure of thylakoid membranes to osmotic stress, particularly in presence of magnesium. The inhibition occurred only in presence of magnesium as indicated by the combinations of several cations/anions (Sundari and Raghavendra, 1987, 1990). Recovery of PSII activity of thylakoid membranes by the addition of exogenous electron donors, hydroxylamine (100% restoration), diphenyl carbazide (60% recovery) and $MnCl_2$ (35-40% recovery), indicated that the water splitting system was the site of inhibition. The loss in PSII activity was associated with conformational changes in thylakoid membranes, as indicated

by changes in absorption spectra. Thylakoid membranes **treated with** hydroxylamine or $CaCl_2$ suggested the 33 kD protein along with manganese might be involved in the inhibition of PSII activity either due to release from the membranes or due to deactivation by some means. Release of manganese from thylakoid membranes (chloroplasts treated with 10 mM $MgCl_2$ was an important factor during suppression of the oxygen evolving complex (Sundari et al., 1994).

Another parameter which readily responds to the onset of osmotic/water stress is the pattern of chlorophyll fluorescence in leaves (Govindjee et al., 1981). Measurement of chlorophyll fluorescence is simple, non-destructive and rapid (Schreiber 1983; Schreiber et al., 1989). Many authors have demonstrated the usefulness of chlorophyll fluorescence in the analysis of photosynthetic response and its application in assessing the plant response to a variety of environmental stresses, such as temperature or light (Critchley and Smillie, 1981; Schreiber and Bilger, 1987; Burke, 1990). The studies are generally on the phenomenon of "Kautsky effect", which contains considerable information on the state of photosynthetic apparatus. The kinetics of "Kautsky effect" are distinguished into two phases. The initial "rapid" phase of induction which occurs within 1 or 2 sec, while the subsequent "slow" phase follows up to a few minutes. These phases of biophysical changes are closely related to the photochemical electron transport and the carbon assimilation pattern (Papageorgiou, 1975; Renger and Schreiber 1986).

Govindjee et al. (1981) reported that fluorescence from chlorophyll *a* was lower in water stressed leaves than in control leaves. The decrease in fluorescence could also be due to an increased pH gradient across the thylakoid membranes, as a consequence of inhibition of photophosphorylation due to water stress (Sharkey and Badger, 1982).

Schwab et al. (1989) demonstrated the reduced rates of PSII activity under water stress in resurrection plants, using the techniques of fluorescence

and luminescence. However, they were doubtful that the PSII donor side inhibition could be a consequence of photoinhibition, because the plants were not completely darkened during dehydration. When leaves *of Arbutus unedo* were dehydrated in complete darkness, variable fluorescence was hardly affected while Calvin cycle activity and intersystem electron flow were inhibited (Schreiber and Bilger, 1987). Under natural conditions, where water stress and high inradiance occur frequently, a limitation primarily at the level of the Calvin cycle can further lead to a serious secondary damage, e.g. photoinhibition of PSII (Björkman and Powles, 1984).

The dehydration of thylakoid membranes by hyperosmotic media had little effect on electron transport, but caused a reversible uncoupling of photophosphorylation (Santarius and Ernst, 1967). Severe reduction of photophosphorylation due to low water potentials is observed by number of workers (Santarius and Ernst, 1967; Keck and Boyer, 1974; Younis et al., 1979; Kaiser et al., 1981a). Younis et al. (1979) have shown that CF| activity was decreased in chloroplasts prepared from wilted leaves. The increase in Chloroplast ATP/ADP ratio upon illumination was slower under osmotic stress than that under isotonic condition, indicating a decrease in photophosphorylation under stress (Kaiser et al., 1981a)

Some of the enzymes of Calvin cycle are quite vulnerable to water stress. Plaut (1971) reported ribose-5-phosphate isomerase, ribose-5-kinase and ribulose-l,5-bisphosphate carboxylase/oxygenase (rubisco) to be such sensitive sites while Berkowitz and Gibbs (1982a) observed fructose-1,6-bisphosphatase (FBPase) and sedoheptulose-1,7-bisphosphatase (SBPase) are markedly inhibited due to osmotic stress.

Under conditions of water deficit, the extent of light induced stromal alkalization was reduced (Berkowitz and Gibbs, 1983). This would lead to a serious decrease in CO_2 assimilation, since the key enzymes of Calvin cycle are activated through light mediated stromal alkalization. Among the photosyn-

thetic enzymes, FBPase and SBPase, particularly require a highly alkaline pH for optimal activity (Baier and Latzko, 1975; Robinson and Walker, 1981). The activities of these two enzymes are markedly reduced under water stress (Sharkey and Badger, 1982; Berkowitz and Gibbs, 1984; Leegood et al., 1985). Boag and Portis (1984) discussed the inhibition of light activation in these enzymes in terms of substrate availability, possible alterations of the redox state of ferredoxin and associated electron carriers, and interactions between enzyme and substrate. Although, rubisco was relatively unaffected by short term water stress, the rate of ribulose-l,5-bisphosphate (RuBP) regeneration was greatly reduced (Farquhar and Sharkey, 1982; Leegood et al., 1985).

In water stressed plants, starch was depleted but sucrose levels were maintained at the same or high level, compared to those in well watered plants (Quick et al., 1989). Increased sucrose levels in water stressed plants could be due to the increased activation of sucrose phosphate synthase (SPS) or decreased rate of export (due to an inhibition of phloem transport) or the compartmentation of sugar away from the site of loading. However, there is an anomaly in the observation on the effects of water stress on SPS activity. Sharkey et al. (1989) showed that under water stress photosynthesis was limited due to the decrease in intercellular CO_2, a consequence of `stomatal closure, while the SPS activity was decreased. The reduced activity of SPS leads to a feed-back inhibition of photosynthesis until new protein synthesis can restore the activity of SPS (Quick et al., 1989).

The sensitivity of different photosynthetic reactions in leaves or chloroplasts to water stress are summarised in Table 1.1.

The literature on the effect of osmotic or water stress on respiration in plant tissues is contradictory, with reports indicating either stimulation or inhibition or no change (Hsiao, 1973; Hanson and Hitz, 1982). On the other hand, the organic solutes are expected to accumulate during water/osmotic stress and maintenance of these may require increased respiratory activity

Table 1.1. *Variation in the sensitivity of different components of photosynthetic process to water stress*

Degree of Sensitivity/Reaction	Reference
Highly sensitive	
Chlorophyll fluorescence induction kinetics	Govindjee et al., 1981
Stromal alkalization	Berkowitz and Gibbs, 1983
Light activation of enzymes	Berkowitz and Gibbs, 1982b
Dark CO_2 fixation	Saradadevi and Raghavendra, 1994
Moderately sensitive	
Thylakoid energization	Dietz and Heber, 1983
Activity of CF_1	Younis et al., 1983
Quantum yield of carbon fixation	Boyer, 1976
Starch/sugar partitioning	Quick et al., 1989
Vulnerability to photoinhibition	Saradadevi and Raghavendra, 1994
Least or transiently sensitive	
PSII dependent electron transport	Saradadevi and Raghavendra, 1994
PSI reactions	Havaux et al., 1986
Chloroplast envelope permeability	Kaiser and Heber, 1981

(Amthor, 1989). In one of the earliest investigations on the effects of water stress on crop respiration, Upchurch et al. (1955) observed a slight increase in white clover shoot respiration during water stress. An increased maintenance respiration, observed with decrease in soil water, was further corroborated with experiments using the starvation method to estimate maintenance respiration (Moldau et al., 1980). The respiration rate of rice increased during a period of water stress in continuous darkness (Kobata andTakahami, 1986). However, a few reports showed a decrease in the rate of respiration at low water potentials. The decreased rate of O_2 uptake during water stress was reported using isolated

mitochondria from maize (Bell et al., 1971). The respiration rate of maize, perennial rye grass and wheat was decreased slightly by water stress induced by the addition of polyethylene glycol (PEG) or mannitol to the culture solution (Penning de Vries et al., 1979).

In any case, the effect of water stress on dark respiration was far less that on apparent photosynthesis. Dark respiration of spinach leaf slices was significantly less sensitive to osmotic stress than photosynthetic carbon fixation. For e.g. dark respiration was inhibited by only 50% at about 50 bar, an osmotic potential sufficient to suppress photosynthesis completely (Kaiser et al., 1983). After rewatering, there was a large and immediate increase in respiration rate, presumably due to rapid conversion of substrate into new structural phytomass, which was corroborated by a rapid increase in leaf area (McCree et al., 1984). The lowest rates of respiration occurred when soil moisture content and air temperature were low (Amthor, 1989). Saradadevi and Raghavendra (1994) reported intriguing responses of respiration in mesophyll protoplasts to osmotic stress. Respiration was stimulated if osmotic stress was induced at room temperature, but was inhibited when protoplasts were subjected to stress at chilling temperature (0 °C).

Another abiotic stress, that plants are often exposed to, is light stress, particularly excess light, leading to the process of 'photoinhibition'. Although light, being the energy source for photosynthesis, is essential for plant life, excess light can inhibit photosynthesis (Powles, 1984; Long et al., 1994). Indeed the phenomenon of photoinhibition can markedly affect plant growth and decrease the yields of agricultural crops (Barber and Andersson, 1992).

Photoinhibition is the phenomenon of severe reduction in the photosynthetic efficiency (quantum yield) under supra-optimal light intensities (Powles, 1984; Kyle and Ohad, 1986; Krause, 1988). The term photoinhibition was earlier used synonymously with PSII damage but is now being defined as a decrease in the efficiency of photosynthetic energy conversion. Photoinhibi-

tion results not only from a reversible damage to PSII but also due to an increase in thermal energy dissipation, which is a photoprotective process and does not represent damage (Demmig-Adams and Adams, 1992).

Photoinhibition of photosynthesis in visible light occurs, not from high light *per se*, but rather an excess of light absorbed by antennae of photosystems beyond the capacity of deexcitation through photochemical reactions (Samuelsson et al., 1985, 1987; Demmig-Adams and Adams, 1992). Under suboptimal conditions (limiting CO_2, water or temperature) which suppress carbon metabolism, the gap between absorption and utilization of quanta increases leading to the over-excitation of the photosynthetic apparatus (Long et al., 1994). Therefore, under such environmental stress, photoinhibition is induced even at moderate light intensity (Briantais et al., 1992; Aro et al., 1993).

From several experimental results, it is confirmed that the PSII is more sensitive than PSI during photoinhibitory process (Critchley, 1981; Powles and Björkman, 1982). There are two possible reasons for such high sensitivity of PSII to light stress: firstly, the generation of highly toxic oxygen radicals from molecular oxygen when pigments are excited by light and secondly, the formation of intermediates within the reaction centre with very high oxidising potentials (>1.0 V) (Barber and Andersson, 1992).

One of the most important proteins of PSII is D1, which harbors functionally important sites involved in PSII mediated electron transfer during photochemical reactions. This protein is quiet dynamic, being continuously synthesised as well as degraded. The half-time for its turnover can be as short as 60 min (Barber and Andersson, 1992). The net result of photoinhibitory damage to PSII is an outcome of the imbalance between the degradation and recovery processes (Ohad et al., 1984). Photoinhibitory light inactivates Dl protein by inducing structural changes, which make the protein sensitive to the membrane bound proteases (Ohad et al., 1984). The vulnerability of Dl

polypeptide can be explained by its proximity to the reaction centres of PSII, which produces highly reactive oxygen radicals (Long et al., 1994).

The photodamage of D1 protein and subsequently of PSII under excess light could be due to the effects on either the acceptor or donor side or both of PSII. Primary charge separation in PSII reaction centre is restricted when the donor side of PSII is more efficient than its acceptor side, results in an impaired electron transport from P_{680} to Q_A (Barber and Andersson, 1992; Demeter et al., 1987). If a charge-recombination occurs, then the P_{680} chlorophyll triplet is generated and it rapidly reacts with oxygen leading to the formation of singlet oxygen. Singlet oxygen species is a highly reactive and toxic, leading to the destruction of the chlorophyll molecule of P680 (Telfer et al., 1990) and results in Dl polypeptide degradation (Shipton and Barber, 1991).

Photoinhibition of photosynthesis also occurs when the donation of electrons from water side to PSII does not keep pace with electron withdrawl. Under these conditions, highly oxidising species $P680^+$ causes damage by oxidation and destruction of accessory chlorophyll, known as Chl670, and of p-carotene (Telfer et al., 1990), and leads to degradation of Dl polypeptide (Shipton and Barber, 1991).

An intrinsic repair mechanism, to cope with such unstable PSII under supra-optimal light, is the ability of chloroplasts to synthesise and replace Dl protein, while removing the damaged protein (Kyle and Ohad, 1986). The exposure of photoinhibited leaf tissues to dim light accelerates the synthesis of Dl protein and helps in the recovery of PSII (Greer et al., 1986). Interference of this process by the use of translational inhibitors, such as streptomycin or chloramphenicol, results in much greater photoinhibition than that in their absence (Schnettger et al., 1994). A transcriptional inhibitor, rifampicin, had no effect on photoinhibition in *Anacystis* (Samuelsson et al., 1985) and *Chlamydomonas* (Lidholm et al., 1987). In contrast, the translational inhibitor, streptomycin, restricted the recovery process and accelerated photoinhibition.

These results indicate that the resynthesis of Dl protein depends on a rapid translation of preexisting stable mRNAs.

Susceptibility of plants to photoinhibition under any photon flux density (PFD) greatly depends also on environmental conditions other than light (Powles, 1984; Krause, 1988). Plants growing under natural conditions have been shown to suffer from photoinhibiton particularly under stresses of drought, high- and low temperatures. Low water potential, i.e. water stress, which can adversely affect the Chloroplast function increases the susceptibilty of photosynthetic apparatus to photoinhibitory injury (Björkman and Powles, 1984; Boyer et al., 1987; Greer and Laing, 1990; Masojidek et al., 1991; Saradadevi and Raghavendra, 1994).

Plants cannot tolerate too low (chilling) or too high temperatures. Chilling can induce photoinhibition of photosynthesis even at moderate light intensities (Kyle, 1987; Somersalo and Krause, 1988, 1989). Chilling at high PFD is associated with increased leakiness of membranes (Garber, 1977). The increased susceptibility to light stress at low temperatures may result from restricted carbon metabolism, which predisposes the photosynthetic apparatus to overenergization and promotes formation of active oxygen species (Schöner and Krause, 1990). Combinations of high PFD and low temperature results in oxygen dependent bleaching of carotenoids, chlorophyll pigments and inhibition of electron transport chain in thylakoids (Öquist et al., 1987). Low temperature also slows down the operation of energy dissipation mechanisms like violaxanthin cycle (Demmig-Adams et al., 1989).

Plants are endowed with several protective mechanisms to avoid the damage to the photosynthetic apparatus due to photoinhibition: photorespiration, mitochondrial respiration, zeaxanthin cycle and dynamism of Dl protein. Photorespiration helps in the disposal of excess photosynthetic energy (Tolbert, 1971), since the overall process of photorespiration requires a high input of energy (Krause and Cornic, 1987).

Mitochondrial dark respiration also plays an important role in protecting the photosynthesis against photoinhibition. When respiration is restricted, photoinhibition was pronounced (Saradadevi and Raghavendra, 1992). Mitochondrial respiration is beneficial for photosynthesis in the plant cell, as shown in leaves and protoplasts (Krömer et al., 1988; Krömer and Heldt, 1991; Vani et al., 1990). Mitochondrial respiration has been proposed to prevent the over-reduction of the photosynthetic electron transport chain in chloroplasts, by providing an outlet for reduced equivalents to the cytosol or mitochondria (Krömer and Heldt, 1991; Raghavendra et al., 1994; Krömer, 1995).

One of the key proteins of PSII, Dl protein, provides another interesting defence mechanism, against strong photooxidative damage to photosynthetic apparatus during photoinhibition. When there is an excess of radiant energy, extensive damage (too difficult to repair) is prevented through quick destruction of Dl protein, thus switching off the electron transport system (Wild et al., 1989).

Recent reports (Rintamäki et al., 1995) suggest that phosphorylation of D1 protein may be involved in regulating the repair of photoinhibited PSII centres. Dl protein of grana stacks is phosphorylated in light. Under photoinhibitory conditions, *in vitro* (Aro et al., 1992; Kettunen et al., 1992) as well as *in vivo* (Syme et al., 1992), the phosphorylated Dl is degraded more slowly than the non-phosphorylated Dl protein. Damaged Dl is protected from degradation through phosphorylation under conditions, where repair cycle can not cope up with the rate of degradation. Phosphorylated Dl could act as a storage form of photodamaged Dl protein (Rintamäki et al., 1995). Further, in *Ceratodon* (a moss), which does not phosphorylate the Dl protein, the degradation of the damaged Dl is very rapid under photoinhibitory conditions (Rintamäki et al., 1994). Photodamage to PSII occurs in appressed region of thylakoids (Cleland et al., 1986) while synthesis of the Dl protein takes place in exposed stromal membranes (Mattoo and Edelman, 1987).

Chloroplast pigments, particularly carotenoids, help in protecting the plants against photoinhibition. One of the most recently discovered mechanisms involved in photoprotection is the controlled thermal dissipation of excessive energy within the photochemical system, presumably in the chlorophyll pigment bed (Demmig-Adams and Adams, 1992). Due to the tight association with chlorophyll molecules, cyclic carotenoids not only act as light harvesting antennae but also can effectively protect the photosynthetic apparatus from photooxidative destruction, by scavenging most of the triplet state of chlorophyll ($^3Chl^*$) and singlet oxygen ($^1O_2$) (Schöner and Krause, 1990; Sandmann et al., 1993).

An active xanthophyll cycle in leaves, helps in the dissipation of excess energyy and protecting the plants against photoinhibition (Demmig et al., 1987; de las Rivas et al., 1989; Demmig-Adams and Adams, 1992). Violaxanthin, one of the xanthophylls, is converted to antheraxanthin and then to zeaxanthin under too high levels of PFD. Zeaxanthin favours a non-radiative energy dissipation of excessive light energy. Thus the pool of pigments within the xanthophyll cycle may constitute a protective mechanism for PSI1, when the input of light energy exceeds the capacity for utilisation (Demmig-Adams et al., 1989; Bilger and Björkman, 1990; Morales et al., 1990).

An important and immediate consequence of water stress is the shrinkage of cells and tissues. Low water potential induced perturbations can be linked to organelle and/or protoplast volume reduction. This hypothesis is supported by the differential effect of non-penetrating (e.g. sorbitol) or penetrating solutes (e.g. ethylene glycol), used to lower the osmotic potential of the external medium (Kaiser and Heber 1981; Berkowitz and Gibbs, 1983). The extent of reduction in size of the protoplast/chloroplast was positively correlated to the inhibition of photosynthesis of plant tissues subjected to osmotic stress (Acevedo et al., 1979; Kaiser, 1982; Sen Gupta and Berkowitz, 1987; Santakumari and Berkowitz, 1989, 1990; Saradadevi et al., 1995).

The strong positive correlation between the reduction in protoplast volume and inhibition of photosynthesis suggests that the primary lesion in cell metabolism may be due to the concentration of regulatory constituents/ions (K^+ in particular) within the cell during dehydration (Kaiser and Heber, 1981). The rise in stromal K^+ in Chloroplast could inturn increase K^+ export and H^+ import into stroma, and cause a reduction in stromal alkalization (Berkowitz and Gibbs, 1983). Ion effects would explain the reduction of photosynthesis with even mild water stress where very little volume change occurs (Sharkey and Badger, 1982). The differences between genotypes in terms of relative sensitivity of photosynthesis to low water potential may be explained by the difference in extent of protoplast volume reduction (Santakumari and Berkowitz, 1990). Osmotic adjustment is an important step during the acclimation of a plant cell to low water potential (Matthews and Boyer, 1984; Berkowitz and Kroll, 1988; Sen Gupta and Berkowitz, 1988; Evans et al., 1992).

Environmental stress conditions are known to alter markedly the molecular organisation of membrane lipid bilayers (Dickens et al., 1980; Raison, 1980). The thylakoids undergo changes in conformation appearing thinner in leaves with low water potentials than those in well-watered controls (Fellows and Boyer, 1976). The reason for low sensitivity of membrane mediated reactions (such as photochemical activities) to low water potential could be that the components of the electron transport chain (except plastocyanin, ferredoxin and ferredoxin NADP-reductase) are embedded in the lipid phase of thylakoid membranes (Kaiser and Heber, 1981). However, maintenance of optimal membrane fluidity is required for successful membrane function.

Water is a major component of any living cell or its membranes. Imposition of water stress is therefore bound to cause water loss and a change in biophysical characteristics of membranes. Compared to the extensive studies made on fluidity of plant cell membranes in response to the temperature stress, similar studies on membrane properties in relation to water/osmotic

stress are quite limited. Further, most of these studies are made on plants subjected to water stress for a long time.

During onset of dehydration in lupin, the level of membrane lipids fell with respect to total leaf lipids, especially in drought susceptible genotypes (Hubac et al., 1989). The ratio of free sterols to phospholipids increases with water stress in *Brassica napus* (Svenningsson and Liljenberg, 1986). There is a correlation between drought tolerance and plasma membrane leakiness (D'Aoust and Hubac, 1986; Hubac et al., 1989). Water stress is expected to increase the apparent fluidity of lipid domain of the cell membranes. The effect is more pronounced in chlorophyll containing mesophyll cells than that in epidermal cell (Gantet et al., 1990). Lipids of the plastidial compartment are also modified by water stress. Although, the plastidial galactolipids and plasma membrane lipids of phosphatidylcholine, phosphatidylethanolamine decreased under stress conditions, the levels of triglyceride content increased by six-fold, in a susceptible genotype of *Lupinusalbus* (Hubac et al., 1989).

Chilling stress is accompanied by the rigidity of cell membranes in contrast to the effect of water stress (Lyons, 1973; Shinitzky, 1984). The difference in tolerance of cells to chilling temperature can be explained on the basis of variation in fatty acid composition of the membrane lipids (Sato et al., 1979; Raison et al., 1982a).

There is a no clear evidence to indicate if lipid mobility within plant membranes is affected by proteins. However, it is known that proteins can influence the thermal and motional properties of lipids in bilayers (Bishop, 1983). Temperature induced changes in lipid ordering can in turn affect the conformation of proteins (Lynch et al., 1987). The ability of proteins to associate with specific lipid components may produce special domains of membrane lipid. The nature and extent of such domains may be a key factor in the differential responses of plant membranes to temperature (Bishop, 1983; Koster

et al., 1994). However, plants possess their own regulatory mechanisms to acclimate/adapt to a wide range of thermal regimes (Raison et al., 1982a,b).

Stress induced alterations in membrane bilayers affect cell function by inducing changes in the structure and function of membrane proteins (Shinitzky, 1984). Activities of some membrane associated enzymes, such as ATPase activity, are quite sensitive to stress conditions, due to changes in lipid component of membranes (Wright et al., 1982). The coupling factor (ATP synthetase of the thylakoid membranes) extracted from water stressed chloroplasts exhibited a loss in binding (affinity) of ADP to the protein, due to conformational changes in the protein (Younis et al., 1979). It is imperative that the conformational status of proteins in membranes also are altered under stress conditions. But again studies on protein conformational changes, on exposure to osmotic stress, are quite a few.

Chloroplasts of higher plants, besides their competence of water-oxidation, are capable of generating several forms of destructive oxygen species (Asada and Takahashi, 1987). Photoreduction of molecular oxygen, the Mehler reaction, is an unavoidable process that produces superoxide anion radicals ($O_2^{\cdot-}$). Dismutation of these radicals results in the formation of hydrogen peroxide (H_2O_2) and the reaction between superoxide radicals and H_2O_2 creates highly reactive hydroxyl radicals (.OH). Excessive levels of these toxic oxygen species can damage the tissues by several mechanisms, for e.g. diversion of metabolic pathways into abnormal routes, inactivation of enzymes and lipid peroxidation (Burke et al., 1985; Demmig-Adams, 1990; Schöner and Krause, 1990; Sharma and Singhal, 1992). Impairment of D1 protein in PSII reaction center could also be due to the oxygen radical attack on amino acid residues of the peptide chain (Kyle, 1987; Ghirardi et al., 1990).

Another type of damage mediated by oxygen is lipid peroxidation, a term that refers to the oxidative degradation of phospholipids and other unsaturated lipids. Among the consequences of lipid peroxidation are: leakiness of

the membranes, cross-linking of lipids and polypeptides and inactivation of membrane enzyme/receptors (Girotti, 1990).

Chloroplast lipids contain a high proportion of unsaturated fatty acids and are therefore susceptible to peroxidation (Halliwell, 1982). Lipid peroxidation in chloroplasts is promoted by singlet oxygen (Takahama and Nishimura, 1975; Girotti, 1990) and is restricted in presence of electron acceptors and electron transport inhibitors, which prevents the formation of singlet oxygen (Heath and Packer, 1968). Lipid peroxidation increases under stress conditions like excess (supra-optimal) light (Heath and Packer, 1968; Robinson, 1988; Vaughn et al., 1988; Sharma and Singhal, 1992) or water stress (Dhindsa and Matowe, 1981; Leprince et al., 1990).

Chloroplasts are equipped with scavengers to minimise the damage caused by toxic oxygen species, produced during photochemical energy conversion. Scavenger systems include either antioxidants such as ascorbate, free thiols, α-tocopherol, carotenoids, flavonoids or enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), mono-dehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR).

Plant cells contain very high levels of reduced glutathione (GSH) and ascorbate (AsA) which are quite effective antioxidants against toxic oxygen species (Asada and Takahashi, 1987). GSH or AsA are present in the Chloroplast stroma at concentrations as high as 3 and 50 mM respectively (Halliwell, 1982). Because of such high cellular concentrations, GSH is more accessible for reaction with O_2 than the thiol groups of enzymes, thereby protecting the enzymes from inactivation by O_2. GSH can also reactivate enzymes by reducing oxidised sulfhydryl groups. The oxidation of GSH by O_2, to form oxidised glutathione (GSSG) occurs rapidly in plants at alkaline pH values (Fahey et al., 1975; Saetre and Rabenstein, 1978). The subsequent reduction of GSSG to GSH, a reaction catalysed by a NADPH-dependent glutathione reductase is

essential for the protection of chloroplasts against oxidative damage, so as to maintain a high ratio of GSH/ GSSG.

AsA participates in several enzymic and nonenzymic systems, that scavenze free radicals, remove peroxide, and quench $^1O_2$ (Hossain and Asada, 1984; Larson, 1988). The role of AsA has been studied extensively in relation to environmental stress factors such as drought and chilling (Levitt, 1972; Chinoy, 1984). AsA also reduces violaxanthin to zeaxanthin in xanthophyll cycle and helps in thermal energy dissipation (Demmig-Adams and Adams, 1992). Increased concentrations of AsA in leaves is one of the mechanisms evolved by the plants for protection against reactive oxygen species generated during illumination, particularly at low temperatures (Schöner et al., 1990).

Tocopherols play an important role in suppressing lipid peroxidation. The process of lipid peroxidation produces several lipid radicals (LOOH) e.g. peroxy radicals and hydroperoxides. These radicals are trapped by tocopherol, thus restricting the propagation of lipid peroxidation. Tocopherols in Chloroplast thylakoids probably participate more in the trapping of the lipid radicals and suppression of lipid peroxidation than in the scavenging of the singlet oxygen (Asada and Takahashi, 1987).

The enzyme SOD, which catalyses dismutation of superoxide radicals to H_2O_2 and O_2, is mostly localised in chloroplasts of the leaves (Jackson et al., 1978). Increase in the activity of SOD has been reported during exposure to high irradiance stress (Schöner and Krause, 1990; Cakmak and Marschner, 1992), drought (Dhindsa and Matowe, 1981; Burke et al., 1985) and chilling (Clare et al., 1984).

The rapid removal of H_2O_2 produced by SOD is important, since otherwise peroxide may inactivate several enzymes by covalent modification of proteins (Bradley et al., 1992). In chloroplasts, which lack catalase (Halliwell, 1982; Asada and Takahashi, 1987), the conversion of H_2O_2, is mediated by a H_2O_2 scavenging system with the involvement of APX, DHAR and GR (Foyer

and Halliwell, 1976; Gillham and Dodge, 1986). Similar H_2O_2 detoxifying system is also present in the cytoplasm (Klapheck et al., 1990).

APX is an important enzyme that scavenges H_2O_2 very effectively in higher plant chloroplasts (Asada, 1992). APX oxidises AsA to monodehydroascorbate while reducing H_2O_2 to water. The enzyme can reduce H_2O_2 at a high rate as its K_m value for H_2O_2 is low. The enzyme is highly specific for AsA. Most of the monodehydroascorbate radicals formed by APX are directly reduced back to ascorbate by MDHAR, using NAD(P)H as the electron donor (Hossain et al., 1984). Under the conditions of limited supply of NAD(P)H, monodehydro-ascorbate radicals would be dismuted to ascorbate and dehydroascorbate, and the dehydroascorbate is reduced to ascorbate by DHAR using GSH (Asada and Takahashi, 1987).

Catalase, localised in the microbodies of higher plants, mediates in the decomposition of H_2O_2, which is also produced by H_2O_2-generating oxidases (Tolbert, 1971). Despite the restricted localisation of catalase, H_2O_2 generated in chloroplasts can readily diffuse across the membranes and get reduced in microbodies. Thus catalase play a significant role in defending against oxidative stress imposed by presence of H_2O_2 (Bowler et al., 1992). However, when plants are exposed to strong light, an apparent loss of catalase activity is observed, since the degradation of catalase exceeds the capacity for repair. Similarly, at chilling temperatures, the rate of catalase-synthesis was slowed down while the degradation of catalase was still high, resulting in a net loss of activity (Mishra et al., 1993).

Under tropical or sub-tropical conditions, plants are often exposed to water/osmotic stress, light stress and often both. These stresses cause a decrease in photosynthesis and respiration, change membrane properties and cause an accumulation of superoxide radicals. On the other hand, plants try to adapt to the imposition of water stress by decrease in the protoplast volume or leaf area, modifying the lipid/protein conformational status in membranes and

developing systems of scavenging superoxide radicals. The present investigation attempts to study some of these features in the system of mesophyll protoplasts isolated from leaves of pea (*Pisum sativum*). A few experiments are performed with leaf discs.

Chapter 2

Approach and Objectives

Chapter 2

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This project is an attempt to study the effect of osmotic stress on: photosynthesis, respiration, select membrane-properties and anti-oxidative agents/ enzymes using mesophyll protoplasts (or leaf discs, for some of the experiments) of pea, *Pisum sativum.* The system of isolated mesophyll protoplasts was used to study also the phenomenon of photoinhibition. Pea (*Pisum sativum)* is a typical C_3 plant, which can be grown easily and the leaf material for experiments can be obtained within 10 to 12 days after sowing the seeds. Further, pea plants are useful and convenient to study the physiological/biochemical behavior of a C_3 plant at different levels of organization like whole plant, leaves/leaf discs, leaf slices, protoplasts and isolated organelles.

The effects of osmotic/water stress on photosynthesis of a plant can be studied with the help of diverse experimental systems, *viz.* intact plant, leaves/leaf discs/leaf slices, cells, protoplasts or isolated chloroplasts. However, each experimental system has its own advantages and disadvantages. An intact plant is too flexible to study the effect of one particular factor. For e.g., the light intensity which is optimal for control plants may be supra-optimal for stressed plants and thus water stress (Boyer et al., 1987) or chilling stress (Öquist et al., 1987) could induce photoinhibition even at moderate light intensity. Another disadvantage with intact leaves is recycling of CO_2/O_2 within intercellular spaces. Water stress often causes non-uniform photosynthesis due to stomatal patchiness. The effects of water stress on biochemistry of photosynthesis are over-estimated due to the superimposition of stomatal closure, which results in limitation of CO_2. On the other hand, the effect of light stress is likely to be under-estimated, when the leaf is illuminated on its adaxial surface, because of the presence of a heterogeneous mixture of upper layer of damaged cells and lower layer of shaded cells in abaxial side (Kyle, 1987).

Using homogeneous suspensions like intact cells, protoplasts or isolated organelles, problems of non-uniform photosynthesis can be avoided and the sample can be exposed uniformly to the given treatment. However, the extraction of functional chloroplasts from water stressed plants is extremely difficult, as the leaf water potentials decrease during wilting far below the water potentials of the standard isolation media (Kaiser et al., 1981a; Sharkey and Badger, 1982). Further, the use of isolated chloroplasts prevents an assessment of water or light stress on the interaction between different organelles within the plant cell.

Isolated mesophyll protoplasts, offer another experimental system to examine the plant metabolism. They do not have any barrier against diffusion of O_2 and allow an evaluation of externally added compounds. Protoplasts offer a system closer to *in vivo* situation than do the isolated organelles since the responses of a leaf to factors like water or light stress can often be quite different from those of isolated organelles. Further, the protoplasts allow us to study the interaction between various organelles of a cell, e.g. chloroplasts and mitochondria. However, some disadvantages of protoplasts are their limited stability at warm temperature, fragile nature and tendency of sedimentation.

We have investigated the effects of water/light stress on photosynthesis and respiration in mesophyll protoplasts of pea, isolated rapidly from normal leaves. These protoplasts are then exposed to either hyper-osmotic concentrations of sorbitol (a non-penetrating polyol) or supra-optimal light intensities or both. Uniformity of the stress treatment was ensured by stirring gently the protoplast suspension. The relative sensitivity of photosynthetic carbon assimilation and photochemical activity was evaluated by monitoring O_2 evolution in presence of either bicarbonate or *p*-benzoquinone.

Plasma membrane of the protoplasts is intact not only under severe hypertonic stress but also after transfer back into an isotonic medium. Experiments were therefore performed to study the reversible nature of the effects of

osmotic stress on photosynthesis and respiration in mesophyll protoplasts, by incubating them in a hypertonic (1.0 M sorbitol) medium and subsequent transfer to isotonic one.

In sub-tropical or tropical regions, water stress often occurs along with excess light leading to photoinhibition. On the other hand, in temperate regions, water stress is associated with chilling, again promoting photoinhibition. The changes induced by osmotic stress in photosynthesis as well respiration of mesophyll protoplasts were evaluated at 0 °C or 25 °C and on further exposure to photoinhibitory light.

Incidentally, this is the first attempt to study in detail the phenomenon of photoinhibition in isolated mesophyll protoplasts. Besides demonstration and characterisation of photoinhibition using mesophyll protoplasts, we have also studied the role of dark respiration (using classic respiratory inhibitors, which inhibit different reactions of respiration), in protecting photosynthesis against photoinhibition.

Among the components of photosynthesis, PSII reactions are the most sensitive to photoinhibition. D1 protein, an important constituent of the photosynthetic electron transport chain, is believed to be the primary target of photoinhibition. We have tried to characterize and understand the role of phosphorylation of highly dynamic Dl protein during photoinhibition, using two types of antibodies SP1 and SP2 (SP1 cross-reacts with only non-phosphorylated protein and SP2 cross-reacts with both nonphosphorylated and phosphorylated forms).

An important and immediate response of plant tissue to osmotic stress is a reduction in symplast volume. There are two widely accepted experimental techniques to monitor the relationship between protoplast volume and water potential: pressure/volume curve analysis and dual label infiltration. However, both these techniques, are time-consuming and have a scope for error. To avoid such problems, we have used a microscope to measure changes in the

volume of isolated protoplasts, subjected to different degrees of osmotic stress. Although the measurements of protoplast diameter under microscope is simple, the technique is nevertheless tedious. Therefore, as an alternative parameter, we have monitored the decrease in area of detached leaf discs, under different degrees of osmotic stress and assessed its correlation with photosynthesis. Most of the earlier studies on leaf area were with intact plants and in the field.

Leaf discs from normal leaves were cut under water and then subjected to osmotic stress by increasing the concentration of sorbitol from water (zero MPa) to 1.0 M (-3.1 MPa) in the surrounding medium. Photosynthesis by leaf discs was examined under high CO_2 (5%, v/v) to avoid stomatal limitation. The area of leaf discs was measured under a stereo microscope using a mm graph paper. A major objective to develop a simple method based on leaf disc area/protoplast volume to monitor and predict the inhibition of photosynthesis due to osmotic stress. Measurement of leaf disc area is indeed easy, simple and quick.

Chlorophyll fluorescence in leaves is known to be altered during osmotic (Ögren, 1990) or temperature stress (Burke, 1990). Therefore, changes in the pattern of chlorophyll fluorescence induction kinetics were examined in detached leaf discs subjected to osmotic stress.

Water stress can be expected to cause marked changes in biophysical characteristics of membranes, since water is major a component of living cell and its membranes. The conformational status of membranes in protoplasts exposed to osmotic and/or chilling stress was examined using three different fluorescent probes, which interact specifically with either lipids or proteins: 1,6-diphenyl-1,3,5-hexatriene (DPH); 1-(4-tri-methylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toulene sulfonate (TMA-DPH) for lipids and fluoresceine isothiocyanate (FITC) for proteins. TMA-DPH is used to study the changes in plasma membrane lipids while DPH reacts with all membrane lipids.

Under stress conditions, leaf tissue is subjected to oxidative damage, i.e. lipid peroxidation, while the tissue attempts to protect itself by elevating its thiols (particularly protein thiols) or increasing the activity of oxidative peroxidase scavenging enzymes or both. The next set of experiments were designed to examine the damage (lipid peroxidation), and protective phenomena (scavenging enzymes and protein/non-protein thiols) in protoplasts under osmotic stress or photoinhibition.

To summarize, major objectives of the present work are:

1. Study the effect of osmotic stress on photosynthesis, respiration in isolated mesophyll protoplasts and detached leaf discs of pea.
2. Assess the reversibility of changes, in photosynthesis or respiration of mesophyll protoplasts, induced by osmotic stress.
3. Characterize the phenomenon of photoinhibition in mesophyll protoplasts.
4. Investigate the role of dark respiration in protecting photosynthesis against photoinhibition in mesophyll protoplasts.
5. Establish the quantitative relationship between the decrease in leaf disc area or protoplast volume and the extent of inhibition of photosynthesis during osmotic stress.
6. Examine the response to osmotic stress of selected membrane properties like chlorophyll fluorescence induction kinetics (in detached leaf discs) and fluidity/conformational changes in lipids/proteins (of protoplasts).
7. Study the damage (lipid peroxidation) and protective phenomena (peroxide scavenging enzymes and protein/non-protein thiols) in protoplasts under photoinhibition and/or osmotic stress.

Most of the experiments were performed with mesophyll protoplasts isolated from pea (*Pisum sativum)* leaves. A few experiments were made with detached leaf discs.

Chapter 3

Materials and Methods

Chapter 3

Materials and Methods

Plant Material

Plants of pea (*Pisum sativum* cv. Bonneville) were raised from seeds, procured from Pocha Seeds Company, Pune, India. The seeds were soaked in water overnight and then surface sterilised with 0.1% (v/v) sodium hypochlorite solution. Big and round seeds were selected and sown in plastic trays filled with soil and farm-yard manure. The plants were grown outdoors under natural photoperiod of approximately 12 h and average temperatures of 30 °C day/20 °C night. The plants were watered daily. The second pair of fully expanded leaves were picked from 8 to 10 day old plants (Plate 3.1) and used for experiments.

Experiments were performed with either mesophyll protoplasts or detached leaf discs.

Isolation of Mesophyll Protoplasts

Mesophyll protoplasts were isolated from leaves by minor modifications of the procedure described by Vani et al. (1990).

The abaxial epidermis of the pea leaves was stripped off with the help of forceps. The stripped leaves were then cut into pieces of *ca.* 0.5 cm^2. The portions containing the midrib were discarded. The leaf pieces were floated with naked abaxial side facing down and in touch with preplasmolysis medium containing 0.3 M sorbitol and 1 mM $CaCl_2$ in 10 mM Mes-KOH buffer pH 6.0. After 30 min, the preplasmolysis medium was removed and the digestion medium, containing 2% (w/v) Cellulase Onozuka R-10, 0.2% (w/v) Macerozyme R-10, 0.25% (w/v) BSA, 10 mM sodium ascorbate, 0.4 M sorbitol and 1 mM $CaCl_2$ in 10 mM Mes-KOH buffer pH 5.5, was added. The leaf pieces

Plate 3.1. A view of 8 to 10-day-old plants of pea (*Pisum sativum* cv. Bonneville) grown in the field (outdoors).

were digested for 30 min at 30 °C under illumination with a tungsten lamp at a light intensity of about 50 $\mu E\ m^{-2}\ s^{-1}$.

After digestion, the digestion medium was gently removed with help of Pasteur pipette. The washing medium (0.4 M sorbitol and 1 mM $CaCl_2$ in 10 mM Mes-KOH pH 6.0) was added to the Petri dish containing the digested leaf strips. The Petri dish was swirled and tapped gently, releasing the protoplasts into the medium. The suspension was filtered through nylon filters of 300 and 60 µrn and centrifuged at 50 g for 5 min. The supernatant was discarded and the pellet was washed twice with the washing medium and once with suspension medium of 0.4 M sorbitol, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ in 10 mM Hepes-KOH pH 7.0. The protoplast pellet was finally suspended in small aliquot of the above medium and kept on ice.

Purity/Intactness of Protoplasts

The viability and intactness of protoplasts were routinely checked using neutral red and Evans blue. The purity of preparation normally ranged from 90-97%. A typical protoplast preparation is shown in Plate 3.2.

Estimation of Chlorophyll

Chlorophyll (Chl) was estimated by extracting into 80% (v/v) acetone (Arnon, 1949).

An aliquot of 25 µl of protoplast suspension was added to 10 ml of 80% (v/v) acetone. The absorbance of acetone extract was measured at 652 nm (A_{652} - to determine Chl) and 710 nm (A_{710} - to correct for turbidity), using a spectrophotometer (Shimadzu UV-160A). The Chl concentration was calculated using the following formula

Chl (mg ml"! of protoplast suspension) = $(A_{652} - A_{710})$ x 11.11

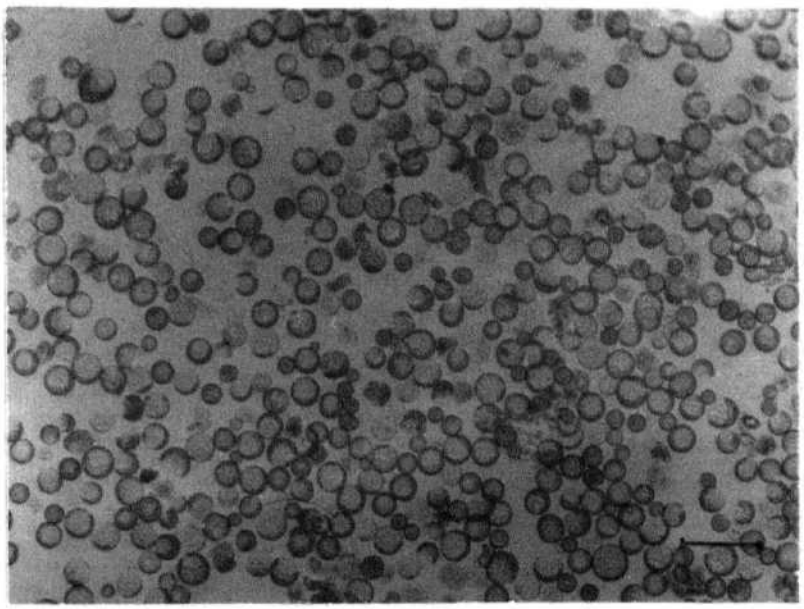

Plate 3.2. Photomicrograph of mesophyll protoplasts isolated from leaves of pea (*Pisum sativum).* The horizontal bar represents 100 μm.

Photosynthetic O_2 Evolution/Respiratory O_2 Uptake

Photosynthetic activity of protoplasts was measured in terms of O_2 evolution on illumination, while respiration was measured by monitoring O_2 uptake in darkness. The evolution or uptake of oxygen was measured at 25 °C using Clark type oxygen electrode (Model DW2, Hansatech Ltd, King's Lynn, U.K.).

The reaction medium of 1 ml containing 0.4 M sorbitol, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM bicarbonate in 10 mM Hepes-KOH, pH 7.5 and protoplasts equivalent to 20 μg Chl. In some of the experiments, 1 mM *p*-benzoquinone was used instead of bicarbonate. Water at a constant temperature of 25 °C was circulated through the outer jacket of reaction chamber. A 35 mm slide projector provided the illumination of 1250 $\mu E\ m^{-2}\ s^{-1}$. Calibration of the oxygen content in the electrode chamber was done with air saturated water, assumed to contain 252 nmoles of oxygen ml^{-1} at 25 °C (Walker, 1988).

Photosynthetic O_2 Evolution *by Leaf Discs*

Leaf discs *of ca.* 0.25 cm^2 were punched under water with a sharp paper-punching machine from either side of the midrib. Photosynthesis by leaf discs was monitored by a computerised leaf disc O_2-electrode system (LD-2, Hansatech Ltd., King's Lynn, U.K.).

Nineteen small discs (each of 0.25 cm^2), used for each measurement, were arranged symmetrically in three successive rings (of 1, 6 and 12 discs) on perforated stainless steel grid kept in the leaf disc O_2-electrode chamber. In the electrode chamber, the components were arranged in the following order (from bottom to top): stainless steel washer, capillary matting, perforated disc (plain centre) foam rubber disc and finally perforated disc on which leaf discs were arranged. The capillary matting was moistened with 150 μl of 1.0 M bicarbonate buffer, pH 9.0, which results in a gaseous atmosphere of 5% (v/v)

CO_2 within the leaf disc chamber. Such high CO_2 helps to avoid stomatal limitation and registers maximal rates of photosynthesis (Walker, 1988).

Photosynthetic O_2 evolution by leaf discs as a function of photon flux density (PFD) was measured at 25 °C by illuminating with a series of step-up light intensities emitted by an array of light emitting diodes (LED). The intensity of the light was increased from 0 to 825 μE m^{-2} s^{-1}, according to a predefined computerised programme (Table 3.1). The light intensity emitted by LEDs was measured with a custom-made quantum meter (SKP 216 Quantum Sensor, Skye Instruments Ltd., UK), which employs a quantum sensor having the spectral response in the range of approximately 550 nm to 750 nm (corresponding to the red light emitted by LEDs). Leaf discs were pre-illuminated with LEDs at an intensity of 200 μE m^{-2} s^{-1} to avoid the initial lag during the photosynthetic measurements.

Leaf disc electrode chamber was calibrated by using 1 ml samples of air, as per the instructions of software supplied by the manufacturer (Hansatech Ltd., King's Lynn, UK). Leaf disc chamber was calibrated for every sample being tested. The data were collected, stored and analysed by an IBM-PC AT386 computer, with the help of software supplied by Hansatech Instruments, King's Lynn, UK.

The typical pattern of automatic measurements of photosynthetic O_2 evolution (or uptake) at increasing light intensity and subsequent plotting of the graph by the computer are illustrated in Fig 3.1.

Exposure of Protoplasts/Leaf Discs to Osmotic Stress

Protoplasts were subjected to osmotic stress by increasing the concentration of sorbitol from 0.4 M (-1.3 MPa) to 1.0 M (-3.1 MPa) while pre-incubating at 25 °C for 10 min (unless otherwise specified) in darkness. In some of the experiments, osmotic stress to protoplasts was imposed at 0 °C to evaluate

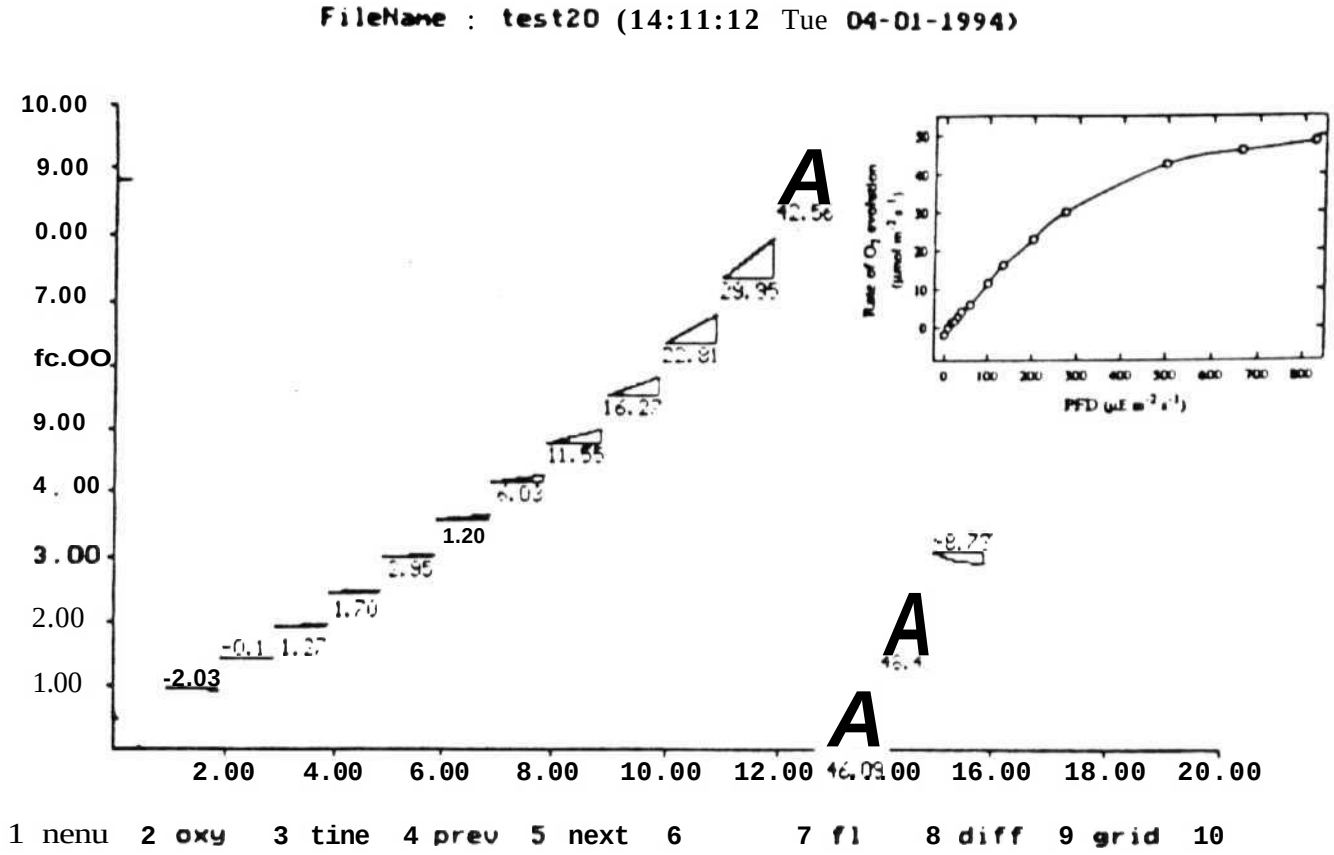

Figure 3.1. A print-out of computer screen display showing traces of O_2 uptake/evolution by detached leaf discs of pea *(Pisum sativum)*, at 25 °C, as a function of photon flux density (PFD). Light intensity was progressively increased from 0 to 825 $\mu E\ m^{-2}\ s^{-1}$, in thirteen steps by a pre-defined computerised programme. The digits near each trace indicate the rate of O_2 uptake (-) or evolution (+), calculated for every 50 sec. The rate of O_2 uptake at 'zero' light intensity (first and the last trace) represents dark respiration by leaf discs. **Inset:** Plot of photosynthetic O_2 evolution *vs* photon flux density (PFD) in a range of 0 to 825 $\mu E\ m^{*2}\ s^{-1}$.

Table 3.1. *The range of PFD values used in a typical measurement of photosynthetic rate by leaf discs of pea.*

S.No.	Output of the Signal from Computer to LED Controller Box (0-4090)	Measured PFD
		($\mu E\ m^{-2}\ s^{-1}$)
1	0	0*
2	39	8
3	74	17
4	106	25
5	138	33
6	170	40
7	200	60
8	317	100
9	630	134
10	980	201
11	1350	272
12	2470	498
13	3250	662
14	4090	826
15	0	0*

* No light; Activity reflects rate of dark respiration

the amplification, if any by chilling. Accordingly, the same osmoticum was maintained in the reaction medium. To evaluate the immediate effect of osmotic stress on protoplasts, the concentration of sorbitol was increased during the assay, i.e. sorbitol concentration of only the reaction medium was increased. Except for sorbitol, the remaining components of suspension/reaction media were unaltered. During pre-incubation the chlorophyll concentration of protoplast suspension was maintained at 200 $\mu g\ ml^{-1}$.

Leaf discs were incubated in varying concentrations of sorbitol from 0.1 M (-0.1 MPa) to 1.0 M (-3.1 MPa) for one hour at 25 °C in darkness. Photosynthetic activity of fully turgid leaf discs, i.e. leaf discs incubated in water for one hour, was considered as control.

Measurement of Protoplast Volume/Leaf Disc Area

The diameter of the palisade and spongy protoplasts, after incubation in varying concentration of sorbitol from (0.4 M to 1.0 M) for 10 min at 0 °C (on ice), were measured under a microscope (Wolfe, Japan) using a pre-calibrated ocular micrometer. Volume was calculated by assuming spherical protoplasts, using the formula of 4/3 πr^3 (r : radius of protoplast). Forty protoplasts were measured during each observation and the experiment was repeated at least four times on different days. Each value of protoplast volume of therefore represents an average of at least 160 measurements.

The diameter of the leaf discs, incubated in varying concentrations of sorbitol from zero (water) to 1.0 M for an hour, were measured under a Stereo microscope (Meopta, Czechoslovakia) by placing the leaf discs on a glass plate below which a cm-graph paper was attached. Area of each leaf disc was calculated using the formula of πr^2 (r : radius of leaf disc). Three leaf discs were measured during each observation and the experiment was repeated for seven times on different days. Each value of leaf disc area of therefore represents an average of at least twenty measurements.

Absorption Spectrum of Protoplasts

The absorbance of protoplast suspension (containing 10 μg Chl ml^{-1}) in varying concentrations of sorbitol from 0.4 M to 1.0 M, was scanned between 250 and 800 nm in a spectrophotometer (Shimadzu UV-160A). Acetone extract of protoplasts, i.e. protoplasts equivalent to 10 μg Chl in 1 ml of 80% (v/v) acetone, also was scanned between the same range.

Chlorophyll Fluorescence of Leaf Discs

The fluorescence induction kinetics of the leaf discs incubated in varying concentrations of sorbitol (0.1 to 1.0 M) or water at 25 °C in darkness

for one hour, was measured using a Chlorophyll Fluorometer (Richard Brancker Research Ltd., Ottawa, Model SF-30).

After the pretreatment with sorbitol solution, the leaf disc was placed on the probe and covered with a black cloth. After 5 min of darkness, the leaf disc was illuminated for 30 sec with a red light (monochromatic light at a wavelength of 670 nm, emitted by a LED) of intensity 6 W m^{-2}. The photocell uses the LED also as a lens to collect the fluorescent radiation from the leaf disc. This light is filtered before detection and amplification for further processing by the control unit. The data on fluorescence emission from the leaf discs were saved in the temporary memory of the instrument.

The fluorescence data were later down-loaded on to a computer (IBM-PC AT386) and splined data were used for further calculation and plotting the graphs of chlorophyll fluorescence vs time. Time taken to reach the fluorescence maximum (F_m) against the concentration of sorbitol during pre-treatment was calculated from the plots. A typical figure obtained from primary data collected by the Chlorophyll Fluorometer and later processed by a computer, is illustrated in Fig. 3.2.

The curve in the Fig. 3.2 has often been designated as OIDPSMT-curve (Labeling indicated in the inset). On illumination, fluorescence rises immediately (in picoseconds but limited by the opening time of the shutter) to O (the level of the "initial fluorescence") and, thereafter, there is an initial rise to level "I", followed by a dip (D), a peak (P) and a fall, via a quasi-steady-state (S) to a terminal steady value (T). Some times, a secondary maximum (M) is seen between S and T.

Potential photosynthesis of the leaf tissue was calculated according to Szigeti et al. (1988) using the formula:

$$\frac{F_m - F_v}{F_v}$$

F_m = maximal fluorescence indicated by 'P' in Fig. 3.2
F_v = variable fluorescence indicated by 'T' in Fig. 3.2

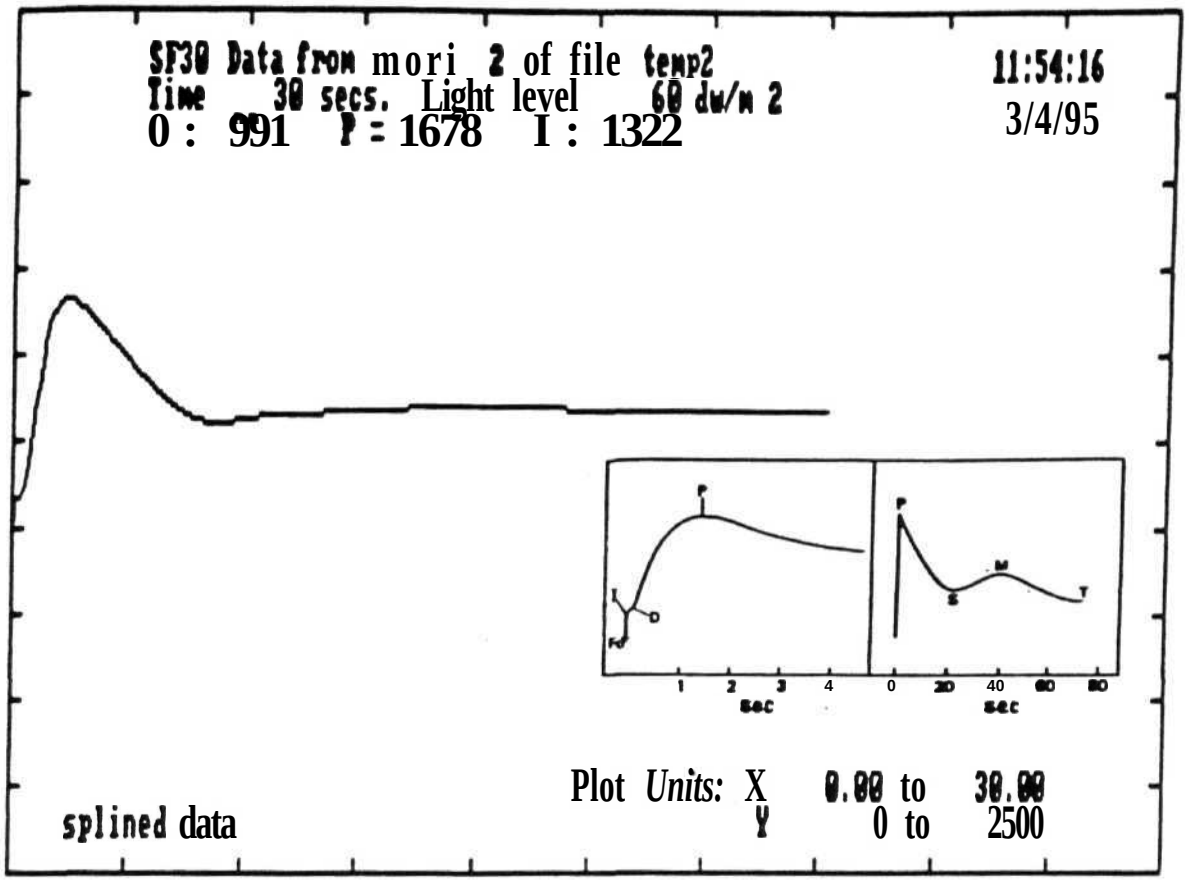

Figure 3.2. A typical chlorophyll fluorescence induction curve of dark adapted leaf disc of pea (*Pisum sativum*), plotted by the computer, using the primary data collected by Chlorophyll Fluorometer. **Inset:** Diagrammatic representation of a typical 'Kautsky' curve indicating the positions of OIDPSMT. On illumination of dark adapted leaf, the fluorescence rises immediately from O (the level of the "initial fluorescence") to I, followed by a dip (D), thereafter to a peak (P) and a fall, via a quasi-steady state (S) to a terminal steady value (T) with a secondary maximum (M) in between S and T.

Membrane Fluidity

Changes in fluidity of protoplast membranes during osmotic stress was studied using different fluorescent probes which interact with lipids or proteins. l,6-diphenyl-l,3,5-hexatriene (DPH) and (1,4-trimethyl-ammonium phenyl)-6-phenyl 1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) are lipophilic dyes, which bind to lipid component of the membranes. On the other hand, fluoresceine isothiocyanate (FITC) binds to the protein component of the membranes.

DPH and TMA-DPH were dissolved in dimethylformamide and FITC in 100 mM carbonate buffer, pH 9.0. The stock solutions were stored at 4 °C in darkness. An aliquot of these dyes was added to protoplast suspension (containing 10 μg Chl ml^{-1}) so as to obtain a final concentration of 0.1 μM DPH, 1.0 μM TMA-DPH and 100 ng ml^{-1} FITC. For all experiments (including bottles to preserve stocks) only glassware were used and plasticware were avoided, as a rule.

Excitation and emission spectra were recorded to identify emission and excitation maxima, respectively under different combinations, using a Fluorescence Spectrophotometer (Hitachi, Model F-3010). The excitation/ emission maxima were 364/430 nm in case of TMA-DPH and 359/430 and 456 nm (two emission peaks) for DPH. The excitation and emission maxima for FITC were 496 and 518, nm respectively. The excitation spectra are shown and the peaks are identified in Figs. 3.3 to 3.5. The emission spectra peaks are included in Chapter 7.

To avoid the problem of protoplast sedimentation of during scanning, the samples were scanned quickly, at a speed of 600 nm min^{-1}. Thus the emission spectra of DPH and TMA-DPH from 400 to 550 nm was done within 15 sec and in case of FITC, scan range was 500 to 600 nm which was completed within 10 sec.

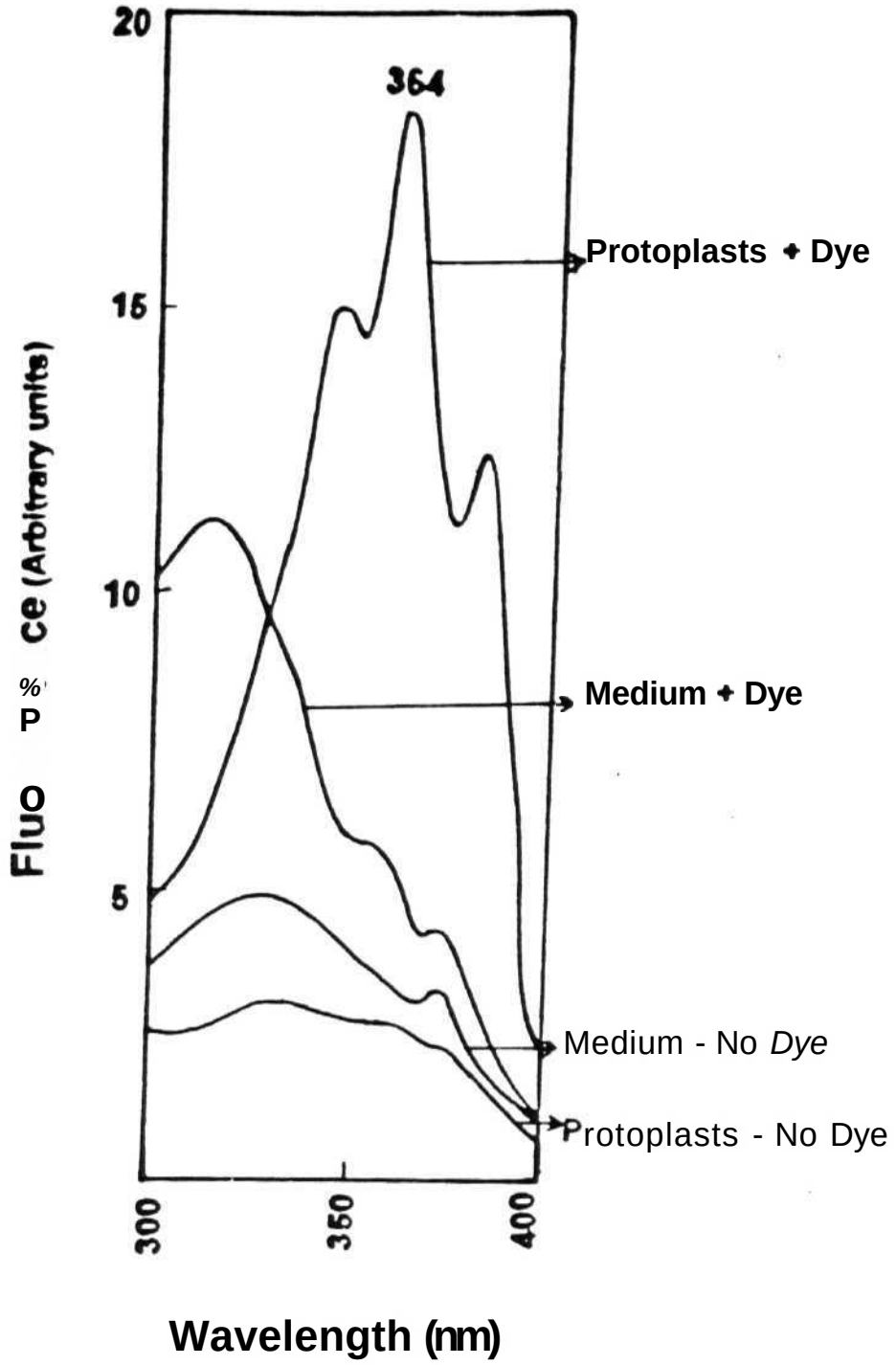

Figure 3.3. Excitation spectra of fluorescence from lipophilic dye, 1.0 μM TMA-DPH (binds to the lipid component of plasma membrane) in the medium without or with protoplasts, equivalent 10 μg Chl ml^{-1}. The excitation maximum of fluorescence from TMA-DPH was at 364 nm.

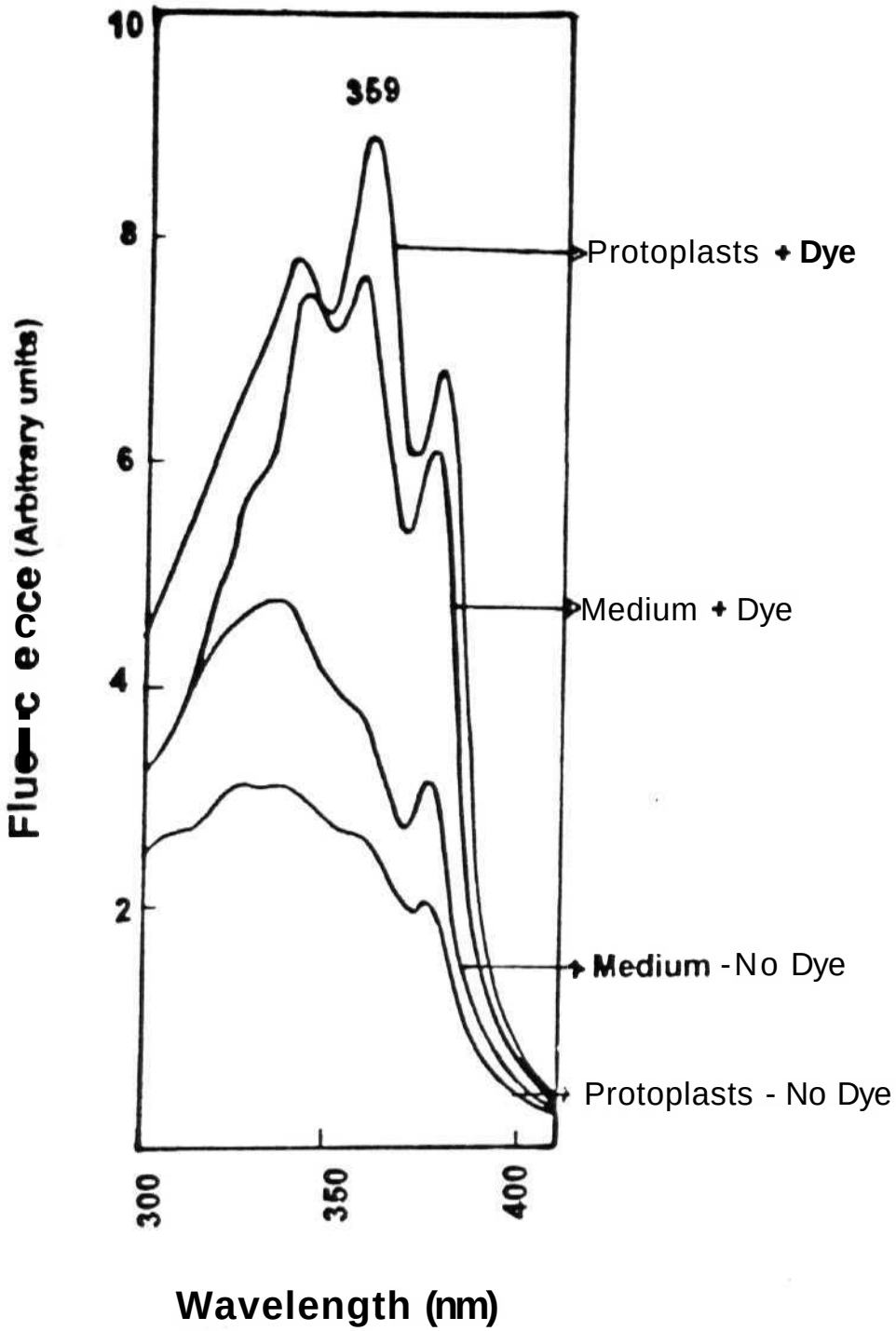

Figure 3.4. Excitation spectra of fluorescence from 0.1 μM DPH (a lipophilic dye which binds to lipid component of all the membranes), without or with protoplasts equivalent 1.0 μg Chl ml^{-1}. The excitation maximum of DPH was at 359 nm.

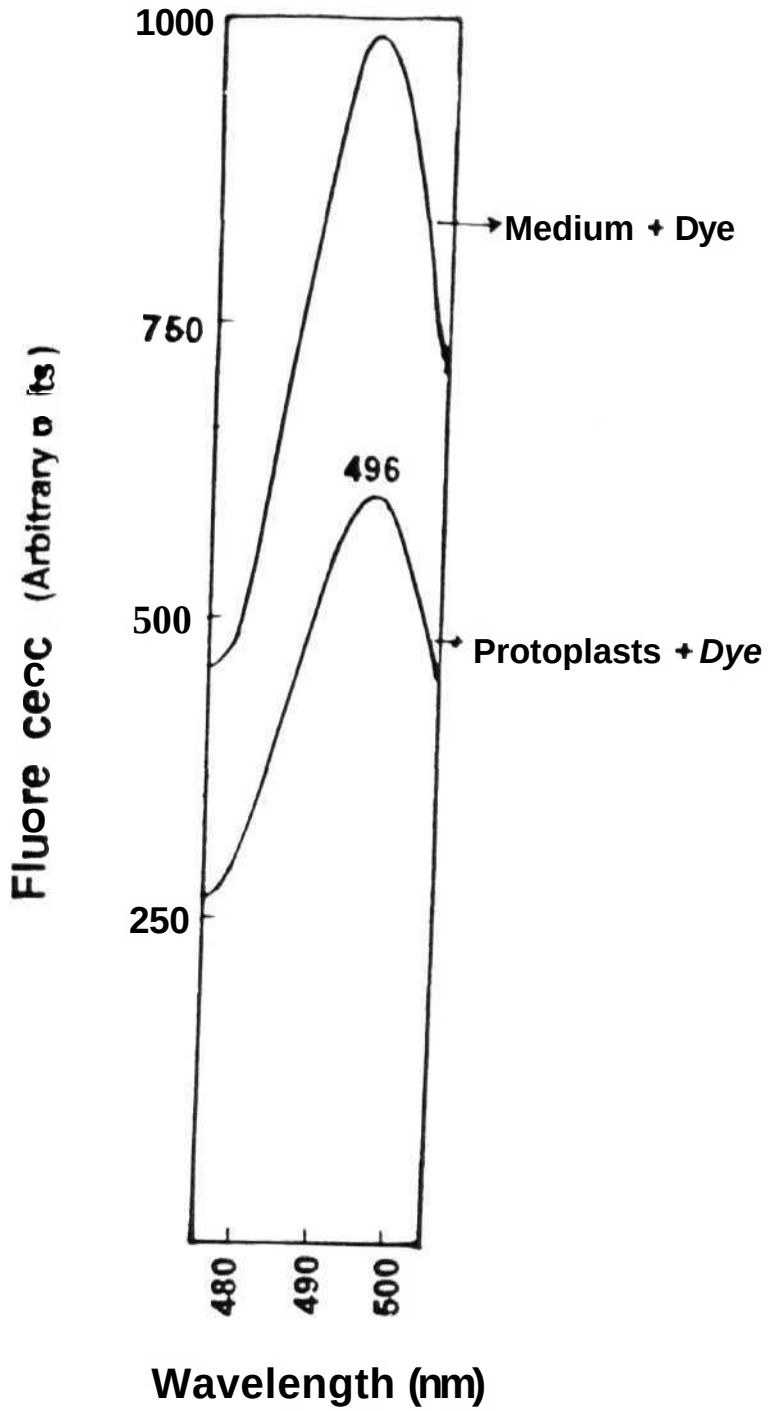

Figure 3.5. Excitation spectra of fluorescence from 100 ng ml^{-1} FITC (a fluorescent dye, which binds to protein component of membranes), without or with protoplasts equivalent 10 μg Chl ml^{-1}. The excitation maximum of FITC was at 495 nm.

The changes in the polarization of the dyes (only in case of TMA-DPH and FITC) during osmotic stress also were studied using excitation as well as emission polarizers. The polarization values (*P') were derived by measurements with vertical or horizontal orientation of excitation/emission polarizers. The polarization value was calculated according to Revathi et al. (1994) using the following equation

$$P = \frac{(I_W - GI_{VH})}{(I_{VV} + GI_{VH})}$$

where I_{VV} and I_{VH} are the fluorescence intensities measured with the vertically oriented excitation polarizer while the emission polarizer is oriented either vertically (I_{VV}) or horizontally (I_{VH}). G is the grating correction factor and is equal to I_{HV}/I_{HH}. These denote fluorescence measurements made with horizontally oriented excitation polarizer, while the emission polarizer is oriented either vertically (I_{HV}) or horizontally (I_{HH}).

The polarization of DPH in protoplast membranes could not be studied because of its slow entry into the protoplasts which resulted in a continuous increase in fluorescence emission of the dye even after 30 min of its addition to protoplast suspension.

Protoplasts were subjected to one or a combination of stresses, namely osmotic and/or chilling stress, by using sorbitol as described earlier. To evaluate the effect of chilling, protoplasts were pre-incubated in 1.0 M at 0 °C. As 1.0 M sorbitol itself gave a high fluorescence emission when compared to 0.4 M sorbitol, the emission or excitation spectra of osmotically stressed protoplasts with dye were corrected with background measurements with 1.0 M assay medium.

Photoinhibition

Protoplasts (containing 200 μg Chl ml^{-1}) were placed in a jacketed chamber maintained at a constant temperature of 25 °C and illuminated with a high light (3000 μE m'^{2} s^{-1}). Illumination was provided by four tungsten bulbs (Philips Comptalux 225 V/75 W), with two each placed on either side of the thermo-jacketed pre-incubation chamber. Additional water filters and a flow of cool air between the lamps and pre-incubation chamber ensured that the chamber was not heated by the lamps. During incubation, the protoplast suspension was stirred gently.

During photoinhibitory treatment, the protoplasts were suspended in either 0.4 M or 1.0 M sorbitol-containing medium to evaluate the synergistic effect of photoinhibition with osmotic stress. In a few experiments, protoplasts were pre-incubated on ice for 10 min in 1.0 M medium and then photoinhibited to evaluate further aggravation, if any, with pre-chilling stress. An aliquot of protoplast suspension equivalent to 20 μg Chl was taken out for measuring photosynthesis or respiration soon after the photoinhibitory treatment. The activity was compared with reference to their respective dark or low light-incubated samples.

For the analysis of D1 (PS II reaction centre) protein, protoplasts were photoinhibited for 10, 20 or 30 min in 0.4 M or 1.0 M sorbitol containing medium. After incubation, aliquots of protoplasts, equivalent to 10 μg Chl (at least in triplicates), were frozen in liquid N_2. One of the samples was used to visualise protoplast membrane proteins on gel and the remaining two samples were used for Western blot analysis (to stain with SP1, SP2 antibodies separately).

SDS-PAGE of Protoplast Membrane Proteins

Frozen protoplast samples were thawed and centrifuged at 10,000 g for 15 min for protein-analysis by SDS-PAGE (Laemmli, 1970).

The pellet was dissolved in 25 ml of sample buffer **containing** 62.5 **mM** Tris-HCl **pH** 6.8, **4%** (w/v) SDS, 5 M urea, 10% (v/v) β-mercaptoethanol, 6% (v/v) glycerol and incubated at 65 °C for 15 min. Polypeptides of these protoplast membranes were separated by denaturing SDS-PAGE. The separating gel contained 12 to 18% (w/v) gradient of acrylamide, 0.32 to 0.48% (w/v) of bis-acrylamide, 0.375 M Tris-HCl pH 8.8, 0.4% (w/v) SDS, 4 M urea and 10% (w/v) sucrose. Stacking gel was made up of 4% (w/v) acrylamide and 0.13% (w/v) bis-acrylamide in 125 mM Tris-HCl, pH 6.8. The electrode buffer contained 0.19 M glycine and 0.1% (w/v) SDS in 25 mM Tris-HCl, pH 8.3. Electrophoresis was performed at 60 V until the dye front migrated into separating gel and later the voltage was increased to 120 V.

At least three gels were run in parallel. One of the gels was stained with coomassie brilliant blue R-250 to visualise the protein separation pattern and, the other two were used for Western blotting. A typical profile of protoplast membranes are shown in Plate 3.3.

The proteins on the gel were fixed by incubating the gel for 15 min in a fixative, containing 40% (v/v) methanol and 7% (v/v) acetic acid. Then the gel was stained with 0.1% (w/v) coomassie brilliant blue R-250 in 50% (v/v) methanol and 12.5% (v/v) acetic acid and destained with a destaining solution containing 50% (v/v) methanol and 12.5% (v/v) acetic acid.

Western Blot Analysis of Dlprotein

After electrophoretic separation, the polypeptides were transferred on to Immobilon-P (Polyvinylidene difluoride, PVDF) membranes with a semi-dry electroblotting unit (LKB, Model, 2117 MultiphorII).

The gel, PVDF membrane and chromatography (Whatman No. 3) papers were soaked in blotting buffer, containing 25 mM Tris-HCl, 192 mM glycine and 20% (v/v) methanol for 30 min. Then the gel and membrane were

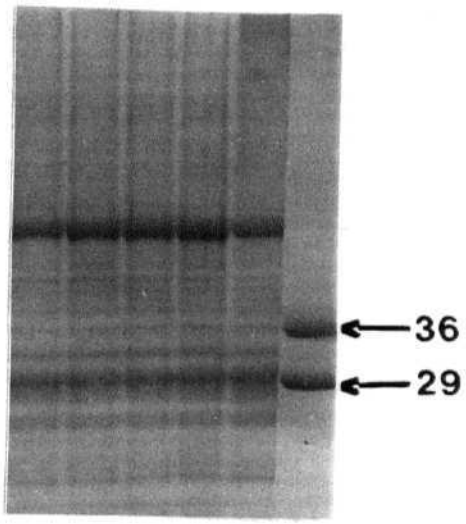

Plate 3.3. A typical protein profile of protoplast membranes separated on SDS-PAGE, using 12 to 18% (w/v) acrylamide and 0.32 to 0.48% (w/v) bis-acrylamide. The two marker proteins (shown by arrows), on the extreme right lane are lactate dehydrogenase and carbonic anhydrase, with molecular weights of 36 and 29 kDa respectively.

sandwiched between the filter papers (three on either side) saturated with buffer. The transfer was done at a constant current of 100 mA for 2 h. After electrotransfer, the membrane was processed, as described by Betz and Dietz (1991).

The membranes were blocked (to saturate nonspecific sites) with 5% (w/v) non-fat milk powder in Tris buffered saline (TBS, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl). The blocked membranes were treated with SP1 or SP2 antibodies of D1 protein (diluted by 1:800 in the blocking solution), for one h. The antibodies were a kind gift from Dr AK Mattoo, Beltsville, Maryland, USA. The membranes were then washed three times (15 min of each wash) with TBS, incubated for one h with secondary antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase) for one hour, and washed for three times. The membranes were developed with 5-bromo-4-chloro-3-indolylphosphate (BCIP) (in a 1:250 dilution of 50 mg ml^{-1} stock solution) and *p*-nitro-blue-tetrazolium chloride (NBT) (used as a 1:125 dilution of 50 mg ml^{-1} stock solution) in 16 mM Tris-HCl buffer (9.5 pH), 4 mM NaCl and 0.2 mM $MgCl_2$.

Preincubation** with Metabolic **Inhibitors

Protoplast suspensions (containing of 200 μg Chl ml^{-1}) were preincubated with different test compounds (antimycin A, sodium azide, Oligomycin, sodium fluoride and sodium malonate) at the concentrations indicated in the text. The preincubation was done in a water jacketed chamber at 25 °C and kept in either darkness or under photoinhibitory light (3000 $\mu E\ m^{-2}\ s^{-1}$). The incubation was for 10 min, unless otherwise specified.

After incubation, protoplast aliquots (equivalent to 20 μg Chl), were removed for assay of their metabolic (respiratory/ photosynthetic) activities (with reference to control samples) or while separate aliquot containing 10 μg Chl were frozen in liquid nitrogen, to be used further for assessing the status of the Dl protein. The effect of the inhibitors of only oxidative electron trans-

port/oxidative phosphorylation (sodium azide, antimycin A and Oligomycin) was examined on the status of D1 protein. In some of the experiments, 10 mM streptomycin was included to block Chloroplast protein synthesis.

Lipid Peroxidation

Peroxidation of protoplast lipids was measured by monitoring the formation of 2-thiobarbituric acid-malondialdehyde (TBA-MDA) adduct, according to Ohkawa et al. (1979) with minor modifications.

After photoinhibitory or osmotic stress treatment, the reaction was arrested by transferring an aliquot of protoplasts (100 µl, equivalent to 20 µg Chl) into a mixture of 0.5 ml of acetic acid (pH adjusted to 3.5 with KOH) and 0.2 ml of 10% (w/v) SDS. Further, 1.5 ml of 0.7% (w/v) thiobarbituric acid (TBA) in aqueous solution was added to the mixture. Samples were incubated for one hour in boiling water bath and were centrifuged at 250 g for 10 min. Absorbance of the clear supernatant was measured at 532 and 600 nm. Absorbance at 600 nm was subtracted from the A_{532} values, to correct for the non-specific turbidity. The amount of MDA formation was calculated using an extinction coefficient of 155 mM cm^{-1}. The extent of lipid peroxidation in protoplasts at different sorbitol concentrations was compared also with respect to their controls (without protoplasts).

Protein and *Non-protein Thiols*

The total soluble protein-SH content of protoplasts was measured according to De Kok et al. (1985), while non-protein thiols were estimated according to Cakmak and Marschner (1992).

Photoinhibited and/or osmotically stressed protoplasts and their respective control samples (100 µl, equivalent to 20 µl Chl) were added to 0.4 ml of 0.15% (w/v) sodium ascorbate and then centrifuged at 30,000 g for 15 min. An aliquot (0.2 ml) of the supernatant was added to a mixture of 0.2 ml of 8%

(w/v) SDS and 0.4 ml of Tris-HCl, pH 8.0. Another aliquot of 0.25 ml (of the supernatant) was de-proteinised by incubating in boiling water bath (100 °C) for 3 min. The de-proteinised samples were centrifuged at 15,000 g for 15 min. The pellet was discarded and 0.25 ml of supernatant was added to 0.25 ml of distilled water (in stead of SDS) and 0.5 ml of Tris-HCl, pH 8.0.

To all the above samples (non-deproteinised as well as de-proteinised), 70 µl of 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), dissolved in 1.0 M Tris-HCl, pH 8.0 was added. The developed yellow color was measured at 412 nm. Absorbance was corrected for the color of the supernatant and for the color of DTNB. Total protein-SH content was determined by subtracting the SH-content measured in a deproteinised supernatant extract from that of a SDS treated supernatant (non-deproteinised) extract.

For estimation of non-protein thiols, protoplast aliquots were extracted with 5% (w/v) meta-phosphoric acid and then extracts were cleared by centrifugation at 15,000 for 40 min. The supernatant of the samples were neutralised with KOH. 0.5 ml of 0.2 M potassium phosphate buffer, pH 8.0 was added to 0.5 ml of neutralised sample, to which 70 µl of 10 mM DTNB (dissolved in 100 mM potassium phosphate buffer, pH 7.0) was added. The developed yellow color was measured at 415 nm as mentioned above.

Centrifugation of all samples/mixtures was performed at 4 °C. Reduced glutathione (GSH) was used as standard for protein and non-protein thiols, and the standard curve was prepared with a range of 1-20 nmol GSH ml^{-1}.

Enzyme Assays

The protoplast samples after the given stress treatment were examined and compared with their respective controls for the levels of different enzymes as described below. All the enzyme assays were done at 25 °C, with the help of a spectrophotometer (Shimadzu UV-160A). Usually protoplasts were present in both the sample as well as reference cuvettes during the assays, while the

substrate was added to only the sample cuvette. 0.02% (v/v) Triton-x was used during the assay so as to solubilise the protoplast membranes.

Ascorbate Peroxidase (EC 1.11.1.11)

Ascorbate peroxidase was assayed by monitoring H_2O_2 dependent oxidation of ascorbate according to Miyake et al. (1993).

The reaction medium (1 ml) contained 50 mM potassium phosphate buffer, pH 7.0, 0.1 mM sodium ascorbate and protoplasts equivalent to 1 fig Chl. The reaction was initiated by adding 0.2 mM H_2O_2 in sample cuvette. The decrease in absorbance at 290 nm was recorded. The reaction was linear for at least 2 min. One unit of ascorbate peroxidase is defined as the amount of enzyme that oxidises 1 μmol of ascorbate per min at 25 °C. An extinction coefficient of 2.8 $mM^{-1} cm^{-1}$ for H_2O_2 was used for calculations.

Guaiacol Peroxidase (EC 1.11.1.7)

The enzyme activity was assayed by monitoring the guaiacol peroxidation, according to the method of Van Huyster and Lobarzewski (1982).

Assay mixture of 1 ml contained 50 mM sodium phosphate buffer pH 7.0, 89 mM guaiacol, and protoplasts equivalent to 1 |ig Chl. The reaction was initiated by adding 5 mM H_2O_2 to the sample cuvette. The formation of tetraguaiacol (increase in absorbance) was immediately measured at 470 nm. The reaction was linear at least for 3 min. Peroxidase activity was expressed as the formation of tetraguaiacol per min using molar extension coefficient of 26.6 $mM^{-1} cm^{-1}$.

Glutathione** Reductase (EC **1.6.4.2)

Glutathione reductase was assayed by determining NADPH dependent reduction of oxidised glutathione with slight modifications of the procedure described by Van Rensburg and Krüger (1994).

One ml of reaction mixture contained 40 mM Tricine-NaOH pH 7.8, 0.1 mM NADPH, and protoplasts equivalent to 2.5 μg Chl. The reaction was initiated by the addition of 0.5 mM oxidised glutathione into sample cuvette. The rate of NADPH oxidation was monitored at 340 nm, was linear at least for 5 min. Enzyme activity was calculated by taking into account the molar extinction coefficient value of NADPH, 6.2 mM^{-1} cm^{-1}.

Catalase (EC1.11.1.6)

Catalase was assayed by monitoring the disappearance of H_2O_2, according to Siminis et al. (1994).

The assay medium (1 ml) contained 50 mM potassium phosphate buffer (pH 7.0) and protoplasts equivalent to 1 μg Chl. The reaction was initiated with 12.5 mM H_2O_2. The decrease in absorbance at 240 nm was monitored quickly and the rate of the reaction in the first min was considered for calculations. One unit of catalase enzyme is defined as the amount of enzyme catalyzing the decomposition of 1 μmol H_2O_2 per min at 25 °C. An extinction coefficient (for H_2O_2 at 240 nm) of 0.036 $cm^{-} mmol^{-}$ was employed for calculations.

Replications and Statistical Analysis

The data presented are the average values (± SE) of results from 3 to 6 experiments conducted on different days. Statistical analysis of data was done using a computerised program written in a basic language. The correlation coefficients (r), and statistical significance (at indicated P values) of observations are included in Tables/Figure legends, wherever relevant.

Materials/Chemicals

Cellulase (Onozuka R-10) and Macerozyme R-10 (pectinase) were procured from Yakult Honsha Co. Ltd., Nishinomiya, Japan. Antimycin A, BCIP,

coomassie brilliant blue R-250, DPH, DTNB, FITC, NBT, NADPH, Oligomycin, PVDF membranes, secondary antibodies, sodium azide and TMA-DPH were from Sigma Chemical Company, USA. All other chemicals were of Sisco Research Laboratories, E. Merck (India) Ltd., Spectrochem Pvt Ltd., Loba Chemie, HiMedia Laboratories Pvt Ltd., or Ranbaxy Laboratories Ltd., all from India.

Antibodies (SP1 and SP2 of D1 protein) were a generous gift from Dr A.K. Mattoo, USDA-ARS Plant Molecular Biology Laboratory, Beltsville Agricultural Research Station, BARC-West, Beltsville, Maryland, USA.

Chapter 4

Inhibition of Photosynthesis by Osmotic Stress in Mesophyll Protoplasts; Intriguing Response of Respiration

Chapter 4

Inhibition of Photosynthesis by Osmotic Stress in Mesophyll Protoplasts; Intriguing Response of Respiration

The imposition of osmotic stress on mesophyll protoplasts of pea caused a significant decrease in the rates of their photosynthetic carbon assimilation as well as on respiration in protoplasts. However, photosynthesis as well as respiration recovered slowly, when the protoplasts were transferred back to iso-osmotic medium. Thus, the effects of osmotic stress on photosynthesis or respiration in mesophyll protoplasts were marked but interestingly reversible.

The average rates of photosynthesis (CO_2 dependent O_2 evolution) by mesophyll protoplasts of pea ranged from 140 to 180 μmol mg^{-1} Chl h^{-1}, when assayed in an isotonic medium containing 0.4 M sorbitol. The photosynthetic rate in mesophyll protoplasts decreased on exposure to solutions of increasing osmolarity. Protoplasts lost over 30% of their photosynthetic capacity, when assayed in a medium containing 1.0 M sorbitol (Fig. 4.1). On the other hand, *p*-benzoquinone dependent oxygen evolution was not much affected (the loss being <10%), even after exposure to 1.0 M sorbitol). The relative insensitivity of PSII reactions, unlike the CO_2 fixation, had been observed by earlier workers (Sharkey and Badger, 1982; Berkowitz and Gibbs, 1984; Kaiser, 1984; Martin and Ruiz-Torres, 1992). Sorbitol is a non-permeating osmoticum and has been used to impose osmotic stress on leaf slices, protoplasts or chloroplasts (Kaiser et al., 1981a,b; Kaiser, 1982; Sen Gupta and Berkowitz, 1987; Sundari and Raghavendra, 1990).

The extent of photosynthetic inhibition in mesophyll protoplasts increased with duration of incubation in hyperosmotic medium at both the temperatures, 25 °C and 0 °C. The reduction in photosynthesis after exposure to 1.0 M sorbitol for 10 min was 25% at 25 °C and 45% at 0 °C (Fig. 4.2B). After 40 min of exposure to 1.0 M sorbitol, the inhibition of photosynthetic

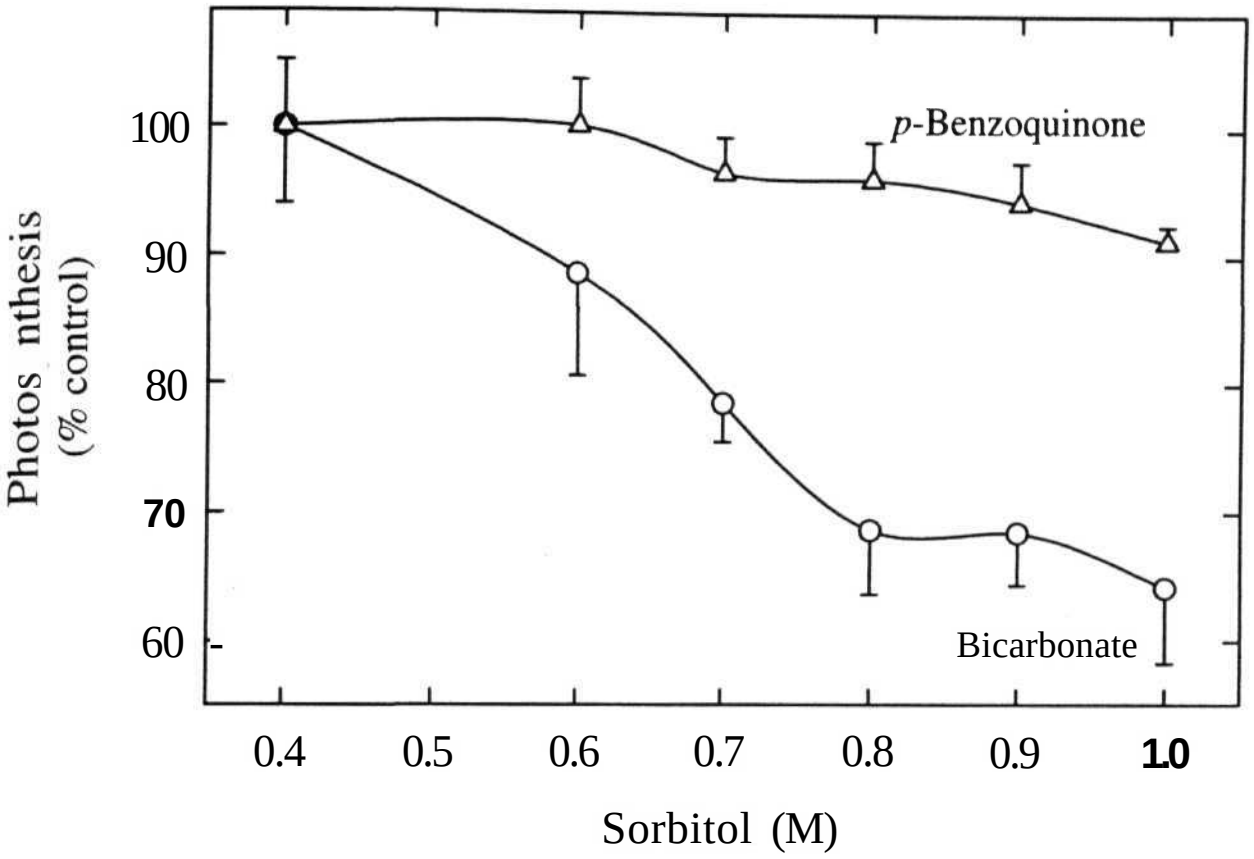

Figure 4.1. Effect of osmotic stress on photosynthetic carbon fixation (bicarbonate-dependent oxygen evolution) or PSIl mediated *p*-benzoquinone dependent O_2 evolution. The stress was imposed by increasing the concentration of sorbitol in the assay medium from 0.4 to 1.0 M as indicated. The rates of *p*-benzoquinone and bicarbonate-dependent oxygen evolution in 0.4 M sorbitol were 290 ± 2 and 150 ± 11 μmol mg^{-1} Chl h^{-1}, respectively, and were taken as the controls (100%).

activity increased further, to about 45% and 55% at 25 °C and 0 °C, respectively. However, the inhibition of photosynthesis by 1.0 M sorbitol (-3.1 MPa) in mesophyll protoplasts was not as severe as that caused by similar water potentials in an intact plant. Similar observations were made by Kaiser et al. (1981a), who suggested that one of the reasons for such difference might be that protoplast photosynthesis was not limited by stomatal or diffusional resistances.

Our results endorse the observations, made using leaf slices, cells and chloroplasts (Sharkey and Badger, 1982; Berkowitz and Gibbs, 1984; Kaiser, 1984), that the biochemical reactions of photosynthesis are affected directly by water stress and are therefore non-stomatal mediated (Sharkey and Badger, 1982; Berkowitz and Gibbs, 1984; Kaiser, 1984). However, photochemical activity such as *p*-benzoquinone-dependent O_2 evolution was not very sensitive to osmotic stress (Fig. 4.1), as observed by earlier workers (Sharkey and Badger, 1982; Berkowitz and Gibbs, 1984; Kaiser, 1984; Martin and Ruiz-Torres, 1992). The inhibition of photosynthesis due to osmotic stress was not only immediate but also persistent, indicating that the osmotic inhibition was not primarily due to a transitory loss of permeability as observed in chloroplasts (Kaiser, 1984).

The slight reduction in photosynthesis in 0.4 M sorbitol at 25 °C in Fig. 4.2A was due to the inherent instability of protoplasts at warm temperatures. Most of the experiments were therefore limited to 10 min. Only a few studies were extended to 20 min or more.

An immediate effect of osmotic stress in protoplasts is the prolongation of the period of photosynthetic induction. Fig. 4.3 demonstrates that the duration of the induction or lag phase of photosynthesis was extended soon after the exposure of protoplasts to 1.0 M sorbitol. The prolongation of the photosynthetic induction period in protoplasts depended on the concentration of sorbitol (0.4 M to 1.0 M) in the assay medium (Fig. 4.4). Such marked

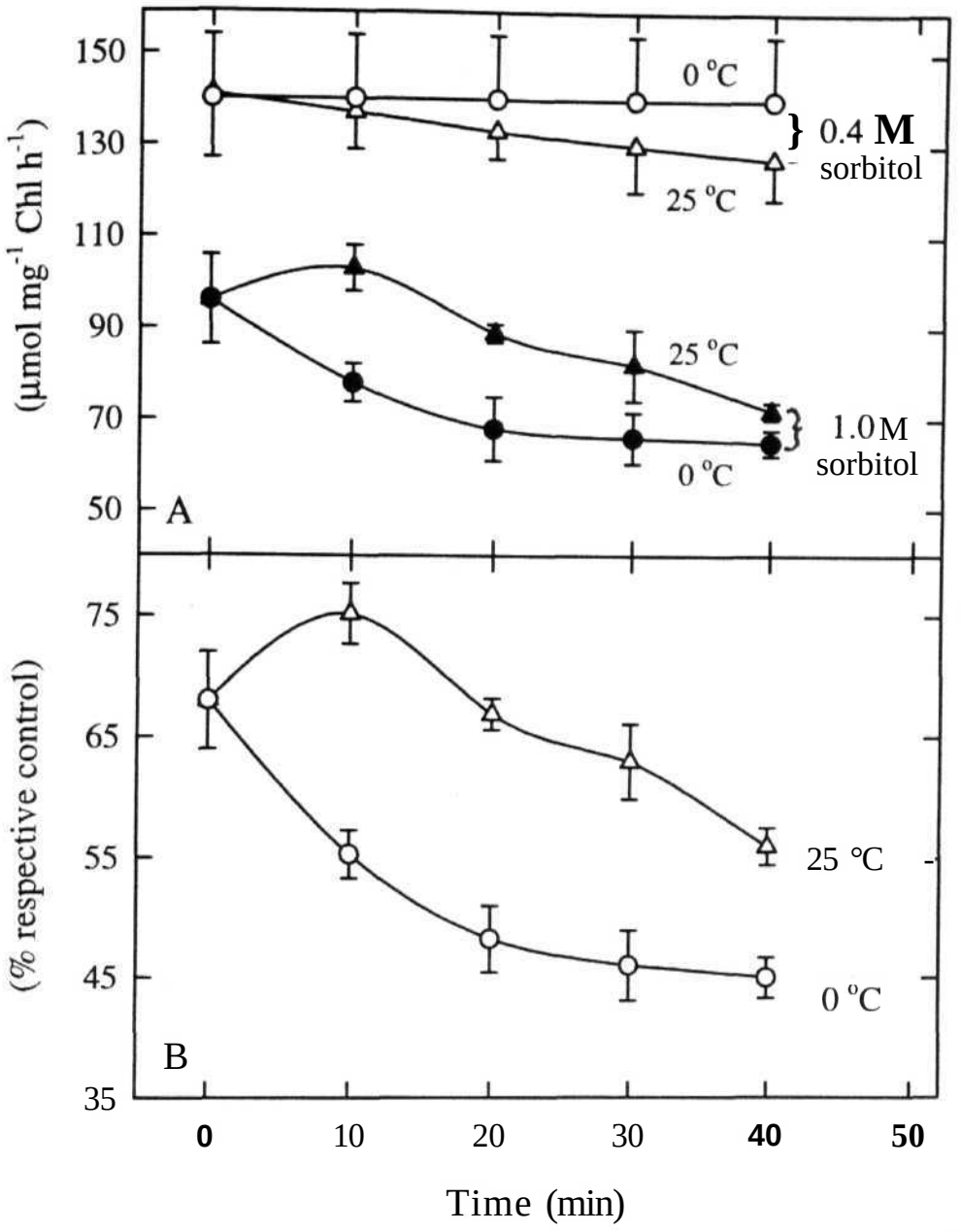

Figure 4.2. Progressive inhibition of photosynthesis with time in pea mesophyll protoplasts on exposure to osmotic stress at either 25 °C or 0 °C. Protoplasts were pre-incubated in 0.4 or 1.0 M sorbitol for varying periods as indicated. A: Rates of photosynthetic oxygen evolution at 25 °C of control and osmotic pre-treated protoplasts at 25 °C or 0 °C. B: Photosynthesis at 1.0 M sorbitol as % of the respective control (0.4 M).

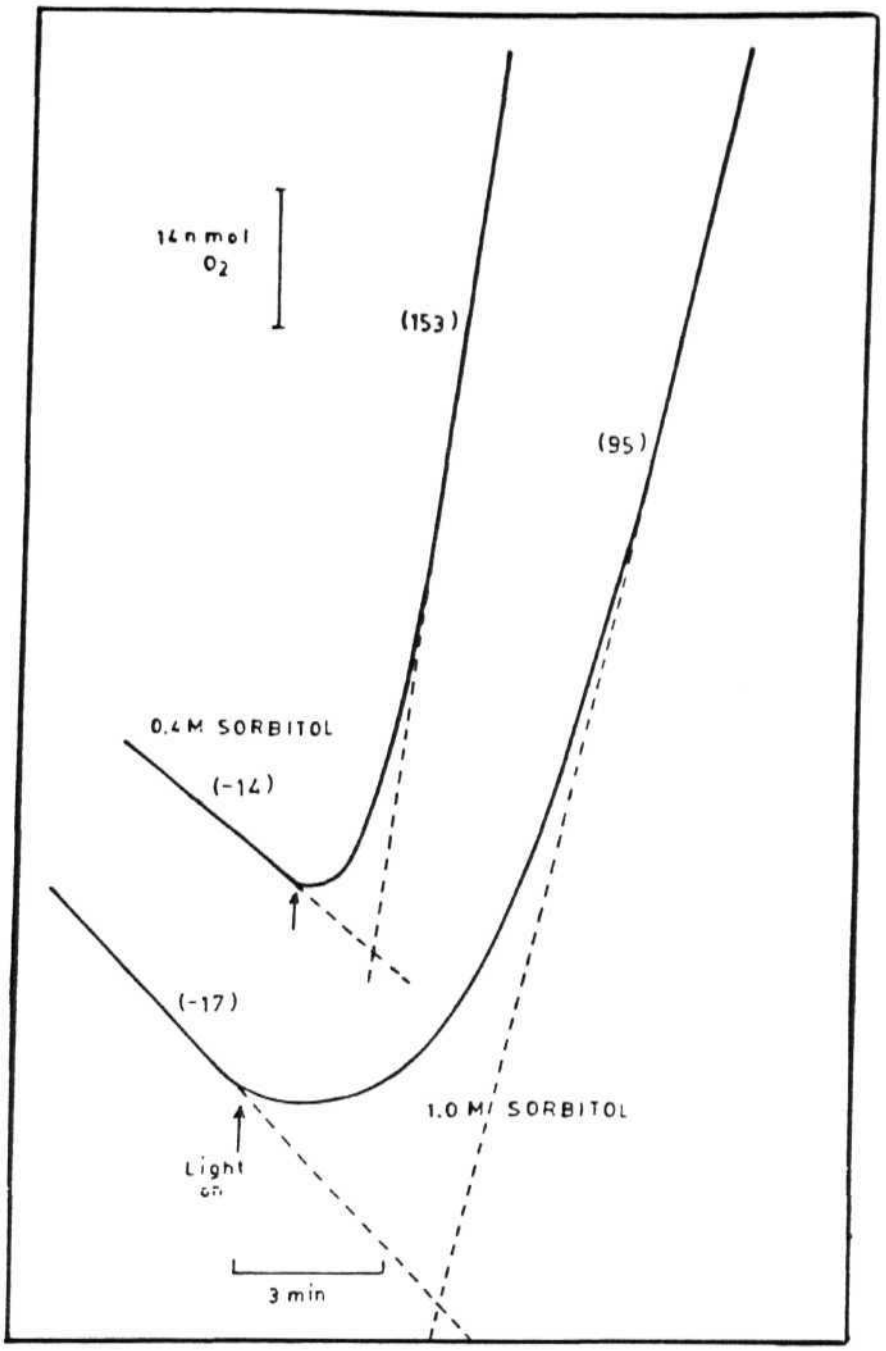

Figure 4.3. Recorder traces of O_2 evolution by protoplasts indicating the prolongation of photosynthetic induction on exposure to osmotic stress. The induction period was about 1.5 min when protoplasts were in 0.4 M sorbitol but extended to >4 min in 1.0 M sorbitol. The figures on the curves represent the rates of photosynthesis or respiration (-values) in μmol O_2 evolution/uptake mg^{-1} Chl h^{-1}. Photosynthetic rate was reduced by 35% in 1.0 M sorbitol.

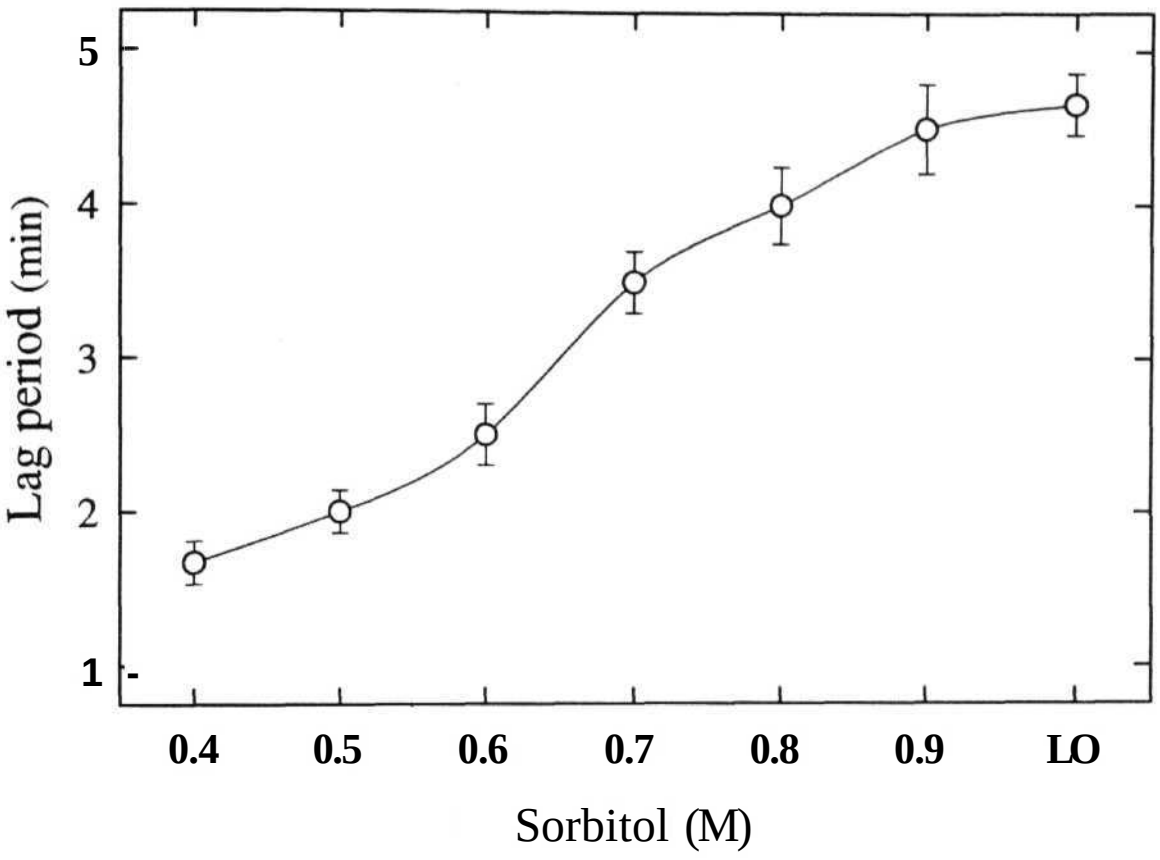

Figure 4.4. The period of photosynthetic induction in protoplasts as a function of sorbitol concentration in the medium. The pattern of lag-period is illustrated in Figure 4.3.

increase in the duration of the photosynthetic induction phase is similar to that found during observations on chloroplasts, and is possibly due to a disturbance in regeneration of Calvin cycle intermediates (Berkowitz and Gibbs, 1984; Boag and Portis, 1985; Leegood et al., 1985). The light activation of FBPase and SBPase is affected as a result of the decrease in the extent of stromal alkalization in osmotically stressed chloroplasts (Sharkey and Badger, 1982; Berkowitz and Gibbs, 1984; Leegood et al., 1985). A leak of Calvin cycle metabolites may occur from such stressed protoplasts as a result of a transient loss of permeability (Kaiser et al., 1981b). However, such leakage may not be crucial, since the recovery of photosynthesis on transfer to isotonic sorbitol is remarkable and nearly total.

The literature on the effect of osmotic or water stress on respiration in plant tissue is contradictory, with reports indicating either stimulation or inhibition or no change (Hisao, 1973; Hanson and Hitz, 1982). However, the rate of respiration was at the lowest level, when soil moisture content and air temperature were low (Amthor, 1989). In our experiments, the change in respiration as a result of osmotic stress was dependent on temperature. The respiratory rate, stimulated at 25 ^{o}C but was decreased on exposure to solutions of increasing osmolarity at 0 ^{o}C (Fig. 4.5). When protoplasts were exposed to 1.0 M sorbitol at 25 °C for 20 min, their respiratory rate was increased by 35%. After a pre-incubation of 20 min in 1.0 M sorbitol at 0 °C, about 25 % of respiratory activity was lost (Fig. 4.6). Our observations demonstrate that osmotic stress affects the function of mitochondria as well as chloroplasts.

Surprisingly, protoplasts did not rupture on exposure to even high concentrations of sorbitol. Protoplasts shrank (decreased in size) but remained intact, as indicated by routine microscope observations (as shown in Plate 6.1, Chapter 6). Kaiser et al. (1981b) also observed that protoplasts were quite good at retaining their integrity under osmotic stress. The possibility of physical shock being an important factor in protoplasts subjected to osmotic

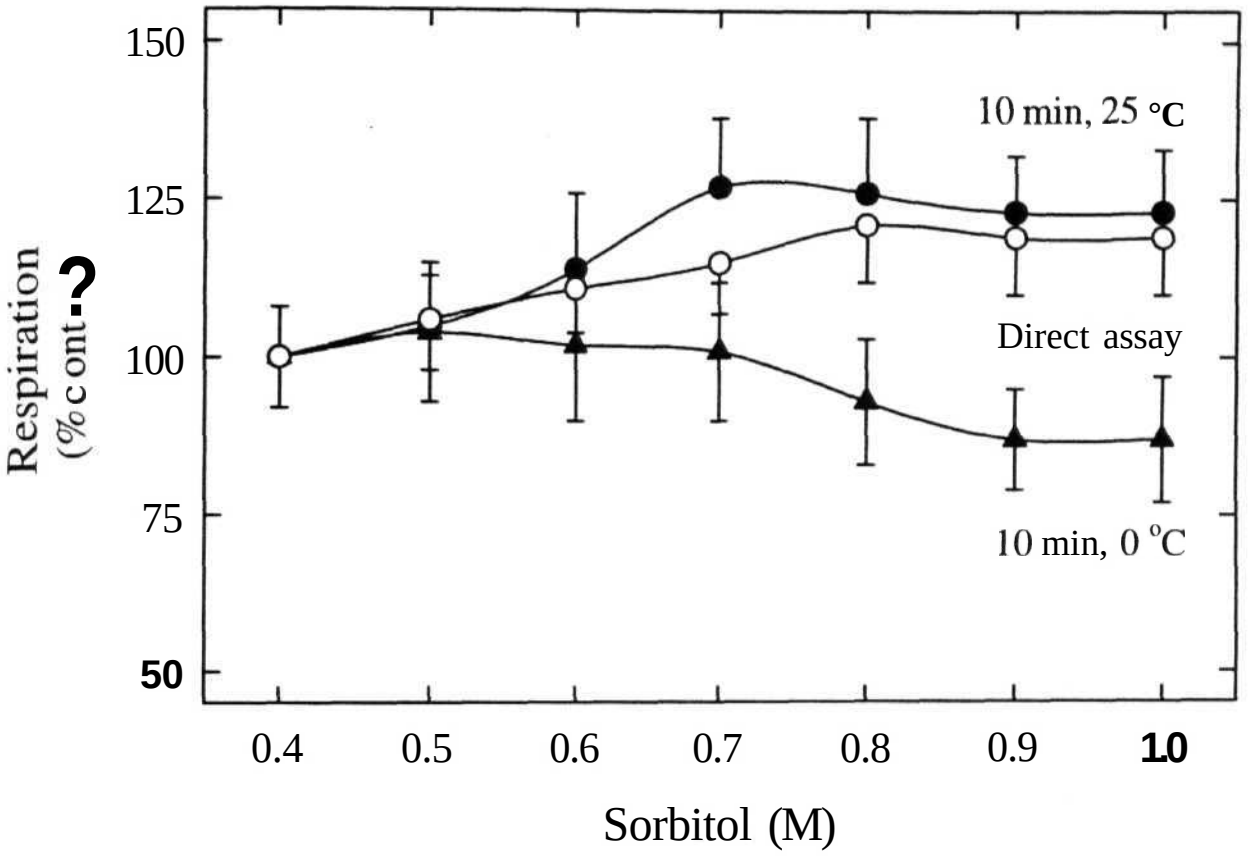

Figure 4.5. Effect of osmotic stress on respiratory O_2 uptake in pea mesophyll protoplasts. Varied levels of sorbitol were present in either assay medium or during pre-incubation of protoplasts for 10 min at 25 °C and 0 °C. Osmotic inhibition of respiration was evident, particularly at 0 °C. There was slight stimulation of respiration when protoplasts were exposed to hyper-osmotic media at 25 °C. The respiratory rate in 0.4 M sorbitol at 25 °C was 16 ± 1.7 μmol of O_2 uptake mg^{-1} Chl h^{-1} (taken as control, 100%).

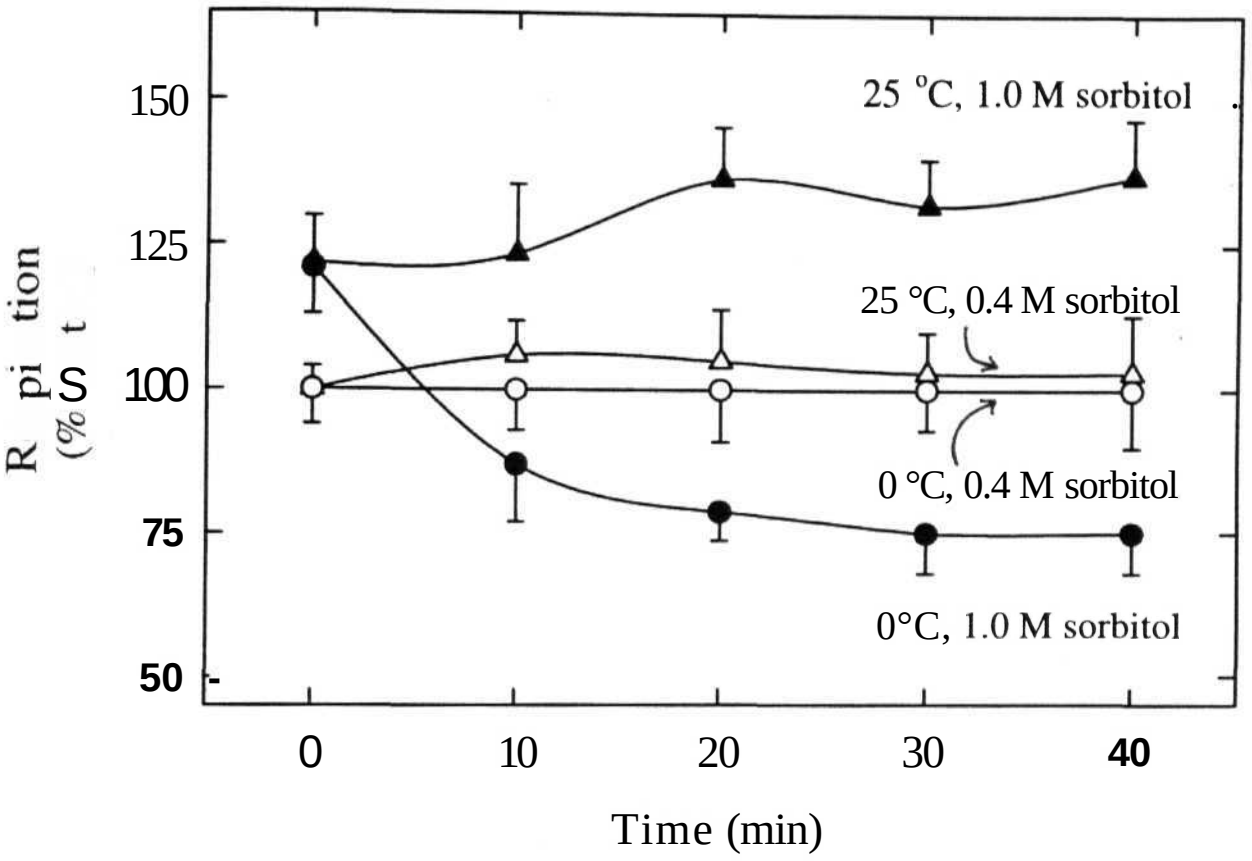

Figure 4.6. Respiration of mesophyll protoplasts during exposure to varied periods of osmotic stress at either 25 °C or 0 °C. Protoplasts were incubated at 25 °C or 0 °C in 0.4 M or 1.0 M sorbitol for varying periods of time as indicated and assayed in the dark at 25 °C. The respiratory rate in 0.4 M sorbitol was 18 ± 0.9 μmol O_2 uptake mg^{-1} Chl h^{-1}.

stress is therefore quite remote. Although it is difficult to envisage changes within a protoplast as a result of osmotic stress, changes in any component (for e.g. the tonoplast membrane) could alter the microenvironment within the cell.

Since the inhibition of photosynthesis or respiration was marked at 0 °C, the reversibility of the effect of osmotic stress at 0 °C was also examined. The inhibition of photosynthesis in protoplasts due to osmotic stress was reversible, as indicated by the marked recovery in the photosynthetic activity when the osmolarity of the medium was decreased from 1.0 M to 0.4 M (Fig. 4.7). The slight loss of PSII mediated activity, as measured by *p*-benzoquinone reduction was completely restored on re-exposure of stressed protoplasts to 0.4 M sorbitol (data not shown).

A small proportion of protoplasts were found broken on re-exposure to 0.4 M sorbitol (data not shown). This may be the reason why the rates of photosynthesis or respiration in stressed protoplasts did not completely recover to equal the control values (without osmotic stress). Nevertheless, the reversibility suggests that the effects of osmotic stress are primarily a result of either the concentration of solutes within protoplasts (Kaiser, 1984) or conformational changes in the intracellular proteins/ membranes (Gantet et al., 1990) or both.

The inhibition of respiration due to osmotic stress was also reversible when the protoplasts were transferred from 1.0 M to 0.4 M sorbitol (Fig. 4.8). However, the inhibition of respiration was less and the recovery faster than those of photosynthesis.

The protoplasts on exposure to 1.0 M sorbitol for 20 min at 25 °C lost <35% of their photosynthetic activity, but the loss of photosynthetic activity was over 50% at 0 °C. The amplifying effect of chilling on the osmotic inhibition of photosynthesis in protoplasts was completely reversible. Transfer of protoplasts from 0 °C to 25 °C after 10 min resulted in a <25% reduction in photosynthetic capacity (Table 4.1). The antagonistic effects of warm

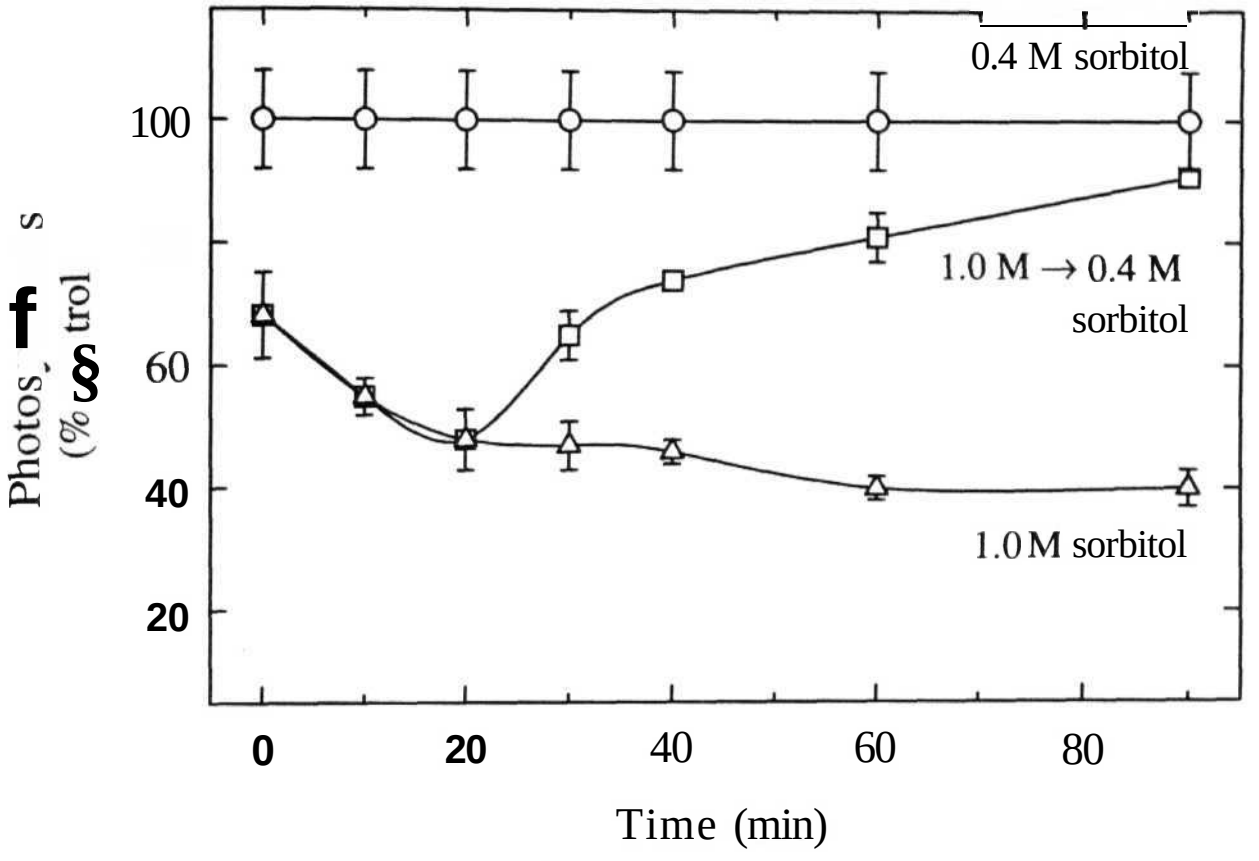

Figure 4.7. The reversal of the osmotic inhibition of photosynthesis on reexposure of mesophyll protoplasts to 0.4 M sorbitol at 0 °C. Protoplasts were exposed to 1.0 M sorbitol and then were transferred back to 0.4 M after 20 min, at 0 °C. An aliquot of a pre-stressed/stress-reversed protoplast preparation was examined for the rate of photosynthesis. Protoplasts were also diluted in 0.4 and 1.0 M sorbitol, incubated and assayed as respective controls to assess the problem of a possible change in activity as a result of dilution and incubation for different periods. The photosynthetic rate in 0.4 M sorbitol at zero time (at 25 °C) was 160 ± 15 μmol of O_2 evolved mg^{-1} Chl h^{-1} (taken as control, 100%).

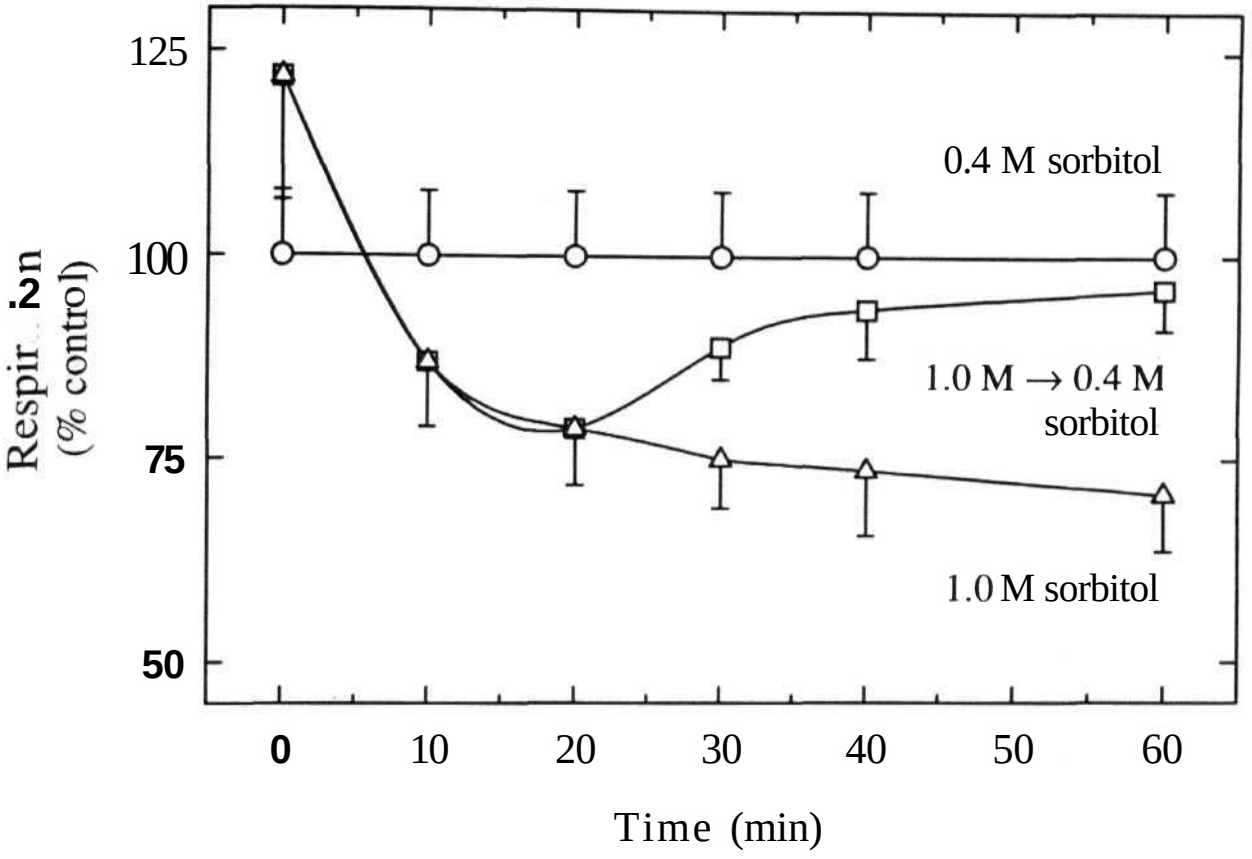

Figure 4.8. The recovery of respiration after osmotic stress inhibition in pea mesophyll protoplasts on re-exposure to 0.4 M sorbitol (at 0 °C). An aliquot of stressed/stress-reversed protoplast preparation was examined for the rate of respiration, at 25 °C. The respiratory rate in 0.4 M sorbitol at zero time was 17 ±2 μmol of O_2 uptake mg^{-1} Chl h^{-1}.

Table 4.1. *The reversible effect of temperature during preincubation on the inhibition of photosynthesis by osmotic stress in protoplasts.*

The inhibition was aggravated on exposure of the protoplasts to chilling at 0 °C, but the activity recovered on re-exposure to 25 °C.

Temperature Treatment	Photosynthesis (Sorbitol)		Inhibition due to 1.0 M Sorbitol
	0.4 M (control)	1.0 M	
	μmol O_2 evolved mg^{-1} Chl h^{-1}		*% inhibition*
Direct assay	114 ± 14	96 ±7	32
Preincubation before assay			
25 °C - 10 min	137 ± 8	103 ±6	25
25 °C - 20 min	133 ± 6	89 ±2	33
0 °C - 10 min	141 ± 14	78 ±4	45
0 °C - 20 min	141 ± 14	68 ±7	52
0 °C - 10 min/25 °C - 10 min	135± 8	105 ± 9	23

temperature on photoinhibition and of water stress on PSII of leaves of several plants have been reported (Havaux, 1992). Our results suggest that low-temperature-induced reversible changes in protoplasts are partly responsible for the inhibition of their photosynthesis. Fluctuations in external temperature cause dramatic, but reversible, changes in lipid structure and membrane fluidity (Shinitzky, 1984; Kasamo et al., 1992).

A consistent feature of mesophyll protoplasts was that the inhibition of photosynthesis as a result of osmotic stress was always much more marked than that of respiration. After a 10 min pre-incubation at 0 °C under osmotic stress (i.e. in 1.0 M sorbitol), there was a reduction of only about 13% in respiratory activity, while there was a 45% loss of photosynthetic activity. Similarly, when protoplasts were relieved of osmotic stress, the recovery of respiration (Fig.

4.8) was faster than that of photosynthesis (Fig. 4.7). Schwab et al. (1989) reported that respiration was less sensitive to dehydration than phtosynthesis and recovered faster during rehydration in resurrection plants like *Craterostigma plantagineum*.

Our results demonstrate that protoplasts can be an additional tool to study photosynthetic responses to osmotic stress. Conventionally, studies on photosynthesis under water stress are carried out using either leaves, chloroplasts or cells (Sharkey and Badger, 1982; Berkowitz and Gibbs, 1984; Kaiser, 1984, 1987). Although there have been a few reports on volume changes in protoplasts under water stress (Dowgert et al., 1987; Sen Gupta et al., 1989), photosynthetic measurements using protoplasts are limited (cf. Kaiser, 1984, 1987).

The study of any stress with isolated protoplasts has several advantages over the use of intact leaves. Data from leaves, often may vary due to the unavoidable variation in the sensitivity of individual leaves, whereas protoplast preparations provide an almost a simulated set-up of mesophyll cell population of a given plant. By using protoplast suspensions supplied with saturating bicarbonate, the diffusional barriers for CO_2 can be eliminated (Rumich-Bayer and Krause, 1986).

One of the disadvantages of protoplasts is their limited instability at room temperature over long periods of time. We have limited most of our experiments at 25 °C to 10 or 20 min. Protoplasts are turgorless and may be slightly dehydrated compared to intact plants. However, their high rates of photosynthesis/respiration (Fig. 4.3) and the reversibility of stress responses (Fig. 4.7 and 4.8) illustrate clearly that the protoplasts retain their metabolic integrity to a great extent. In spite of their fragility and limited stability, protoplasts are useful for experiments involving rapid fractionation of organelles, metabolite analysis and membrane fluidity during osmotic stress.

Chapter 5

Photoinhibition of Photosynthesis in Mesophyll Protoplasts: Aggravation by Osmotic Stress and Protection by Dark Respiration

Chapter 5

Photoinhibition of Photosynthesis in Mesophyll Protoplasts: Aggravation by Osmotic Stress and Protection by Dark Respiration

Photoinhibition is the phenomenon of severe reduction in photosynthetic performance under supra-optimal light intensities, particularly in the absence of CO_2 and O_2 (Powles, 1984; Kyle and Ohad, 1986; Krause, 1988). The phenomenon of photoinhibition has so far been demonstrated in leaves, algal cells, chloroplasts and thylakoid membranes (Kyle and Ohad, 1986; Kyle, 1987; Lidholm et al., 1987; Krause, 1988; Wild et al., 1989). The present report characterises and describes the phenomenon of photoinhibition in another system, namely mesophyll protoplasts of pea. The optimal light intensity required for maximal rates of photosynthetic oxygen evolution at 25 °C (ranged from 140-180 μmol mg^{-1} Chl h^{-1}) by mesophyll protoplasts of pea was 1250 μE m^{-2} s^{-1}. Exposure of isolated mesophyll protoplasts from pea leaves to supra-optimal light intensities resulted in photoinhibition of photosynthesis.

The pattern of photosynthesis after exposure to photoinhibitory light was compared to those kept either in darkness or optimal light intensity of 1250 μE m^{-2} s^{-1}. There was a slight decrease in the photosynthetic rate of protoplasts when kept at 25 °C, in darkness or normal light. On the other hand, when protoplasts were exposed to supra-optimal light of 3000 μE m^{-2} s^{-1}, their photosynthetic rates declined rapidly with time (Fig. 5.1). Protoplasts lost nearly 35% of their photosynthetic activity after 10 min and approximately 60% of the activity by 20 min, with respect to dark-incubated samples, demonstrating that photoinhibition of photosynthesis occurred when protoplasts were exposed to strong light.

The present article, therefore, establishes that the protoplasts can be employed to study the process of photoinhibition. There was only a brief report

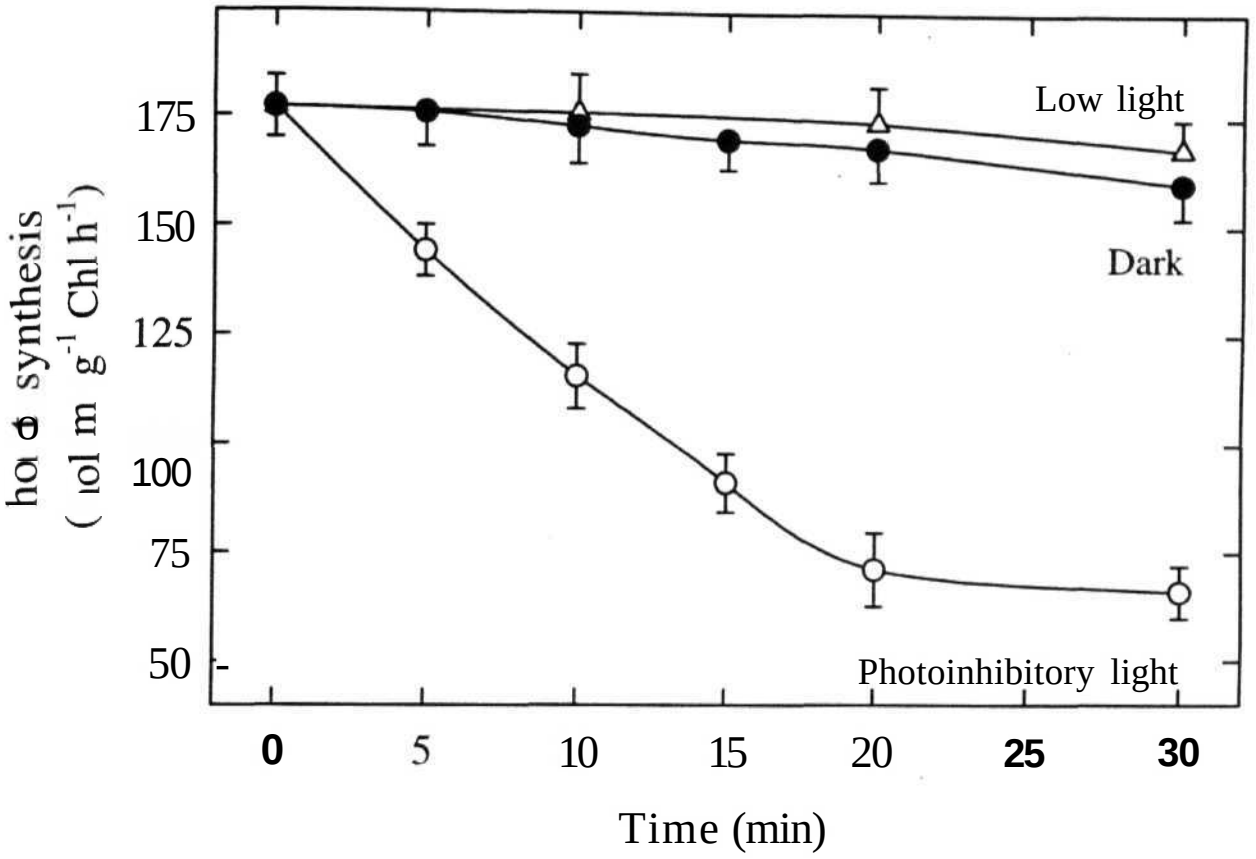

Figure 5.1. The rates of photosynthesis by mesophyll protoplasts at different times of incubation at 25 °C in either darkness or low light (100 μE m^{-2} s^{-1}) or photoinhibitory light (3000 μE m^{-2} s^{-1}). The loss of photosynthetic activity was negligible when protoplasts were in darkness or low light. But photosynthetic rate declined steeply on exposure to supra-optimal or photoinhibitory light. The Chl concentration during preincubation was 200 μg ml^{-1}.

on photoinhibition in protoplasts describing changes in fluorescence induction curves (Baker and Horton, 1987).

Each experimental system, however, has its own advantages and disadvantages. For example, leaves have a non-uniform light profile between adaxial and abaxial surfaces and exhibit variation in partial pressures of CO_2/O_2 levels within intercellular spaces (Kyle, 1987). The interaction between different organelles of a cell can not be assessed while using chloroplasts. The system of protoplasts offers an additional tool to study the phenomenon of photoinhibition. An advantage is the possibility of assessing the interaction between organelles and testing the effect of exogenously added metabolites/ inhibitors. The major disadvantage, however, is the limited stability of protoplasts, particularly at room temperature. Most of the experiments in the present report were limited to 10 min. Within this period, the loss in photosynthetic capacity of pea mesophyll protoplasts was negligible (Fig. 5.1).

Among the components of photosynthesis, PSII reactions are the most sensitive to photoinhibition (Powles, 1984; Kyle, 1987). Protoplasts lost 30% of their PSII activity within 5 min and the inhibition increased to 55% after 30 min of exposure to photoinhibitory light, compared to their dark-incubated samples (Fig. 5.2A). The marked sensitivity of PSII dependent reactions to photoinhibition is in a marked contrast to the effect of osmotic stress alone. In fact, PSII photochemical activities were reported to be much less sensitive to osmotic stress than that of carbon fixation in protoplasts, under iso-osmotic conditions (Saradadevi and Raghavendra, 1994).

Both PSII activity and the carbon fixation capacity of protoplasts were more sensitive to photoinhibitory light when they were in 1.0 M sorbitol, than their response when the protoplasts were in 0.4 M sorbitol.

Protoplasts lost >50% of their PSII activity (as indicated by their benzoquinone dependent O_2 evolution) after 5 min and 85% after 30 min of exposure to photoinhibitory light in 1.0 M sorbitol containing medium

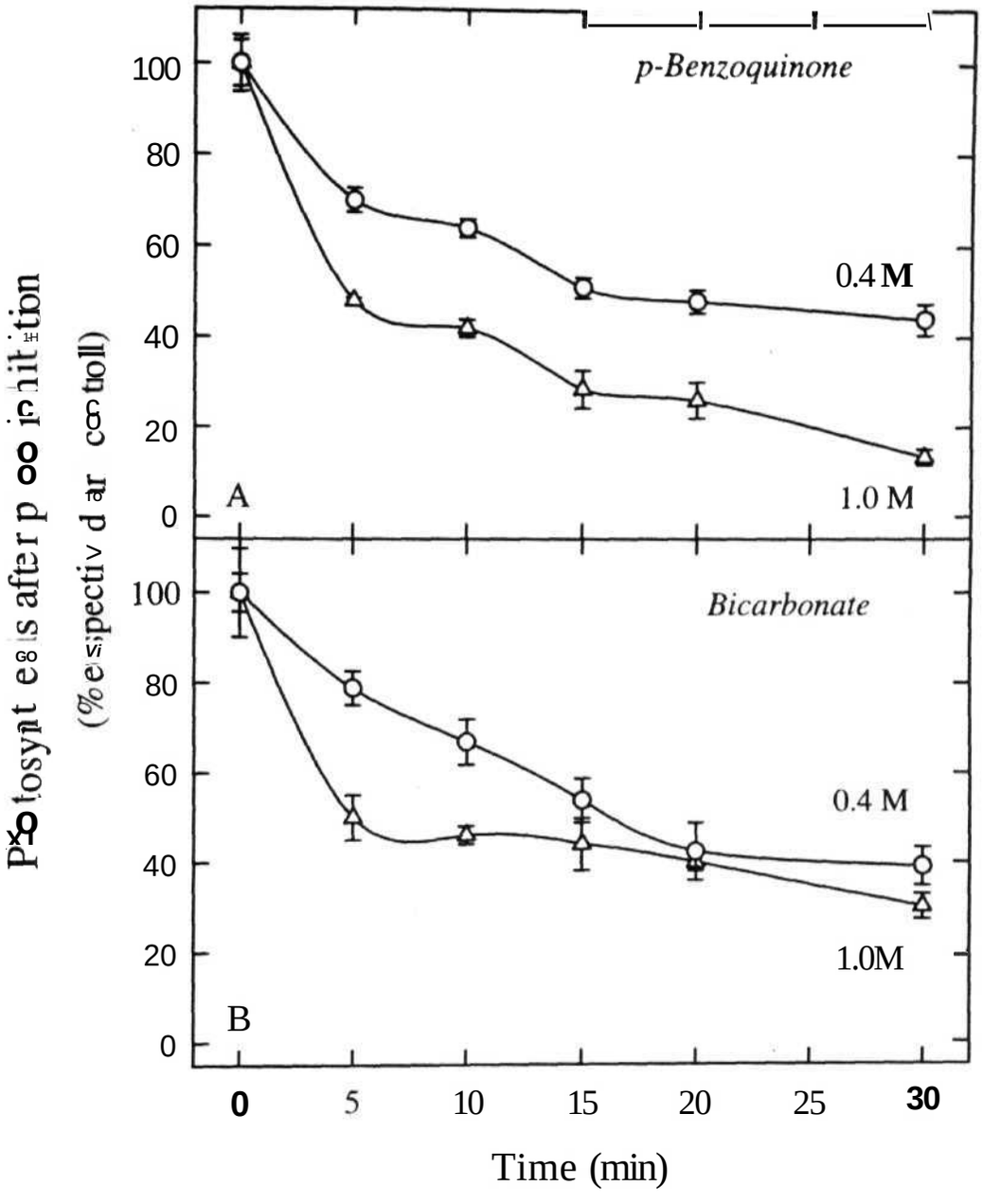

Figure 5.2. Effect of photoinhibitory light on photosynthesis by protoplasts in 0.4 M or 1.0 M sorbitol. Protoplasts were exposed to photoinhibitory light (3000 $\mu E\, m^{-2}\, s^{-1}$) for different periods of time, in either 0.4 M or 1.0 M sorbitol, at 25 °C. An aliquot of protoplasts was examined for A: PSII activity (*p*-benzoquinone dependent O_2 evolution), or B: photosynthesis (bicarbonate dependent O_2 evolution), The activity of protoplasts after photoinhibition is expressed in relation to the corresponding samples kept in darkness.

Fig. 5.2A). The inhibition of bicarbonate dependent O_2 evolution of protoplasts due to photoinhibitory light also increased under osmotic stress conditions. The loss in photosynthetic activity of protoplasts increased from 35% (in 0.4 M sorbitol) to 50% when the protoplasts were subjected to photoinhibitory light in 1.0 M sorbitol (Fig. 5.2B). The loss soared to nearly 70% if protoplasts were preincubated at 0 °C for 10 min before exposure to photoinhibitory light (Table 5.1).

The extent of photoinhibition is amplified under adverse environmental conditions (Powles, 1984; Barber and Andersson, 1992; Long et al., 1994). Plants growing under natural conditions have been shown to suffer from photoinhibiton under stresses of drought (Björkman and Powles, 1984) or low temperature (Somersalo and Krause, 1988, 1989). The synergistic effect of photoinhibition and water stress in leaves is well documented (e.g. Masojídek et al., 1991). The present results demonstrate that the sensitivity of protoplast photosynthesis to photoinhibitory light was further aggravated on exposure to chilling or high irradiance or both (Table 5.1). We suggest that this property can be used for amplifying the photosynthetic responses in protoplasts to photoinhibition and vice versa (i.e. to amplify osmotic inhibition of photosynthesis by exposure to supra-optimal light).

The primary target of photoinhibition is believed to be the D1 protein, an important constituent of the photosynthetic electron transport chain (Kyle et al., 1984; Ohad et al., 1984; Powles, 1984). This highly dynamic protein is synthesised as well as degraded continuously. Photoinhibition becomes apparent when the damage caused by light exceeds the extent of repair (Ohad et al., 1984). Our experiments confirm that there are marked changes in not only the level of Dl protein in protoplast membranes but also within Dl protein, which exists in either phosphorylated or non-phosphorylated form.

We have used two types of antibodies, against Dl protein, in our experiments: SP1 and SP2 (SP1 cross reacts with only non-phosphorylated

Table 5.1. *Photoinhibition of photosynthesis in mesophyll protoplasts in relation to osmotic stress and chilling at 0 °C*

Preincubation/ Osmotic Treatment*	Photosynthesis		Photoinhibition of Photosynthesis
	Dark (control)	Photoinhibitory light**	
	μmol O_2 evolved mg^{-1} Chl h^{-1}		*% inhibition {respective dark treatment)*
No preincubation			
0.4 M sorbitol	137 ±8	89 ±7	35
1.0 M sorbitol	103 ±6	47 ±5	54
Preincubation for 10 min at 0 °C			
1.0 M sorbitol	105 ±9	35 ±9	67

•At 25 °C.

**At an intensity of 3000 μE m^{-2} s^{-1} for 10 min at 25 °C.

protein and SP2 cross reacts with both non-phosphorylated and phosphorylated forms). The level of non-phosphorylated Dl protein, probed with SP1 antibody, decreased on exposure of protoplasts to photoinhibitory light. The extent of decrease in non-phosphorylated protein was further increased, when protoplasts exposed to photoinhibitory light under osmotic stress conditions. The non-phosphorylated Dl protein (located with SP1) almost disappeared, after 30 min of exposure of protoplasts to photoinhibitory light in 1.0 M sorbitol medium (Plate 5.1B). On the other hand, the level of Dl protein, when probed with SP2 antibody, was visibly not affected with photoinhibitory treatment in either iso-osmotic (0.4 M sorbitol) or hyper-osmotic (1.0 M sorbitol) media (Plate 5.1C). These observations reveal that only non-phosphorylated D1 protein (but not the phosphorylated form) decreased during photoinhibiton.

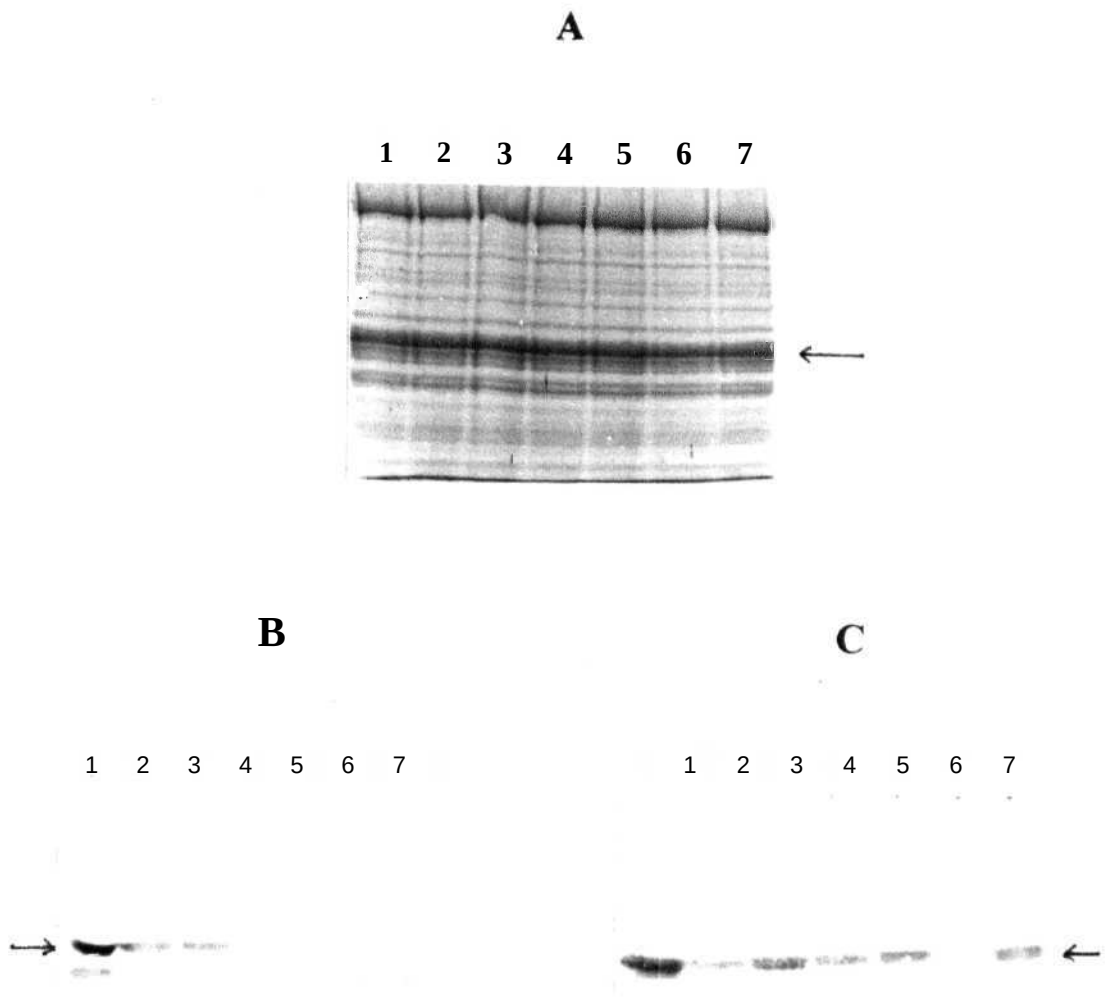

Plate 5.1. A: Protein profile of protoplast membranes indicating the position of D1 protein on the gel stained with coomassie brilliant blue R-250. B: Western blot developed after probing with SP1 antibody, which reacts with only non-phosphorylated form of Dl protein. C: Western blot probed with SP2 antibody, which detects both non-phosphorylated and phosphorylated forms of Dl protein. Lane 1 represents control protoplasts. Lanes 2, 3 and 4 represent protoplasts in iso-osmotic (0.4 M sorbitol) medium exposed to photoinhibitory light for 10, 20 and 30 min respectively. Lanes 5, 6 and 7 represent protoplasts in hyper-osmotic (1.0 M sorbitol) medium and subjected to photoinhibition for 10, 20 and 30 min respectively.

Recent reports (Rintamäki et al., 1995) suggest that phosphorylation of D1 protein in higher plants is involved in the regulation of the repair cycle of photoinhibited PSII centres. Tyystjärvi et al. (1992) have shown that *in vivo* photoinhibited PSII centres have their Dl protein in phosphorylated D1* state, which seem to result in marked restriction of degradation of Dl in the appressed membranes. *In vitro* (Aro et al., 1992; Kettunen et al., 1992) as well as *in vivo* (Syme et al., 1992) experiments done under photoinhibitory conditions indicated that the degradation of phosphorylated D1* is slower than that of non-phosphorylated Dl protein. Phosphorylated D1* is reported to be present only in grana stacks. Damaged Dl is protected from degradation through phosphorylation under conditions, where repair cycle can not cope up with the rate of degradation and phosphorylated D1 acts as a storage form of photodamaged Dl protein (Rintamaki et al., 1995). Further, in the moss *Ceratodon,* which does not phosphorylate the Dl protein, the degradation of the damaged Dl is very rapid under photoinhibitory conditions resulting in significant net loss of Dl protein from thylakoid membranes (Rintamaki et al., 1994).

Since phosphorylated Dl is a poor substrate for D1-specific proteinase, dephosphorylation of the damaged D1 protein probably precedes the proteolytic degradation (Aro et al., 1993). Dephosphorylation of thylakoid phospho-proteins is catalysed by membrane bound phosphatase(s) (Bennett, 1980). Elich et al. (1993) reported that dephosphorylation of PSII core proteins is controlled by excitation of PSI *in vivo.* Both appressed and non-appressed thylakoid domains participate in PSII repair cycle. Photodamage to PSII occurs in appressed region of thylakoids (Cleland et al., 1986), while synthesis of the Dl protein takes place in stromal membranes (Mattoo and Edelman, 1987).

On exposure of protoplasts to photoinhibitory light, not only their photosynthesis but also respiration was markedly inhibited. In fact, the rate of respiration was slightly stimulated when protoplasts were allowed to

photosynthesise under optimal light intensity of 1250 $\mu E\ m^{-2}\ s^{-1}$ (Fig. 5.3). Due to photoinhibitory light, the protoplasts lost about 18% and 40% of their respiratory activity after 10 and 20 min of their exposure respectively. Such loss in protoplast respiratory activity was almost doubled (32%), when they were exposed to photoinhibitory light for 10 min in the hyper-osmotic medium of 1.0 M sorbitol.

Apart from rapid responses in turnover of D1 protein (Wild et al., 1989), plant systems have additional defense mechanisms to minimize the damage of photoinhibition. Carbon dioxide and oxygen, the basic substrates for photo-synthesis and photorespiration respectively, protect plant cell against photoinhibition (Krause and Comic, 1987). The operation of PCR cycle facilitates a continuous supply of terminal electron acceptor of photochemical reactions (NADP) and permits a steady rate of photochemical deexcitation of reaction centers (Krause and Cornic, 1987). In the absence (or limitation) of CO_2, the protection against photoinhibtion is provided by photorespiratory carbon metabolism (Osmond, 1981; Heber et al., 1990). Some of the other mechanisms that alleviate the effects of photoinhibition include the violaxanthin cycle (e.g. Montanes et al., 1989).

Our interest was aroused to assess the role of dark respiration in protecting photosynthesis against photoinhibiton. Therefore we have examined the response of protoplasts to photoinhibitory light in presence of classic inhibitors known to suppress different components of respiration: antimycin A, sodium azide, Oligomycin (oxidative electron transport/phosphorylation), sodium fluoride and sodium malonate (glycolysis/tricarboxylic acid cycle).

Photosynthetic activities of protoplasts were determined after incubation in either darkness or photoinhibitory light for 10 min, in presence or absence of low concentrations of three classic respiratory inhibitors of oxidative electron transport/oxidative phosphorylation (Fig. 5.4). Photoinhibition was remarkably

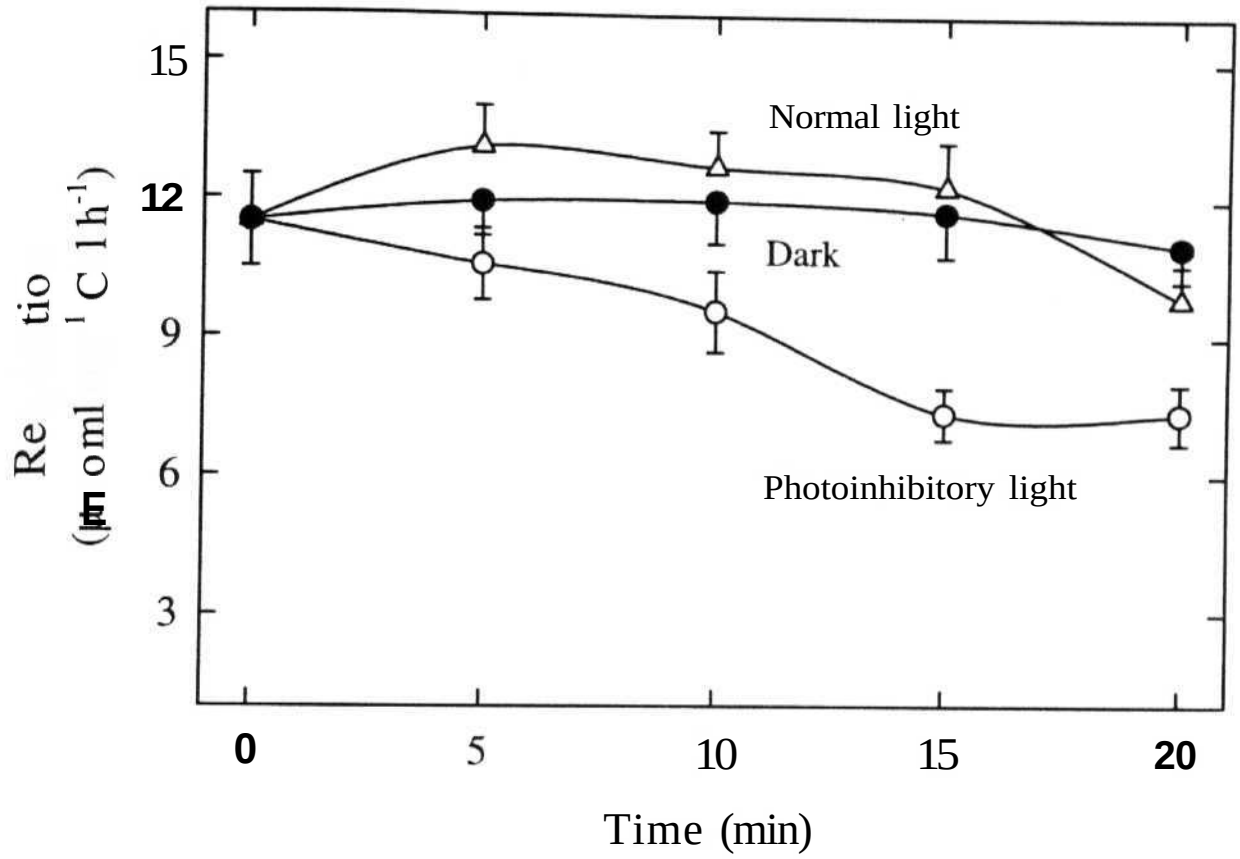

Figure 5.3. Respiratory oxygen uptake in mesophyll protoplasts on exposure to photoinhibitory light, compared to the samples kept in darkness or normal light. Further details were as in Fig. 5.1.

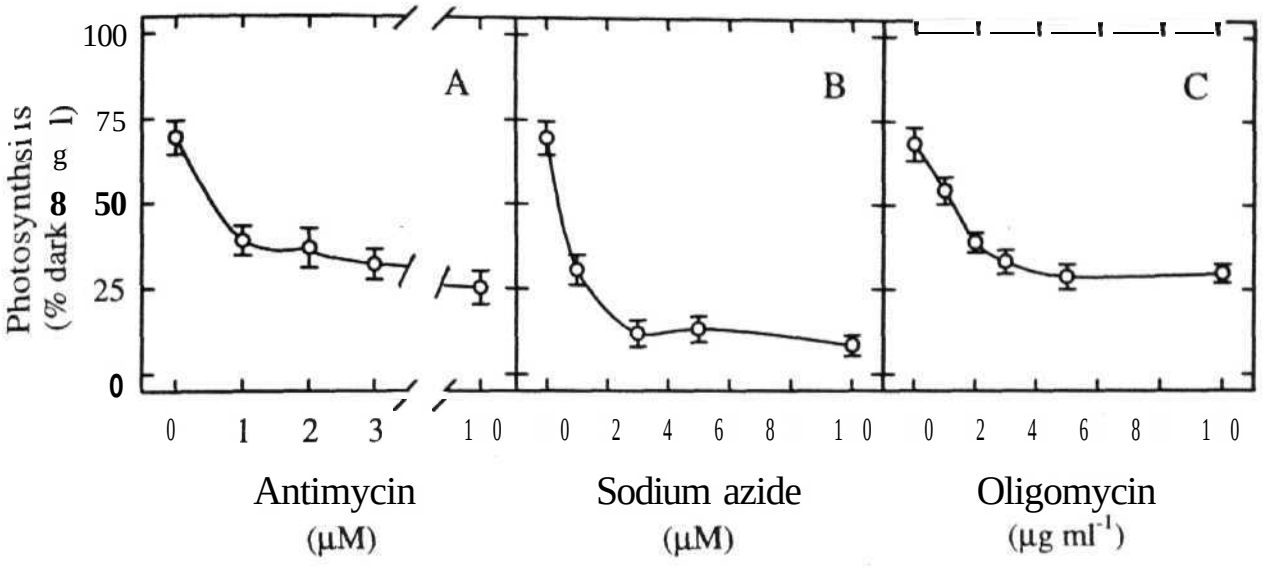

Figure 5.4. Promotion of photoinhibition of photosynthesis in mesophyll protoplasts by respiratory inhibitors. Protoplasts were either kept in darkness or exposed to photoinhibitory light for 10 min at 25 °C. The test compounds were included in the medium during pre-incubation. The figures represent the activity after photoinhibition compared to the corresponding samples kept in darkness. The photosynthetic activity after photoinhibition in the absence of inhibitors (indicated at zero concentration in A, B and C) ranged from 65 to 74% of that in dark-incubated sample.

enhanced by the presence of even 1 μM antimycin A (Fig. 5.4A), 1 μM sodium azide (Fig. 5.4B) and 1 μg ml^{-1} of Oligomycin (Fig. 5.4C).

The possibility that these metabolic inhibitors could exert a direct inhibitory effect on photosynthesis, was checked by including the inhibitor in the assay medium while measuring photosynthesis. However, antimycin A, sodium azide or Oligomycin did not affect photosynthetic O_2 evolution by protoplasts at these low concentrations (data not shown, but see Table 5.2 for result of a similar experiment).

Table 5.2. *A comparison of the effect of metabolic inhibitors on photosynthesis and photoinhibition in protoplasts*

These compounds affect different components of respiration: sodium fluoride (inhibits glycolysis), sodium malonate (tricarboxylic acid cycle) antimycin A, sodium azide (oxidative electron transport), and Oligomycin (oxidative phosphorylation). The protoplasts were examined for their photosynthetic activity after a preincubation (with or without inhibitors) for 10 min at 25 °C in either darkness or photoinhibitory light.

Respiratory Inhibitor	Photosynthetic Rate after Preincubation		Direct Effect on Photosynthesis	Photoinhibition of Photosynthesis
	Dark	Photoinhibitory light*		
	μmol O_2 evolved mg^{-1} Chl h^{-1}		*% inhibition*	*% inhibition (respective dark treatment)*
None (control)	122 ±8	78 ±5	0	36
1 μM antimycin A	100±4	38 ± 5	18	62
1 uM sodium azide	113 ±6	38±4	7	66
1 μg ml^{-1} Oligomycin	105 ±4	48 ±5	20	54
10 mM sodium fluoride	117±5	70±4	4	40
10 mM sodium malonate	120 ±5	69 ±3	2	42

*At an intensity of 3000 μE m^{-2} s^{-1} for 10 min at 25 °C.

Antimycin A, sodium azide and Oligomycin inhibit oxidative electron transport and phosphorylation. The effect of two more inhibitors, sodium fluoride and sodium malonate, which interfere with glycolysis and the tricarboxylic acid cycle, respectively, were also examined. However, sodium fluoride and sodium malonate did not have much effect on photoinhibition (Table 5.2). On the other hand, antimycin A, sodium azide or Oligomycin markedly promoted photoinhibition of photosynthesis. At all these concentrations and experimental incubatory conditions, the inhibition by test compounds of the respiratory activity was quite marked and ranged from 29 to 46% (Table 5.3).

These respiratory inhibitors decreased markedly the level of non-phosphorylated D1 protein, as revealed the western blots with SP1 (Plate 5.2A) while having negligible effect on phosphorylated form, as reflected in the

Table **5.3.** *Effect of inhibition of dark respiration by test compounds under the present experiment conditions*

The protoplasts were preincubated (with or without inhibitor for 10 min at 25 ^{o}C in darkness and were examined for their rate of respiratory oxygen uptake.

Respiratory Inhibitor	Respiratory Activity	
	Rate	Inhibition
	μmol O_2 uptake mg^{-1} CM h^{-1}	*% control*
None (control)	8.4 ±0.3	0
1 μM antimycin A	6.0 ±0.5	30
1 μM sodium azide	4.5 ±1.3	56
1μg ml^{-1} Oligomycin	5.1 ±1.0	39
10 mM sodium fluoride	5.9 ± 1.1	30
10 mM sodium malonate	5.0 ±1.3	40

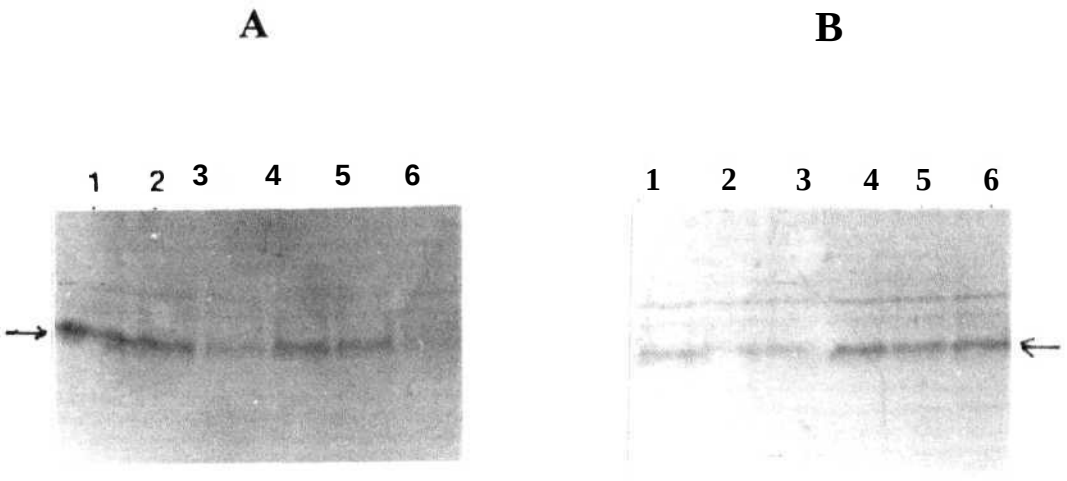

Plate 5.2. A: Western blot developed after probing with SP1 antibody, which reacts with only non-phosphorylated form of D1 protein. B: Western blot probed with SP2 antibody, which detects both non-phosphorylated and phosphorylated forms of Dl protein. Lane 1 represents the extracts from control protoplasts and lane 2 is of protoplasts exposed to photoinhibitory light for 10 min at 25 °C without any inhibitor. Lanes 3 to 6 represent protoplasts exposed to photoinhibitory light in presence of 10 mM streptomycin, 1 μg ml^{-1} Oligomycin, 1 μM sodium azide or 1 μM antimycin A respectively.

western blot with SP2 (Plate 5.2B). Only streptomycin, an inhibitor of Chloroplast protein synthesis decreased the level of both forms of D1. Schnettger et al. (1994) reported recently that the presence of streptomycin suppressed the levels of Dl protein and promoted photoinhibition in protoplasts of *Valerianella locusta.*

Our results revealed that dark respiration forms an additional defense mechanism to protect the leaf cells against photoinhibition. This suggestion is based on three observations: restriction of respiration by test compounds (Table 5.3), decrease in respiratory rates due to photoinhibitory light (Fig. 5.2) and marked promotion of photoinhibition, even at very low concentrations of classic inhibitors of mitochondrial metabolism (Figs. 5.4A, B and C; Table 5.2).

The effect of O_2 on photoinhibtion is intriguing. In an excess of light, O_2 forms $O_2^{\cdot-}$, which are harmful to the photosynthetic apparatus (Egneus et al., 1975). Leaf cells are vulnerable to photooxidative damage because they are exposed to bright light while they produce dioxygen. The leaves are equipped to suppress the production and/or to remove immediately the $O_2^{\cdot-}/H_2O_2$. SOD and catalase are among the important factors that help the plant cells in scavenging of $O_2^{\cdot-}$, thus avoiding further photoinhbition (Asada and Takahashi, 1987).

Some of these inhibitors (antimycin A, sodium azide and Oligomycin) could affect the scavenging of $O_2^{\cdot-}$ by suppressing the activities of catalase or peroxidase to make the photosynthetic system more vulnerable. However, antimycin A or Oligomycin did not have any effect on catalase or peroxidase at the concentrations used during photoinhibitory treatment, even at concentrations as high as 100 μM or 100 μg ml^{-1} (data not shown). Catalase is known to be inhibited by sodium azide (Nakano and Asada, 1980), but at much higher concentration than that required to promote photoinhibition (Fig. 5.4B; Table 5.3). Sodium azide did not inhibit peroxidase, but suppressed about 15 and 40 % of catalase activity at concentrations of 1 and 10 μM, respectively.

We do not know of any report on inhibition of catalase/peroxidase by antimycin A, or Oligomycin. Antimycin also may affect photosynthetic carbon metabolism, but again at a high concentration and at certain conditions (Slovacek and Hind, 1980). Therefore, we suggest that the promotion of photoinhibition by low concentrations of antimycin A, sodium azide or Oligomycin is basically due to their interference with respiratory metabolism.

There are three possible factors that could facilitate respiration to protect the mesophyll protoplasts against photoinhibition (Saradadevi and Raghavendra, 1992). Respiratory metabolism could either (a) elevate the level of intracellular CO_2, particularly at CO_2-limiting conditions, (b) provide extra energy toward the turnover of D1 protein required to prevent photoinhibition or (c) help to maintain an optimal redox state in chloroplasts or cytosol of the cells. Sodium fluoride and malonate, which inhibit glycolysis and tricarboxylic acid cycle, respectively, would decrease the decarboxylation of carbon compounds but could not affect the extent of photoinhibition. Similarly, Oligomycin, which inhibits oxidative phosphorylation, was not as effective as sodium azide or antimycin A (inhibitors of oxidative electron transport) in enhancing the extent of photoinhibition. The present results demonstrate strongly that oxidative electron transport in mitochondria plays a much stronger role than the oxidative phosphorylation during protection of protoplasts against photoinhibition.

Active photosynthetic carbon metabolism in light promotes respiration during the subsequent dark period. This phenomenon of light enhanced dark respiration (LEDR) is already demonstrated in leaves and protoplasts (Stokes et al., 1990; Reddy et al., 1991). The decrease in respiratory oxygen uptake after photoinhibitory treatment could, therefore, be a consequence of restriction in the photosynthetic capacity during the preceding light period.

Oxidative metabolism in mitochondria has recently been demonstrated to play a beneficial and essential role in the photosynthetic process as well

(Krömer et al., 1988; Vani et al., 1990; Krömer and Heldt, 1991; Raghavendra et al., 1994; Krömer, 1995). It is possible that the marginal interference by respiratory inhibitors of photosynthetic metabolism (Table 5.2) makes protoplasts highly susceptible to photoinhibitory light. Respiration has been proposed to prevent the over-reduction of the photosynthetic electron transport chain in chloroplasts by providing an outlet for reduced equivalents to the cytosol or mitochondria (Krömer and Heldt, 1991). We suggest that oxidative metabolism protects the plant cell against photoinhibition possibly by preventing the over-reduction of electron transport chain in chloroplasts. Dark respiration, possibly through the supply of ATP, could modulate remarkably the process of photoinhibition of photosynthesis (and subsequent reactivation) in the cyanobacterium, *Anacystis nidulans* (Shyam et al., 1993).

The metabolic movement between chloroplasts, mitochondria and cytosol as the biochemical basis of the beneficial interaction between photosynthetic carbon assimilation and dark respiration is discussed in detail in two recent reviews (Raghavendra et al., 1994; Krömer, 1995).

Chapter 6

Correlation Between the Inhibition of Photosynthesis and the Decrease in Leaf Disc Area or Protoplast Volume under Osmotic Stress

Chapter 6

Correlation between the Inhibition of Photosynthesis and the Decrease in Leaf Disc Area or Protoplast Volume under Osmotic Stress

This chapter presents the results of our studies on photosynthesis and its response to osmotic stress in another experimental system: detached leaf discs of pea. Besides demonstrating the inhibition of leaf disc photosynthesis by osmotic stress, data are presented to indicate a highly significant correlation between the decrease in leaf disc area and inhibition of photosynthesis under different degrees of osmotic stress. Further, a strong positive correlation is established also between the sensitivity of photosynthesis and decrease in the volume of protoplasts, when subjected to stress by incubation in a range of hyper-osmotic media.

Photosynthetic rates in leaf discs of pea were quite sensitive to osmotic stress. The rate of photosynthesis decreased by nearly 70% at 1.0 M sorbitol (Fig. 6.1 A). There was a marked reduction also in the area of these leaf discs under hyper-osmotic concentrations of sorbitol (Fig. 6.1B). There was reduction of up to 40% in leaf disc area when exposed to 1.0 M sorbitol for an hour as compared to fully turgid leaf discs. Protoplasts shrank in their size (Plate 6.1) and lost >50% of their photosynthetic capacity when they were incubated in 1.0 M sorbitol containing medium for 10 min (Fig. 6.2A). The degree of shrinkage (decrease in size) of protoplasts increased along with the concentration of sorbitol in the medium (Fig. 6.2B). The volume of the protoplasts decreased by >70%, when they were exposed to 1.0 M sorbitol for 10 min at 0 °C in comparison with protoplasts in 0.4 M sorbitol. The degree of shrinkage was similar in both palisade and spongy mesophyll protoplasts (data not shown).

Several observations in literature suggested that a decrease in photosynthesis was associated with a reduction in protoplast/chloroplast volume (Kaiser,

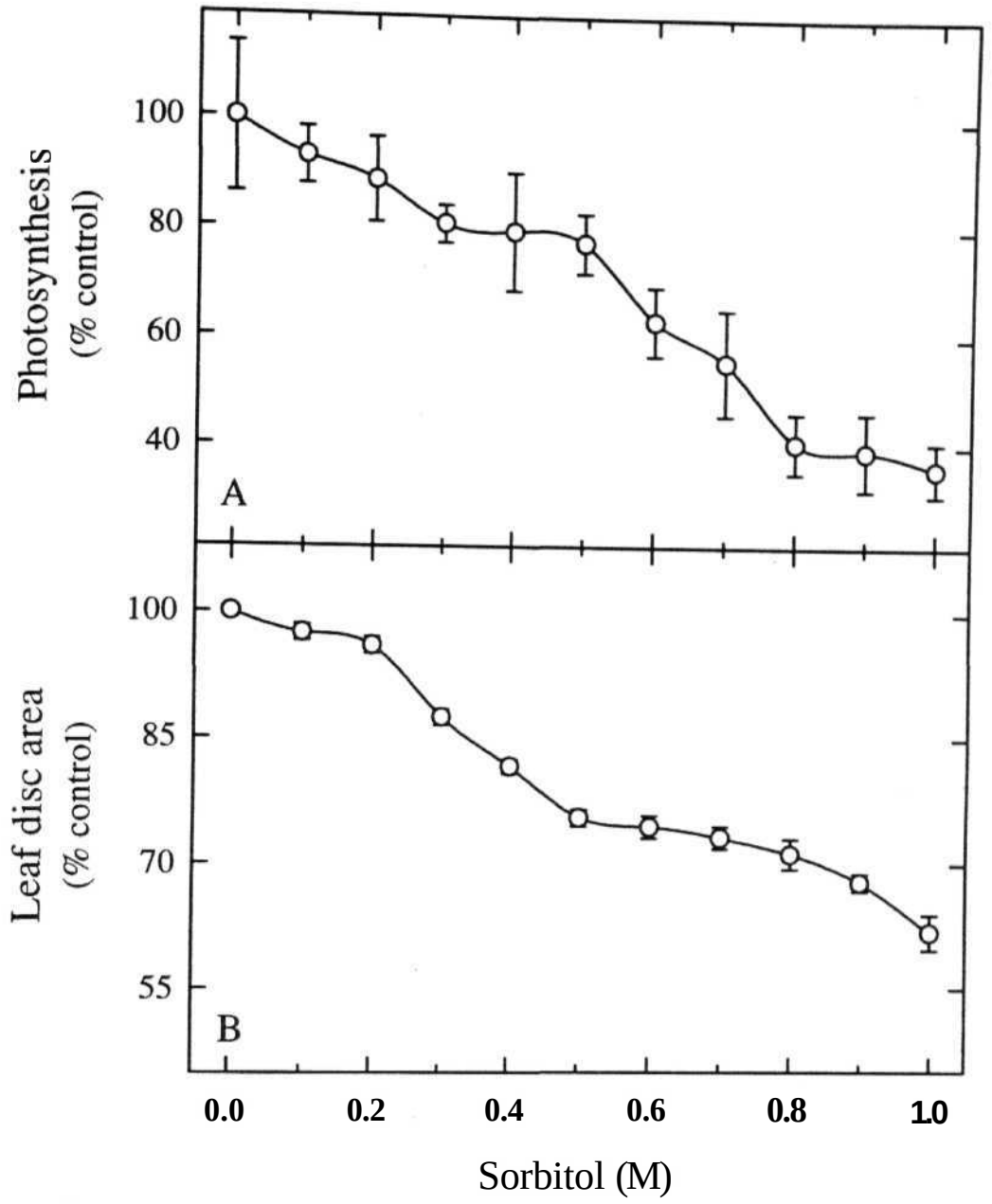

Figure 6.1. Inhibition of photosynthetic oxygen evolution (A) and the decrease in the area (B) of detached leaf discs of pea after pre-incubation for 1 h in increasing concentration of sorbitol from 0.1 to 1.0 M (-0.1 to -3.1 MPa). Photosynthesis was measured in a leaf disc O_2 electrode with *ca.* 5% (v/v) CO_2 at 25 °C. The photosynthetic rate of fully-turgid leaf discs (pre-incubated in water for 1 h) was 46.4 ± 4.9 μmol O_2 evolved m^{-2} s^{-1} (control - 100%). The average area of a fully turgid leaf disc was 0.27 ± 0.01 cm^2 (control - 100%).

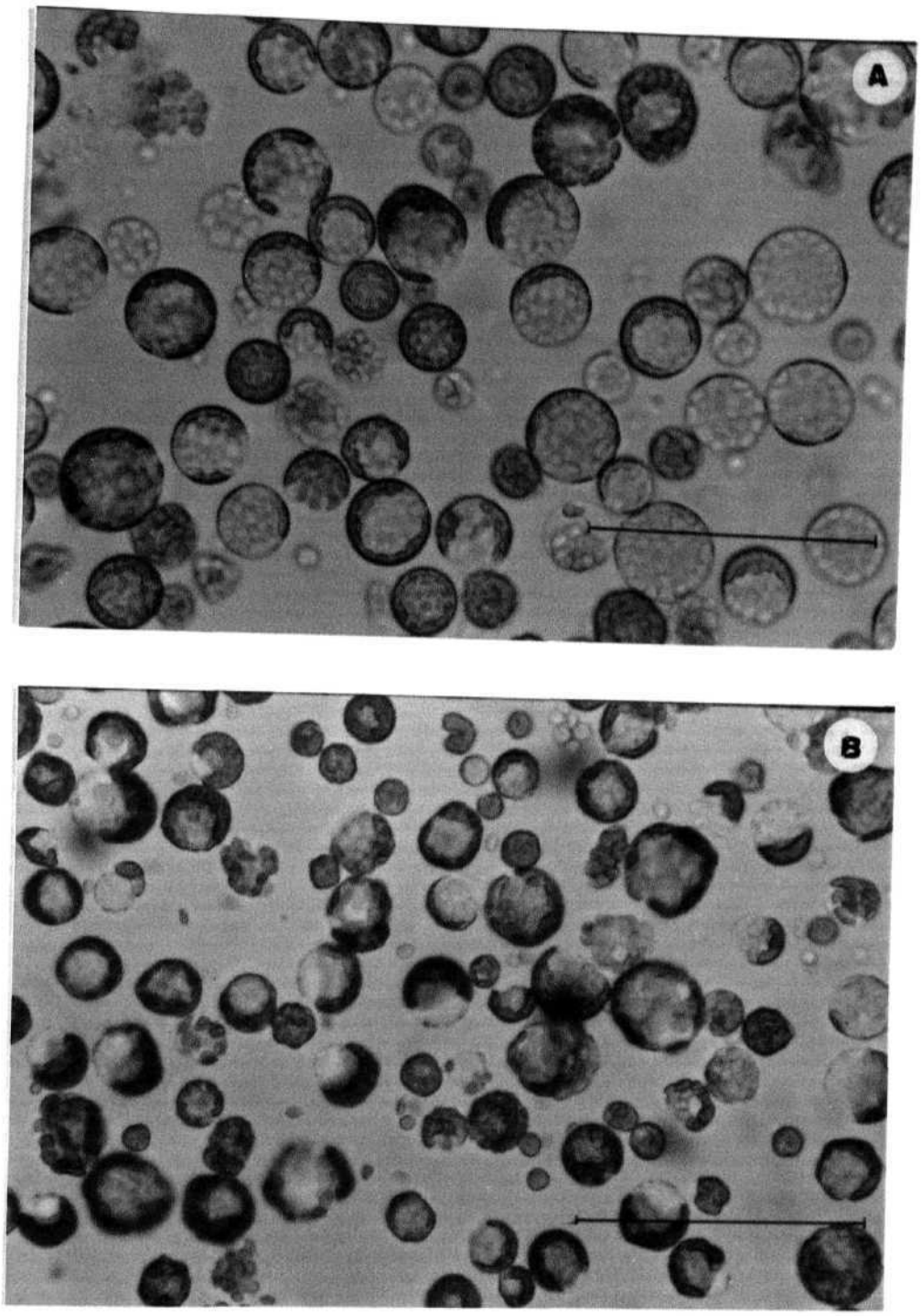

Plate 6.1. A: Photomicrograph of isolated mesophyll protoplasts suspended in 0.4 M sorbitol (iso-osmotic) containing medium. The Chl concentration of protoplast suspension was 200 $\mu g\ ml^{-1}$. B: Same protoplast preparation after incubation in 1.0 M sorbitol (hyper-osmotic medium) for 10 min on ice (i.e. at 0 °C). Horizontal bar represents 100 μm.

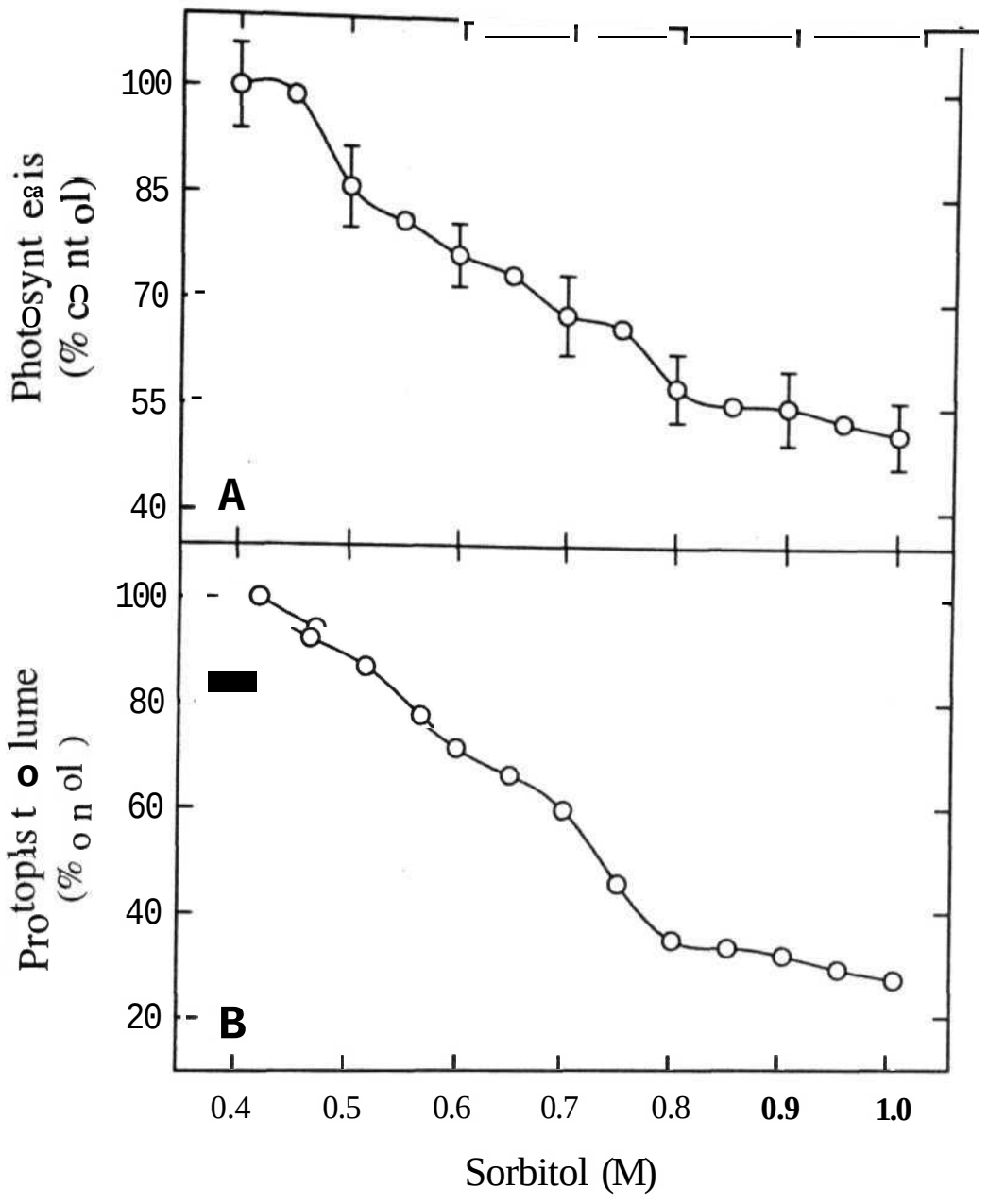

Figure 6.2. A: Inhibition of photosynthetic oxygen evolution, and B: Reduction in volume of pea mesophyll protoplasts, on exposure to osmotic stress. Sorbitol concentration in the suspension as well as reaction media was increased from 0.4 to 1.0 M (-1.3 MPa to -3.1 MPa). The rate of bicarbonate dependent oxygen evolution by protoplasts suspended in 0.4 M sorbitol was 170 ± 8 μmol mg^{-1} Chl h^{-1} (control - 100%). Average volumes of palisade and spongy mesophyll protoplasts in 0.4 M sorbitol (control) were $2.6 \times 10^{-5} \pm 0.5 \times 10^{-5}$ mm^3 and $0.8 \times 10^{-5} \pm 0.2 \times 10^{-5}$ mm^3, respectively.

1982; Santakumari and Berkowitz, 1989, 1990). Drought resistant varieties undergo osmotic adjustment, at low water potentials, to maintain their photosynthetic efficiency as well as to minimize the reduction in volume of protoplast/chloroplast (Acevedo et al., 1979; Morgan, 1984; Sen Gupta and Berkowitz, 1987; Berkowitz and Kroll, 1988; Santakumari and Berkowitz, 1990).

We have also observed a marked decrease in the absorbance of protoplasts on exposure to 1.0 M sorbitol for 10 min (Fig. 6.3B). The typical absorption spectrum of protoplasts in 0.4 M sorbitol is shown in Fig. 6.3 A. The difference spectrum of osmotically stressed protoplasts (suspended in 1.0M sorbitol) against control (0.4 M sorbitol) presented a mirror-image of the spectrum of 80% acetone extract of protoplasts, i.e., Chloroplast pigments (Fig. 6.3C), indicating that most of the decrease in protoplast size was accounted for by the chloroplasts. This idea is supported by the recent study of Winter et al. (1994), which revealed that besides the vacuoles, chloroplasts constitute the second largest compartment, amounting to 16% of the total cellular volume in spinach leaf.

The shrinkage of protoplasts is due to the loss of water as well as the decrease in the size of cellular organelles. As per Fig. 6.3B nearly two-thirds of the decrease in absorbance of protoplasts appears to be due to chloroplasts, while the remaining one-third is due to other components. Obviously, the contribution of chloroplasts to the absorbance of protoplasts is much more predominant than that of vacuoles. However, this is not surprising, as chloroplasts are known to be green while vacuoles are not brightly coloured.

An important reason for inhibition of photosynthesis in chloroplasts under osmotic stress has been proposed to be the reduction in stromal volume (Kaiser, 1982; Sen Gupta and Berkowitz, 1987). The volume reduction occurs when stromal ψ_w equilibrates with external ψ_s by dehydration and the resulting biophysical changes inhibit photosynthesis (Santakumari and Berkowitz, 1990).

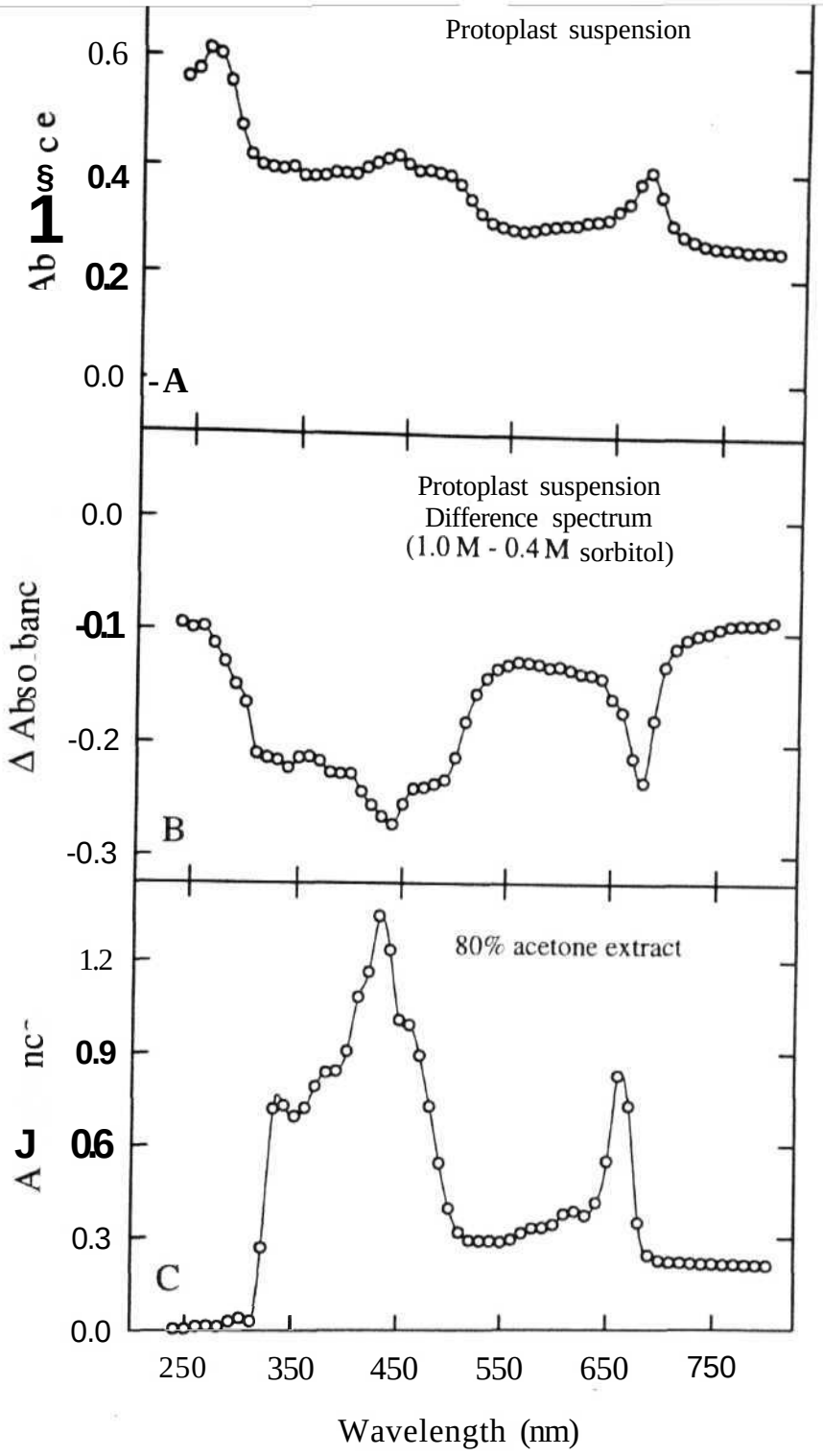

Figure 6.3. **A:** Absorption spectrum of a protoplast suspension, or C: its acetone extract, containing protoplasts equivalent to 10 µg Chl ml^{-1}. B: Difference spectrum of protoplasts indicating the decrease in protoplast absorbance on incubation in hyper-osmotic concentration of 1.0 M sorbitol (sample cuvette) compared to that protoplasts in 0.4 M (iso-osmotic) sorbitol (reference cuvette). The decrease in absorbance was a reflection of the spectrum of Chloroplast pigments.

During osmotic dehydration, intracellular solutes are concentrated. The accumulation of solutes in the chloroplasts can affect the activity of stromal enzymes, leading ultimately to a decrease in the rate of photosynthesis (Kaiser and Heber, 1981; Kaiser, 1982).

In the next set of experiments, the quantitative relationship was evaluated between the photosynthetic inhibition and the decrease in leaf disc area or the protoplast volume (indicated by either their absorbance at 440 nm or calculated from the diameter measurements). The extent of decrease in photosynthetic rates (even under very high CO_2 concentration, *i.e.* 5%) in leaf discs on exposure to osmotic stress was positively correlated to reduction in leaf area (Fig. 6.4A). The extent of shrinkage in protoplast volume (in response to the increasing concentrations of sorbitol) was positively correlated with the inhibition of photosynthetic activity. The correlation coefficient of photosynthetic inhibition versus reduction in volume was 0.987 (Fig. 6.4B).

There was a positive correlation between the inhibition of photosynthesis and decrease in A440 of protoplasts on exposure to osmotic stress (Fig. 6.5A). However, the correlation coefficient between the photosynthetic inhibition and the decrease in absorbance of protoplasts corrected for turbidity (i.e. $A_{440} - A_{750}$) and its statistical significance (Fig. 6.5B) were much greater than those in case of A_{440} alone. We consider this quite logical because the absorption of protoplasts at 440 nm, when corrected for non-specific turbidity at 750 nm, indicates the contribution of mainly chloroplasts. Obviously, the degree of Chloroplast shrinkage is highly correlated with the extent of inhibition in photosynthesis due to osmotic stress (Fig. 6.5B).

The highlight of the present work is the identification of two simple criteria, based on protoplast volume/leaf disc area, which can be easily used to monitor/predict the inhibition of photosynthesis due to osmotic stress: spectrophotometric measurement of the absorbance of protoplasts and determination of the area of detached leaf discs under a stereo dissecting microscope.

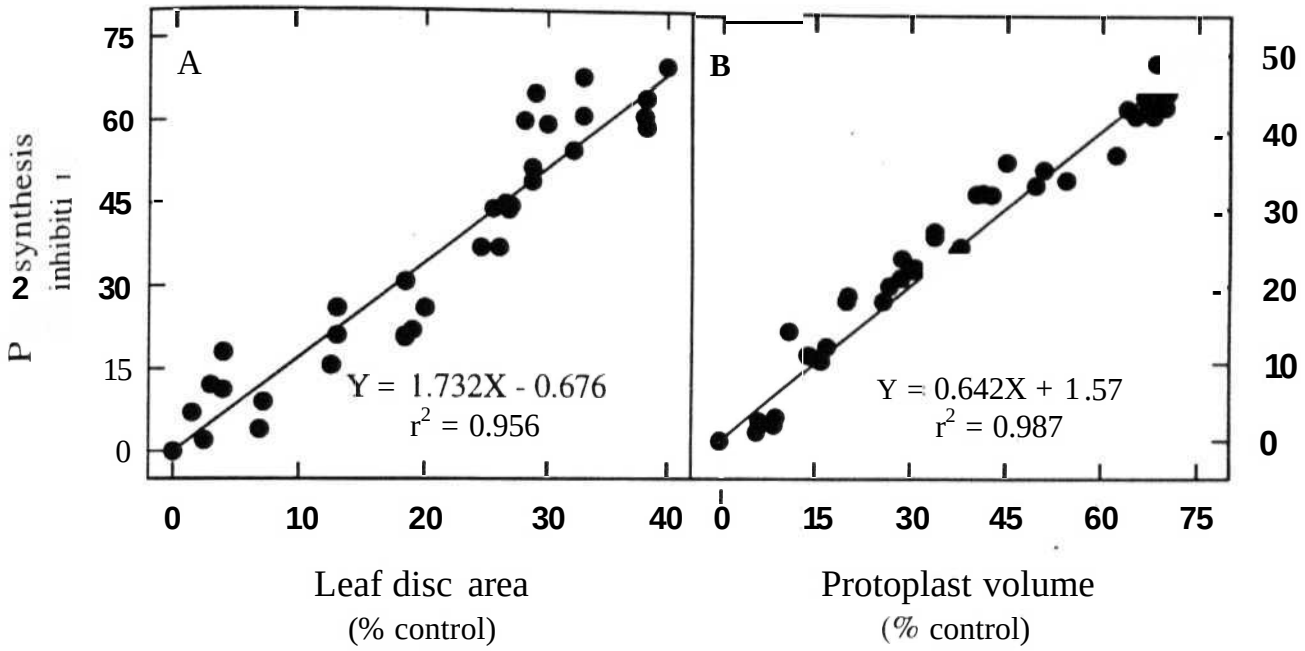

Figure 6.4. Very high positive correlations between inhibition of photosynthetic capacity on exposure to osmotic stress and the decrease in leaf disc area (A) or protoplast volume (B). Both the correlation coefficients (r^2) were statistically significant ($P < 0.001$). Other details were as described in Figs. 6.1 and 6.2.

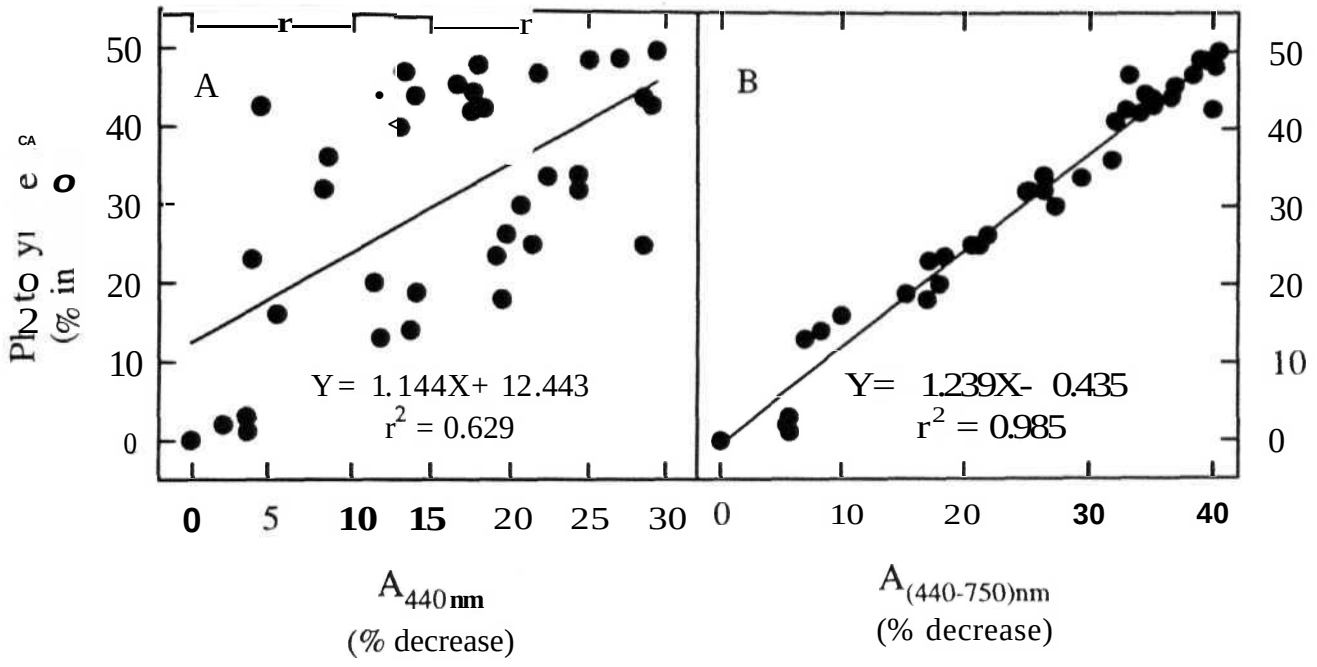

Figure 6.5. **A:** Positive, but limited correlation between the decrease in absorbance of protoplasts at 440 nm and inhibition of their photosynthetic capacity on exposure to osmotic stress. The average absorbance of protoplasts (equivalent to 20 μg Chl ml^{-1}) at 440 nm was 0.91 ± 0.05. B: Very high positive correlation between the decrease in absorbance of protoplasts at 440 nm (corrected for turbidity at 750 nm) and inhibition of their photosynthesis on exposure to osmotic stress. The average absorbance ($A_{440-750}$) of protoplasts (equivalent to 20 μg Chl ml^{-1}) was 0.35 ± 0.01. Both correlation coefficients were statistically significant ($P < 0.001$).

There are two widely accepted experimental techniques to monitor the relationship between protoplast volume and water potential. One of them, pressure/volume curve analysis depends on an accurate estimate of a theoretically derived value (Santakumari and Berkowitz, 1989). But several hours are needed to obtain the data for a single pressure/volume curve. The second technique is the use of dual label infiltration, developed by Kaiser (1982). However, a disadvantage with this technique is the scope for large error because of the equilibration of ^{14}C-sorbitol into the symplast due to the loss of membrane integrity during vacuum infiltration (Santakumari and Berkowitz, 1989). As per the present observations, a decrease in absorbance of protoplasts at 440 nm, corrected for turbidity at 750 nm, represents an accurate, yet convenient measure of protoplast volume (Fig. 6.5B).

The measurement of protoplast size/volume under a microscope is much more tedious than determining the absorption of protoplast suspension. We therefore suggest that the decrease in absorbance at 440 nm (corrected for turbidity at 750 nm) indicating the protoplast size, can be used as a simple criterion to monitor and/or predict the inhibition of photosynthesis under osmotic stress in mesophyll protoplasts.

Most of the earlier studies on leaf area were with intact plants and in the field. We now demonstrate a rapid decrease in leaf disc area along with their photosynthetic rate in detached leaf discs, which can be monitored in the laboratory (Figs. 6.1 A, B). The decrease in leaf disc area is significantly correlated with the extent of decrease in their photosynthetic capacity (Fig 6.4A). We suggest that the area of leaf discs also forms a very convenient parameter to monitor and evaluate inhibition of photosynthesis by osmotic stress. This technique of measuring leaf disc area is simple, easy and quick. It would be of great interest to evaluate this concept with agronomic cultivars of crop species. This would be taken up in the next phase of our work.

Analysis of Chl *a* fluorescence *in vivo* has been a powerful tool in probing the functional properties of the photosynthetic apparatus without disrupting the intactness of the system (Melis and Zeiger, 1982). When part of the light is absorbed by the photosynthetic pigments (chlorophylls or carotenoids), a major part is used for photosynthesis, while the remainder is re-emitted as fluorescence. Thus, fluorescence information from photosynthetic systems provide information about the rate and yield of photochemical performance in plants (Walker, 1988; Joshi and Mohanty, 1995). Hardly any change in photosynthetic reactions will escape from being reflected in one or the other parameter of Chl fluorescence.

For almost half a century, Chl fluorescence has been a tool to study the primary processes of photosynthesis (Schreiber and Bilger, 1987; Govindjee, 1995). Being an intrinsic indicator of the photosynthetic reactions in the chloroplasts of green plants, Chl fluorescence has been recommended for use as an indicator of the state of the leaf adenylate system (Kobayashi et al., 1982) and for early detection and detailed analysis *in vivo* of stress effects on plants (Schreiber and Bilger, 1987; Lichtenthaler, 1988). However, Chl *a* fluorescence is so rich in information (and is affected by several deexcitation pathways) that it also becomes an ambiguous signal. Therefore, the data on Chl fluorescence must be used with caution and in combination with other signals to provide a proper interpretation (Govindjee, 1995).

When a dark adapted photosynthetic cells are illumnated, the yield of Chl *a* fluorescence follows a particular pattern. These transient changes in the fluorescence are known as "Kautsky" phenomenon. Typical kinetics of such Chl fluorescence induction are illustrated in the 'inset' of Fig. 6.6. The kinetics are distinguished into two major phases. The initial "rapid" phase of induction which occurs within 1 or 2 sec, while subsequent "slow" phase follows up for several minutes. The 'O' or F_o represents the basal level of fluorescence emissions, originating from light harvesting antenna (LHCII)

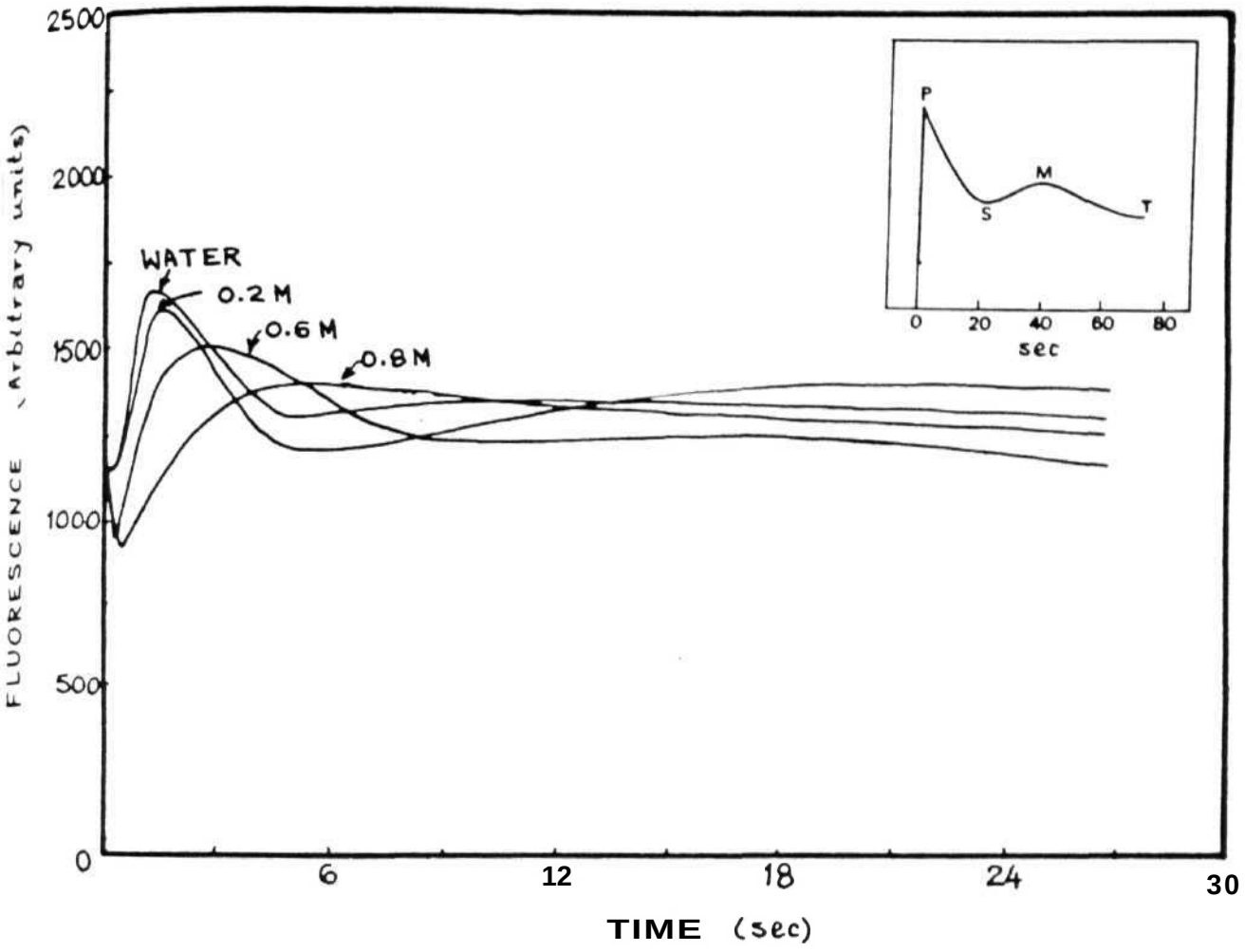

Figure 6.6. Fluorescence induction curves of detached leaf discs of pea after incubating in different concentrations of sorbitol ranging from zero (water) to 1.0 M sorbitol for 1 h. The 'P' value decreased as the concentration of sorbitol was increased. The time taken to reach peak (P or F_m) was increased due to osmotic stress indicating that significant changes in the fluorescence induction curves occurred during osmotic stress. **Inset:** Diagrammatic representation of typical fluorescence induction curves **labeled as OPSMT.**

when all PSII reaction centres are in the open state. When such sample is illuminated, the excitation of Chl results in a fast rise of the fluorescence to 'P' and then quenches to 'S' state, which partially reverses to 'M' state. Thereafter M quenches to a final steady state level of T. The fluorescence pattern of the first few seconds of excitation ('OPSM') reflects electron transfer reactions, and in the next phase ('MT'), the interplay between overall electron transport and the carbon reduction cycle (Shreiber and Bilger, 1987; Govindjee, 1995; Joshi and Mohanty, 1995).

There are several aspects and parameters of the chloroplyll fluorescence, which can be used for stress detection in intact plants. These are:

(i) Fast and slow phase of light induced fluorescence induction kinetics, used to assess the effect of drought in willow leaves (Ögren, 1990).

(ii) Ratio of maximum fluorescence F_m to ground fluorescence F_o: (F_m/F_o) is used to asses the effect of stress on photosynthesis in *Digitalis lanata* (Stuhlfauth et al., 1988).

(iii) Registration of the fluorescence induction kinetics (slow component of Kautsky curve) with determination of Rfd-values as vitality index. This provides information on the physiological state of photosynthesis and has been applied with great success in forest decline research, ecophysiology and stress detection in plants (Lichtenthaler 1988).

(iv) Stress-adaptation index (Ap), measured by simultaneous registration of the induction kinetics in the 690 and 730 nm region, and contains additional information on the photosynthetic apparatus and its degree of damage (Lichtenthaler, 1988).

We have tried to correlate decrease in photosynthesis due to osmotic stress with different types of derivations/equations from 'O', 'P' and 'T' values. Amongst several equations tried, the ratio of P/O (F_m/F_o) in leaf discs decreased with increasing sorbitol concentration (Fig. 6.7). However, there was a lot of deviation in the measurement. The variation/inconsistency could be due to

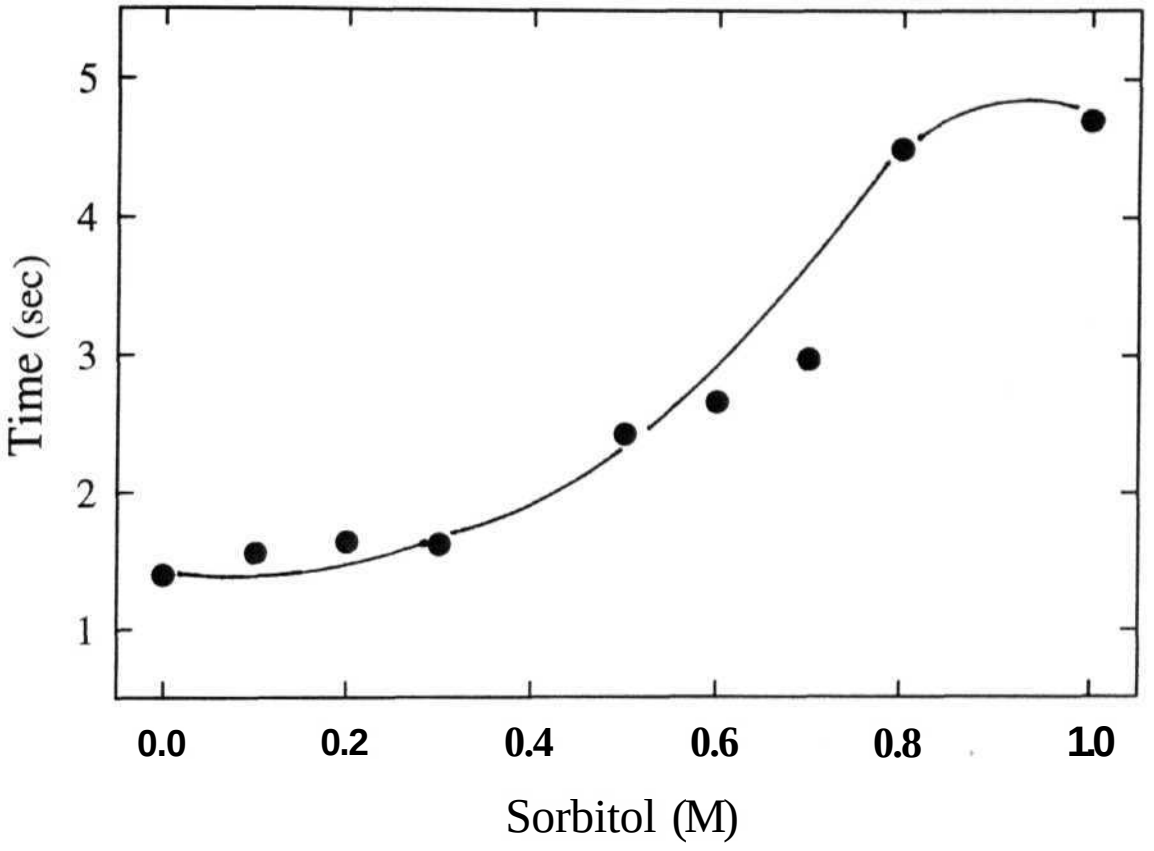

Figure 6.7. Effect of osmotic stress on time (in sec) taken to reach 'P' value or F_{max} of fluorescence induction curves of detached leaf discs of pea. It increased with increase in sorbitol concentration during pre-incubation of leaf discs.

either the low sensitivity of photochemical reactions to the osmotic/water stress, or the limited efficiency of the instrument itself, which we have used (as discussed further in the following pages). Stuhlfauth et al. (1988) have reported that in water stressed *Digitalis lanata*, the levels of F_o and F_m were not significantly changed throughout the period of stress. The peak F_p value slightly decreased with increasing water stress. Even in our experiments, the 'P' value decreased with increasing stress, i.e. high sorbitol concentation during pre-osmotic treatment of detached leaf discs (Fig. 6.6).

The pattern of Chl fluorescence transients in leaves is known to change markedly in response to osmotic/water stress conditions (Schreiber and Bilger, 1987; Stuhlfauth et al., 1988; Ögren, 1990) and are therefore used to analyse responses of plants to water (Stuhlfauth et al., 1988; Ögren, 1990; Jefferies, 1992) or temperature stress (Peeler and Nay lor, 1988; Burke, 1990).. In our experiments, the time taken to reach peak ('P') value from origin ('O') of fluorescence was extended with increase in concentration of sorbitol during preincubation of leaf discs (Figs. 6.6 and 6.8).

During fluorescence measurements, F_o and F_m cover only few seconds of induction kinetics, and may not fully represent the photochemical process in a leaf, it is therefore better to use the whole fluorescence induction kinetics and the determination of Rfd values for screening of stress effects (Lichtenthaler 1988). Ögren (1990) has shown that, the slow (but not the rapid), phase of fluorescence induction is altered in leaves suffering from drought. Even at a relatively low water content, where electron transport is severely affected, the variable fluorescence is almost unchanged, suggesting that the PSII donor side and the primary reaction are not affected (Schreiber and Bilger, 1987).

The height of the Rfd-values indicates the potential photosynthetic activity of a leaf, as was confirmed by parallel measurements with an infra-red gas analyzer (IRGA) system. Under water stress, the Rfd values (fluorescence

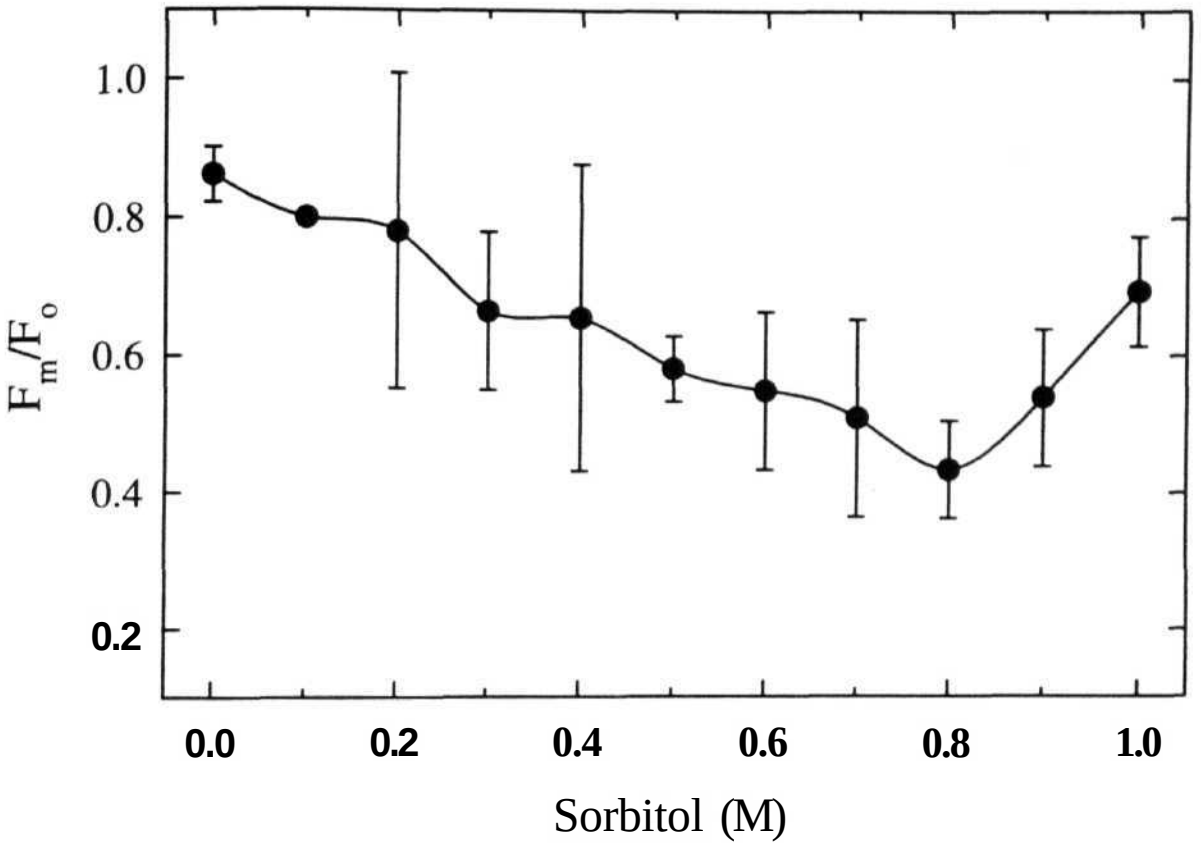

Figure 6.8. Effect of osmotic stress on F_m/F_o or P/O ratio. F_m/F_o was calculated from fluorescence induction curves measured after pre-incubating leaf discs in different concentrations of sorbitol. The F_m/F_o ratio decreased with increasing concentration of sorbitol during pre-incubation of leaf discs. But the decrease was not linearly related to sorbitol concentration and there was a lot of variation in the measured values.

decrease from the maximum to the steady state, which indicates potential photosynthetic activity of a leaf) are lowered (Lichtenthaler, 1988). Therefore, we have used the formula to calculate potential photosynthesis from Rfd-vitality index. The calculated potential photosynthesis (from Chl fluorescence induction curves), decreased with increase in osmotic concentration, from water (zero MPa) to 1.0 M sorbitol (-3.1 MPa) (Fig. 6.9). Measurement of Rfd-value with a portable field fluorometer, permits a fast and extensive outdoor screening of the vitality of trees and agricultural plants, and can even be used for remote sensing of vegetation (Lichtenthaler, 1988).

We could measure only simple Kautsky induction curves using our Chlorophyll Fluorometer (Richard Brancker Ltd., Ottawa, Model SF-30). We could not differentiate photochemical quenching from non-photochemical quenching. The instrument does not have accessory devices like pulse modulation frequency control. We could not assess PS1 and PSII activities separately with the information obtained from this instrument. The intensity of the excitation beam was very weak (only 6 W m^{-2}) and may not be sufficient to open all the reaction centres which were closed during dark adaptation of leaf discs. As a result, the F_{max} measured with this instrument may be an under-estimate.

To make full use of Chl fluorescence as an indicator, a fluorometer with improved performance, like PAM (pulse amplitude modulation) fluorometer is needed. Fluorescence measurements using PAM fluorometer are comprehensive and useful for early detection and detailed analysis of stress effect on photosynthesis (Schreiber and Bilger, 1987).

Fluorescence measurements are very useful and give valuable information provided the instrument is sophisticated and is equipped with all accessories. However, if the instrument (chlorophyll fluorometer) is too simple, it is difficult to rely on fluorescence measurements to assess inhibition of

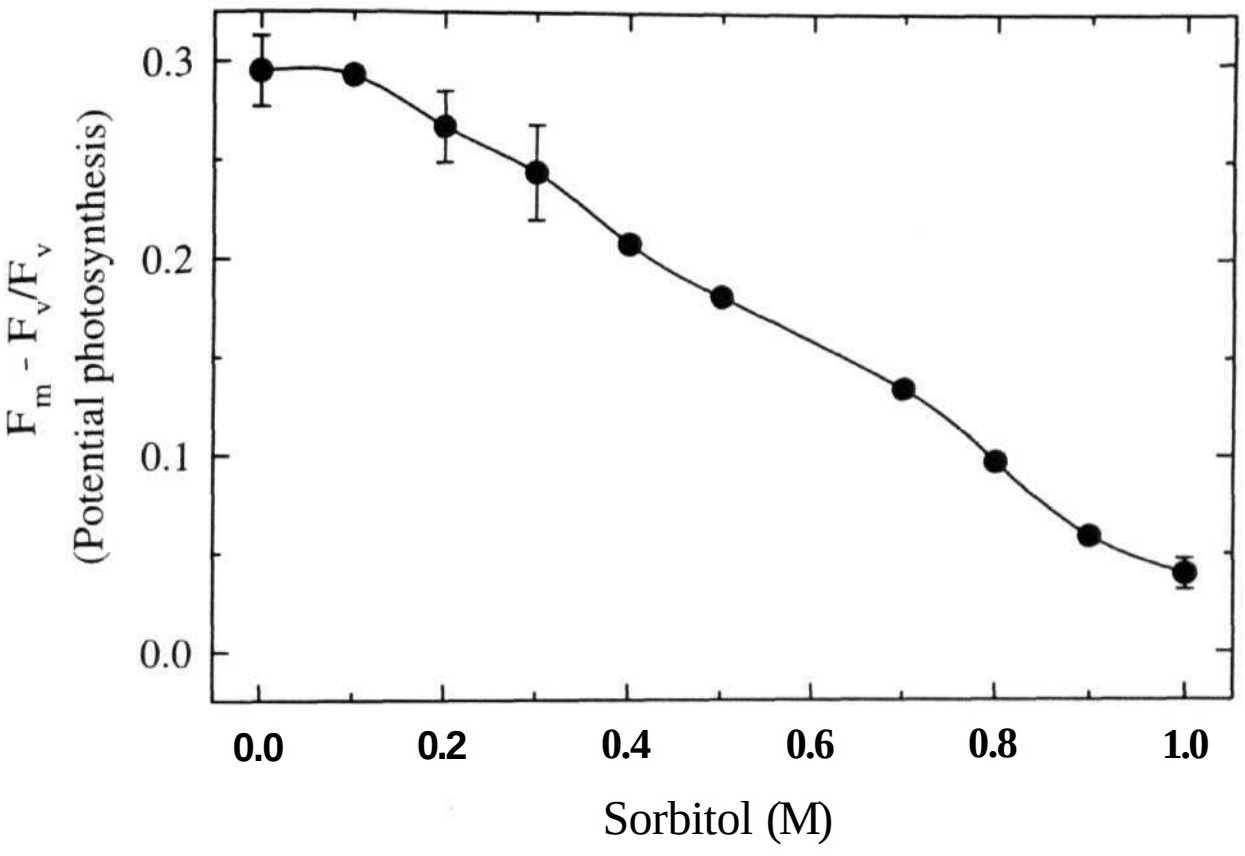

Figure 6.9. Effect of osmotic stress on potential photosynthesis of detached discs of pea. Leaf discs were pre-incubated in different concentration of sorbitol for 1 h in darkness and checked for their fluorescence induction curves. The potential photosynthesis calculated from the formula of $(F_m/F_o)/F_v$ or (P- T)/T decreased as concentrations of sorbitol was raised from zero (water) to 1.0 M (-3.1 MPa) during pre-incubation of leaf discs.

photosynthesis due to osmotic/water stress. We conclude that measurement of leaf area under osmotic stress conditions is much simpler and appropriate to predict the sensitivity of photosynthetic activities to decrease in water potential (i.e. water/osmotic stress).

Chapter 7

Conformational Status of Lipids and Proteins in Membranes of Protoplasts under Osmotic stress

Chapter 7

Conformational Status of Lipids and Proteins in Membranes of Protoplasts under Osmotic Stress

Since water is major component of the living cell and membranes, water/osmotic stress can be expected to change the conformational status of lipids as well as proteins in the membranes. A common and well-established response of a cell to water- or chilling stress is a marked change in fluidity of the membranes (Caldwell and Whitman, 1987; Gantet et al., 1990). Biophysical techniques, capable of sensing the short-range molecular motions within lipid bilayer, can be employed to study the effects of water or temperature stress on membrane fluidity. Some of these techniques are fluorescence polarization, flow cytometric fluorescence anisotropy, differential scanning calorimetry and electron spin resonance (Murata and Yamaha, 1984; Raison and Orr, 1986; Caldwell and Whitman, 1987; Lynch et al., 1987; Gantet et al., 1990).

Isolated protoplasts facilitate an easy study of their interaction with external medium, because of the absence of cell wall, which otherwise intervenes between plasma membrane and the ambient medium (Struck et al., 1994). Protoplasts have earlier been used for studies on polarization of fluorescent probes (Borochov et al., 1978; Vigh et al., 1979; Legge et al., 1986; Gantet et al., 1990). However, the present work is unique in showing conformational changes of lipids as well as proteins, using relevant fluorescent probes.

We report quick changes (within sec or min) in membranes, in response to osmotic and/or chilling, by monitoring fluorescence of three different probes, which get incorporated into different components of cell membranes. DPH, **a** neutral membrane probe and its cationic derivative TMA-DPH are known to fluoresce on interaction with membrane lipids, while FITC binds to proteins in biomembranes (Haugland, 1992). TMA-DPH labels only plasma membrane

lipids, where as DPH labels lipids of all the membranes including those of organelles, within the protoplasts (Gantet et al., 1990).

The dye, DPH or TMA-DPH, in the assay medium alone had only a very small level of fluorescence emission. The fluorescence emitted from these dyes was enhanced remarkedly when protoplasts also were present in the medium, confirming that these two dyes fluoresced only on interaction (presumbly bound to the lipid component) with protoplasts (Figs. 7.1 and 7.2). There was an increase in fluorescence emission of DPH at both 430 and 456 nm, but the response of fluorescence at 430 nm was maximum. Therefore the fluorescence emission at 430 nm was considered in all experiments. On the other hand, the fluorescence of FITC decreased by >45% in presence of protoplasts, demonstrating that the FITC- fluorescence was quenched on binding to the membrane proteins (Fig. 7.3).

Incubation of protoplasts with fluorescent dyes revealed that TMA-DPH and FITC labeling were quick and stable within 15 sec, while the increase in DPH fluorescence continued even at 30 min. There were two phases of DPH binding to the membranes, as indicated by the initial steep increase within 5 min, followed by a slow increase till 30 min (Fig. 7.4). These two phases appear to correspond to different components of cells. The increase in fluorescence of the dye, DPH during the first phase could mostly be due to the outer plasma membrane and the increase during the second phase reflects changes in membranes of other internal organelles (thylakoids/mitochondria). It is known that DPH equilibrates rapidly with plasma membrane within few minutes, while being slow to incorporate into other membranes (Gantet et al., 1990).

Interpretation of data from DPH on protoplasts is rather difficult due to two factors: (i) the labeling of membrane lipids in different cell components (Gantet et al., 1990) and (ii) non-equilibrium of fluorescence signal even after 30 min (Fig. 7.4). The use of TMA-DPH to study membrane lipids of plant

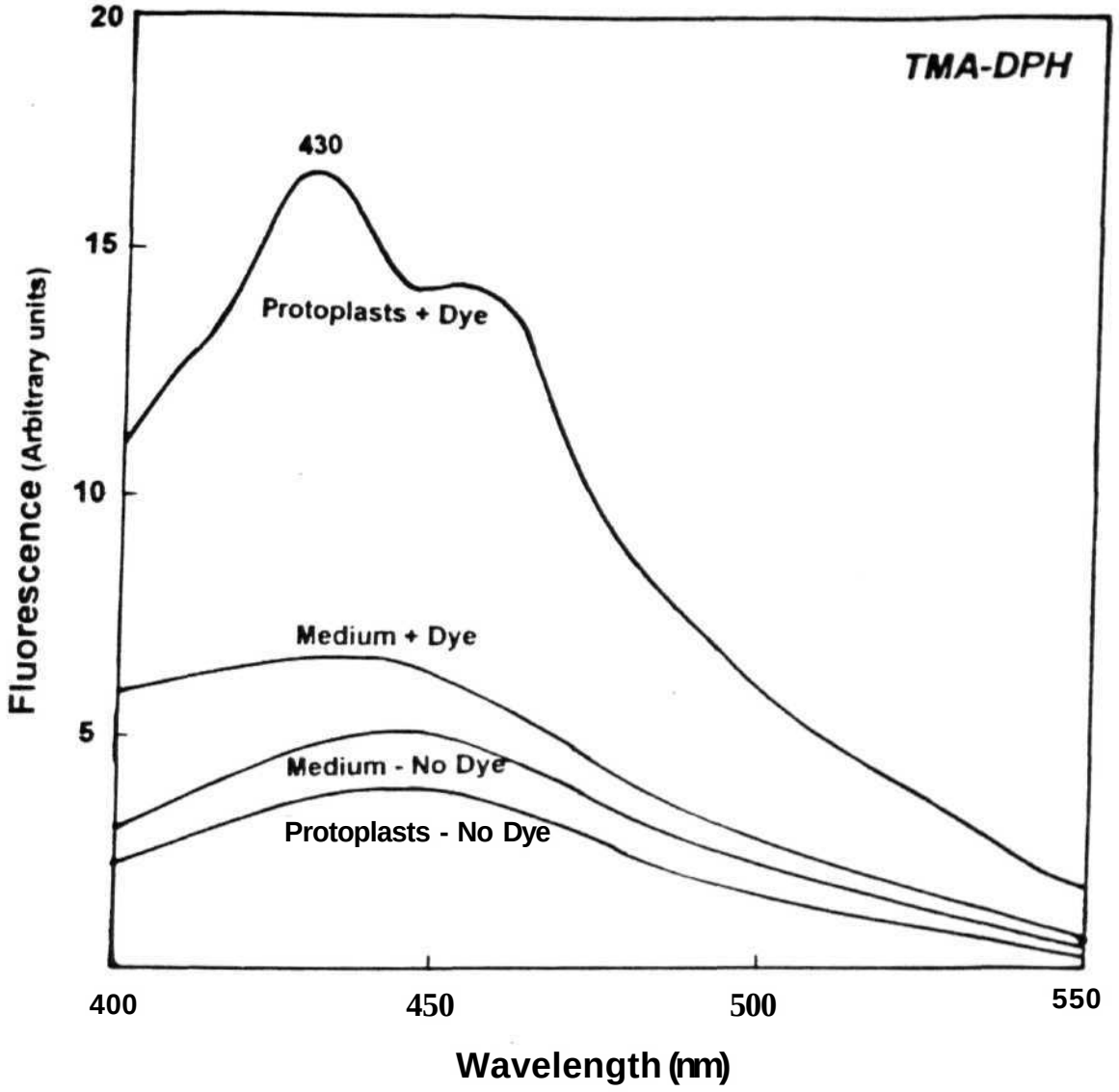

Figure 7.1. Emission spectra of fluorescence from the lipophilic dye, 1.0 μM TMA-DPH (binds to the lipid component of plasma membrane) without or with protoplasts equivalent 10 μg Chl ml^{-1}. The emission maximum of fluorescence from TMA-DPH was at 430 nm. The peak of excitation was at 364 nm.

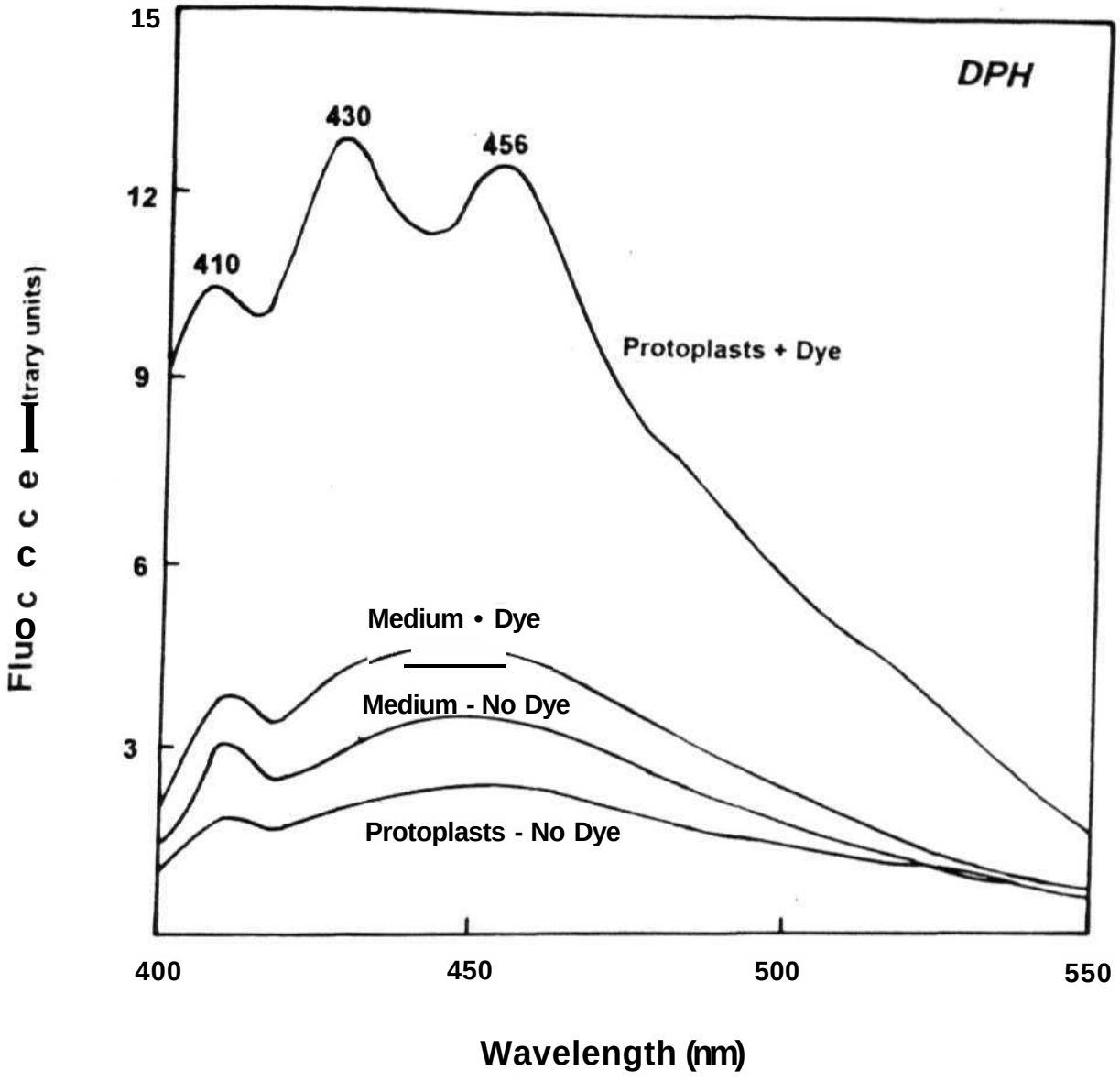

Figure 7.2. Emission spectra of fluorescence from the 0.1 μM DPH (a lipophilic dye which binds to lipids of all membranes) without or with protoplasts equivalent 1.0 fig Chl ml^{-1}. The two peaks of emission were at 430 nm and 456 nm. The peak of excitation was at 359 nm.

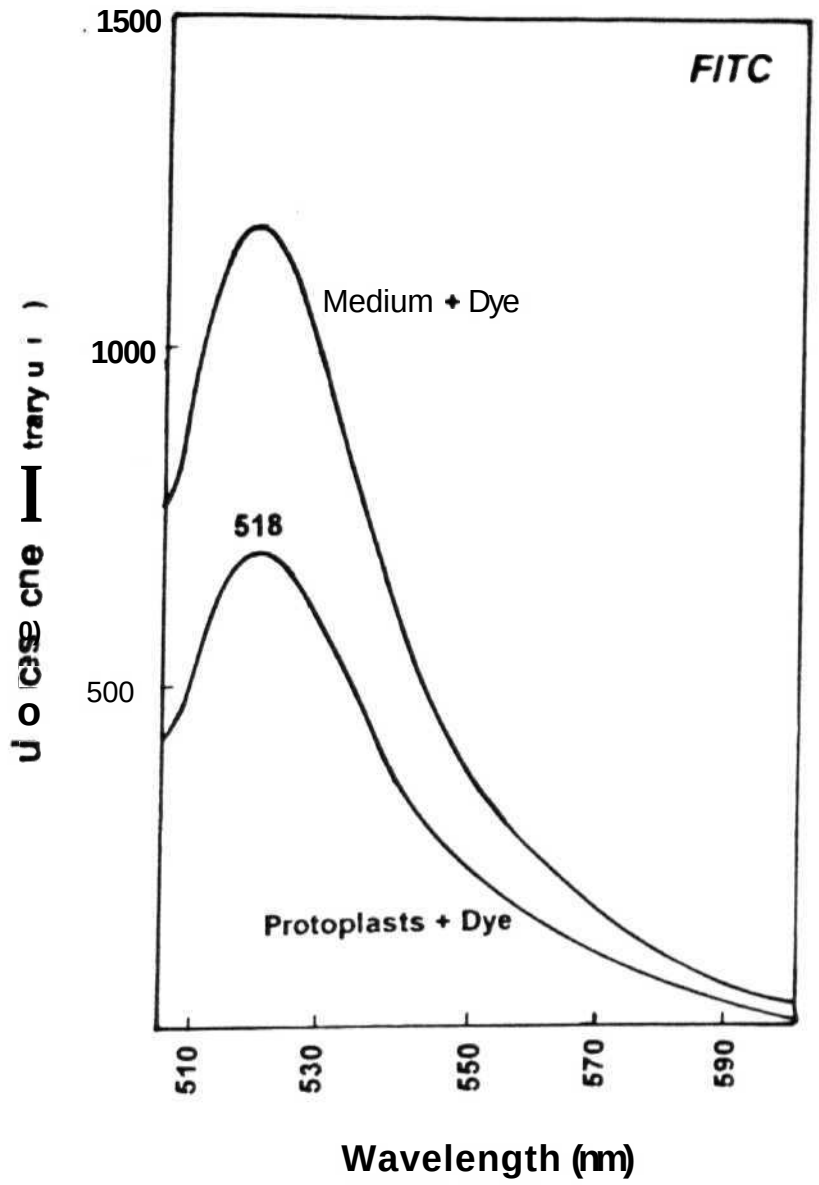

Figure 7.3. Emission spectra of fluorescence from 100 ng ml^{-1} FITC (a fluorescent dye, which binds to protein component of membranes) without or with protoplasts equivalent 10 µg Chl ml^{-1}. The emission maximum of FITC was at 518 nm. The peak of excitation was at 495 nm.

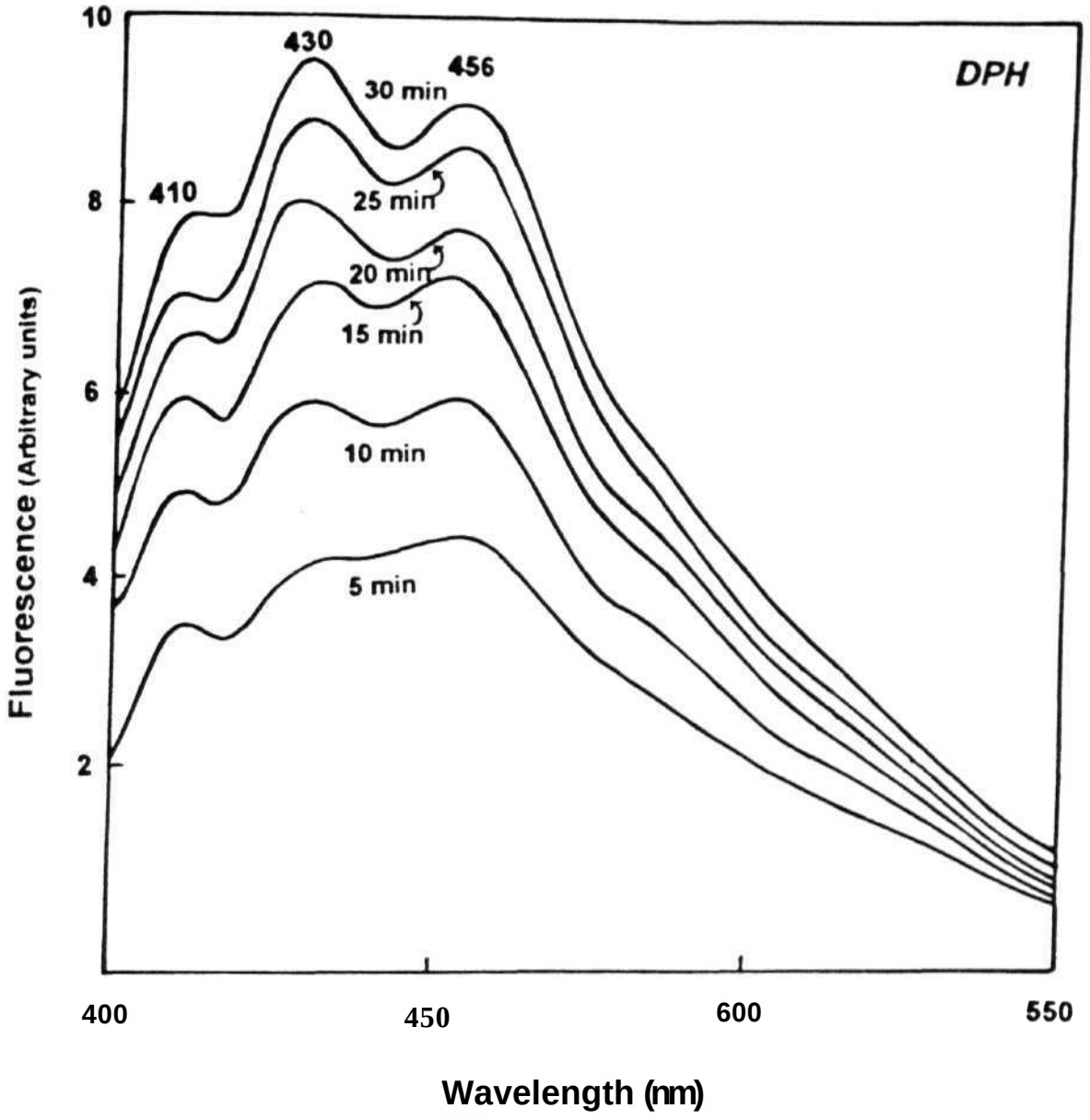

Figure 7.4. The progressive increase in fluorescence of DPH during incubation with protoplasts. The increase in fluorescence could be seen at 430 nm as well as 456 nm and continued up to 30 min of incubation. The increase in fluorescence of DPH due to its binding to protoplasts was corrected for its background (very small and almost negligible) fluorescence. Difference spectrum was obtained from (protoplasts with dye - protoplasts) - (medium with dye - medium).

protoplasts is appropriate, due to the more specific localization of TMA-DPH than that of DPH (Gantet et al., 1990). There was a marked decrease (>25%) in fluorescence of TMA-DPH, on exposure of protoplasts to hyper-osmotic medium of 1.0 M sorbitol, particularly at 0 °C (Fig. 7.5) indicating changes in plasma membrane. Such decrease in fluorescence of TMA-DPH due to osmotic stress was immediate and did not change with increase in the duration of pre-incubation of protoplasts in 1.0 M medium. Since TMA-DPH is considered to be a probe reasonably specific for plasma membrane lipids (Gantet et al., 1990), our observations confirm that plasma membrane lipids are affected soon after the exposure of protoplasts to osmotic stress.

Amongst the cellular membranes, plasma membrane is involved in a number of important cellular functions (Navari-Izzo et al., 1993). The first component that senses the external stress is the plasma membrane of the cell (Revathi et al., 1994). Water stress in plants is accompanied by the formation of a pronounced water vapour gradient across the cell's plasma membrane. One can therefore speculate that the plasma membrane contains a sensory apparatus that responds to water stress which would in turn trigger a cascade of events (Drory et al., 1992).

A decrease or increase in the extent of fluorescence could suggest merely a change in the binding pattern of fluorescent dye to either lipids or proteins and possibly a reflection of the surface property . The polarization of fluorescence signal reflects, further, the conformation of these lipids or proteins within membrane. The marked changes in the fluidity of plasma membrane are clearly indicated by changes in their polarization, ('P' values) of both TMA-DPH or FITC. The changes in polarization of DPH due to osmotic stress in protoplasts could not be studied as the fluorescence signal was not steady even after 30 min of incubation with protoplasts (Fig. 7.4). The significant decrease in 'P' value of TMA-DPH particularly at 25 °C (Table 7.1), indicated

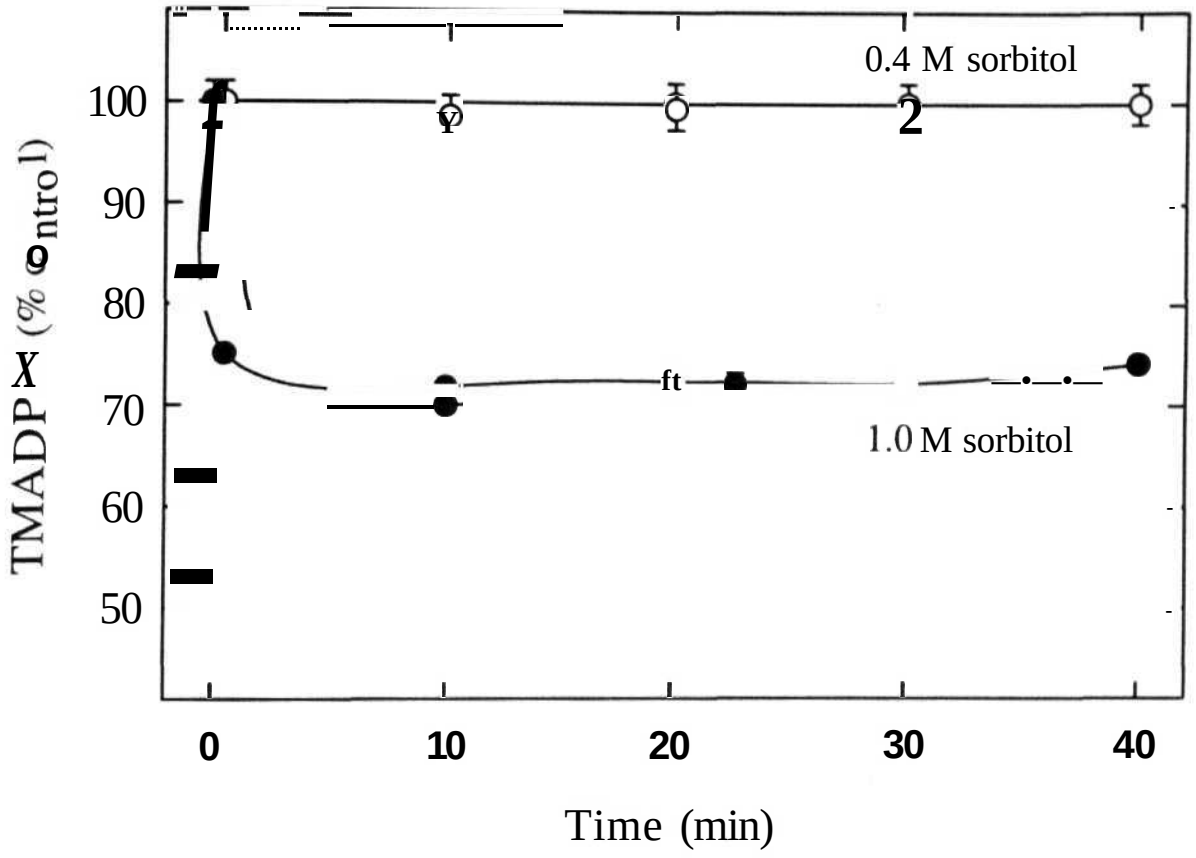

Figure 7.5. Effect of osmotic stress on fluorescence emission from TMA-DPH incorporated into pea mesophyll protoplasts, indicating the status of plasma membrane lipids. The decrease in fluorescence emission of probe due to 1.0M sorbitol was almost immediate (within 30 sec) and did not change much with further increase in time of incubation. The fluorescence from 1.0 μM TMA-DPH incorporated into protoplast membranes in 0.4 M sorbitol medium was 12.4 ± 0.28 arbitrary units (considered as control -100%).

Table 7.1. *Fluorescence polarization of TMA-DPH incorporated into mesophyll protoplasts suspended in 0.4 M or 1.0 M sorbitol*

Measurements were made after 10 min of incubation in 0.4 or 1.0 M sorbitol at 0 °C or 25 °C.

Temperature	Polarization Value (P)		ΔP
	Sorbitol		
	0.4 M	1.0 M	
0 °C	0.352 ±0.022	0.347 ±0.018	0.005
25 °C	0.304 ±0.008	0.233 ±0.015	0.071

an increase in fluidity of plasma membrane under osmotic stress conditions. An increase in membrane fluidity of plasma membrane of protoplasts during water stress was briefly reported by Gantet et al. (1990), using fluorescence of TMA-DPH.

The fluorescence emission of DPH (on binding to protoplasts) showed a remarkable decrease (at least by 25%) due to 1.0 M sorbitol. However, this decrease in fluorescence was only during the initial phase of DPH binding, and its fluorescence emission rised slowly with time of incubation of protoplasts with DPH (Fig. 7.6), indicating that the intracellular (presumably Chloroplast, mitochondrial and other) membranes are less sensitive to osmotic stress than the plasma membrane.

Studies on photochemical activities of protoplasts (*p*-benzoquinone dependent oxygen evolution) indicated the relatively low sensitivity of Chloroplast membranes to osmotic stress. The PSII activity in protoplasts decreased by only <10% (Fig. 7.7) unlike the carbon fixation which was inhibited by >30% soon after their exposure to 1.0 M sorbitol containing medium (see Fig. 4.1 in Chapter 4). However, the inhibition of PSII activity due to osmotic stress

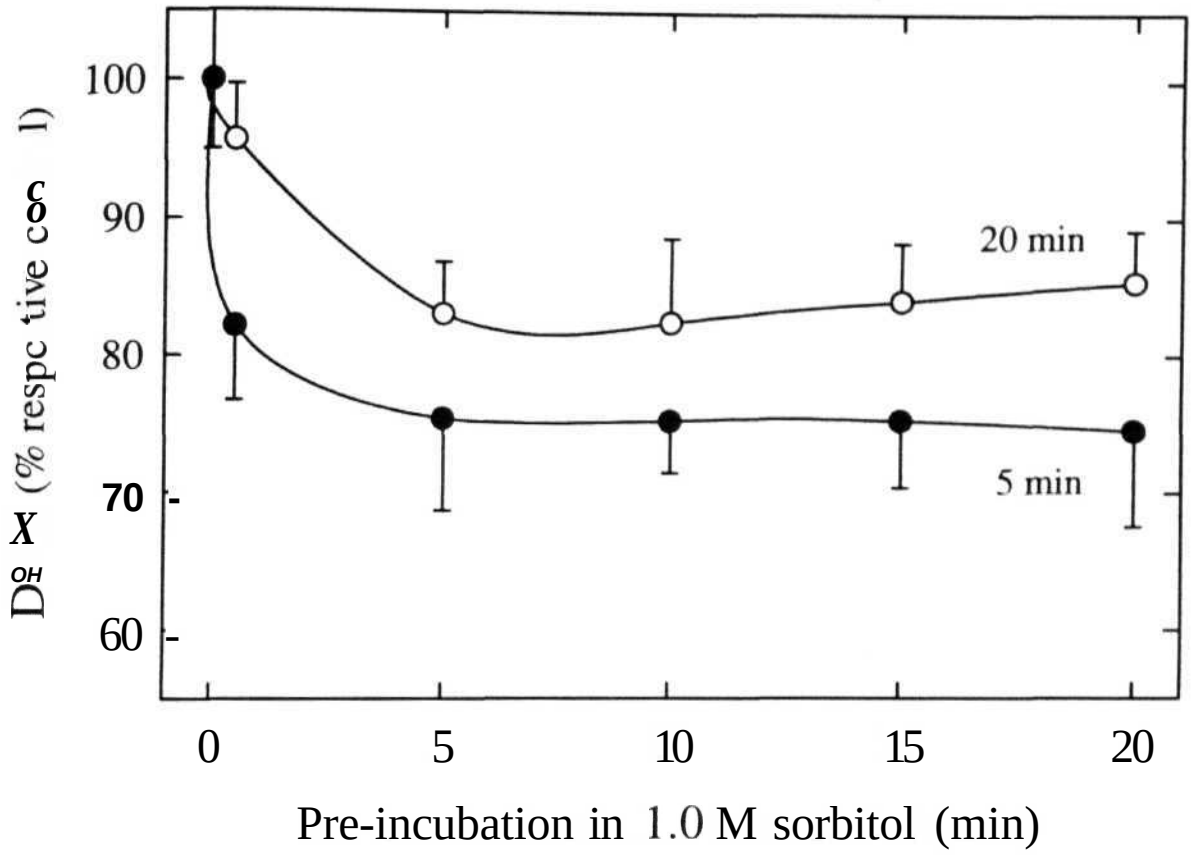

Figure 7.6. Effect of osmotic stress on fluorescence emission at 5 and 20 min respectively from DPH incorporated into pea mesophyll protoplasts indicates the status of plasma membrane and intracellular (chloroplastic, mitochondrial and other) membrane lipids. The fluorescence emission from 0.1 μM DPH and protoplasts in 0.4 M sorbitol medium were 5.0 ± 0.8 and 9 ± 1.0 arbitrary units, after 5 and 20 min of equilibration respectively (considered as control - 100%).

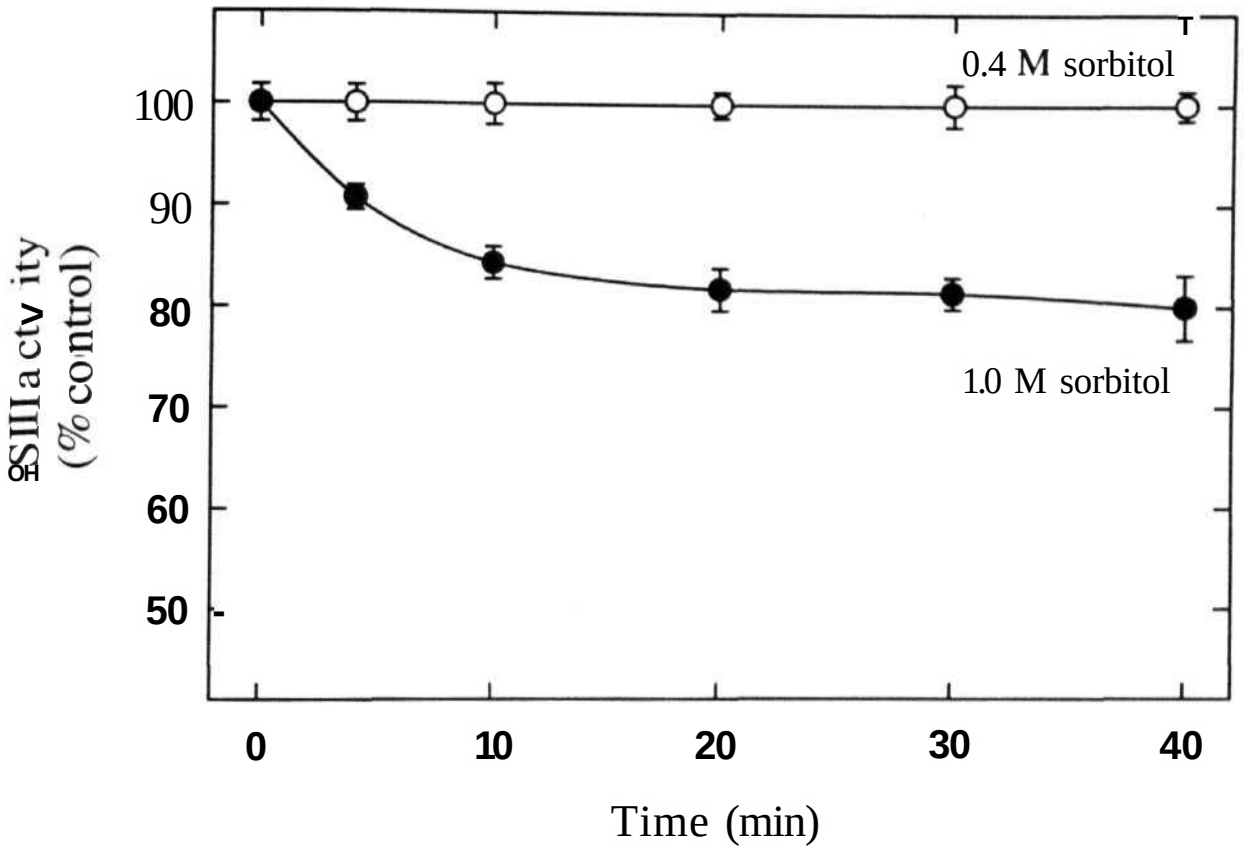

Figure 7.7. The effect of osmotic stress at 0 °C on PSII activity (*p*-benzoquinine dependent O_2 evolution) of pea mesophyll protoplasts. The rate of *p*-benzoquinone dependent O_2 evolution in 0.4 M sorbitol containing medium was 300 ± 10 μmol mg^{-1} Chl h^{-1} (control - 100%).

(1.0 M) increased from 10% to 20% with incubation for one hour in 1.0 M sorbitol. There are a few reports indicating that the Chloroplast lipids are affected by water stress (Hubac et al., 1989).

Studies on rapid responses in membrane lipids on exposure to osmotic stress are quite limited. On the other hand, there is an extensive literature on changes in lipid composition/membrane properties in leaves on exposure to water stress. Most of these studies are on long term basis as the observed changes occurred over several days. Adaptation of plants in response to perturbed environmental conditions, such as drought or chilling, is brought out (among others) by changes in membrane composition and phase behaviour in order to maintain optimal fluidity and minimize the damage to the cell (Vigh et al., 1979; Hubac et al., 1989; Wang et al., 1992; Navari-Izzo et al., 1993; Quartacci et al., 1995).

Water stress increased the apparent fluidity, particularly in the susceptible genotype, and this effect was more pronounced in the chlorophyll containing mesophyll cells than in the epidermal cells (Gantet et al., 1990). The nature and extent of such changes depend on not only the intensity and duration of stress, but also the genetically determined ability of plant to cope up with the environment (Navari-Izzo et al., 1995). Typically, a drought-resistant plant maintains its membrane integrity for a longer period during water stress.

In drought tolerant varieties and in resurrection plants, there is a decrease in MGDG and an increase in DGDG following dehydration, leading to a rise in DGDG/MGDG molar ratio (Stevanovic et al., 1992; Monteiro de Paula et al., 1993; Navari-Izzo et al., 1995). Because of the importance of MGDGs and DGDGs in the structure of thylakoid membrane, a change in their proportion is likely to alter the physical properties of thylakoids. An increase in DGDG/MGDG molar ratio, upon dehydration, may help to maintain the structure and function of the chloroplasts and thus the survival of the plant (Quinn and Williams, 1983). A shift is observed towards abundance of

unsaturated fatty acids in all lipid classes of membranes, during water stress (Navari-Izzo et al., 1995). Another factor which contributes to the reduction in membrane lipid fluidity, is the high sterol to phospholipid ratio, which occurs during long term water stress (Drory et al., 1992).

High temperature increases the fluidity of the membranes (Santarius, 1980) and low temperature provokes rigidity of cell membranes (Lyons, 1973; Shinitzky, 1984; Wang et al., 1992; Koster et al., 1994). Membranes, including the plasma membrane, are expected to contain domains having differing diffusional characteristics, due to the coexistence of patches of gel- and fluid-phase lipids in the membrane (Metcalf et al., 1986). Increase in fluidity observed at high temperatures is believed to be a result of immobile gel-phase lipids melting into fluid domains (Koster et al., 1994).

Our results are in agreement with these observations. The decrease in polarization value of TMA-DPH when protoplasts were transferred to 25 °C from 0 °C (on ice) even in iso-osmotic conditions (Table 7.1), confirms that there is an increase in fluidity of plasma membrane due to warm temperature.

Isolated protoplasts are preserved at 0 °C (on ice) to maintain their stability. Low temperatures are known to stabilize biological systems and are often used for preservation (Heber et al., 1981). Although lowering of temperature is a simple physical process, it produces several effects. The decrease in membrane fluidity (i.e. increase in rigidity) at low temperature is considered often to be a primary response to chilling (Raison and Orr, 1986; Caldwell and Whitman, 1987; Koster et al., 1994). In addition to changes in the lipid unsaturation in response to drought, there was an increase in the lipid to protein ratio in thylakoids of a wheat cultivar Ofanto (Quartacci et al., 1995). Such an increase in lipid to protein ratio may rise the fluidity of membranes (Chapman et al., 1983).

Besides lipids, proteins constitute nearly half of all biological membranes. Naturally, membrane-proteins can be also expected to be affected by stress conditions. Further, the organization of proteins in a biological membrane will depend on the interaction of proteins with other components, such as lipids or even proteins themselves (Munkonge et al., 1988). Within the thylakoids, the membrane lipids have an important role to play in integrating and stabilizing the protein complexes (Li et al., 1989). Pick et al. (1985) suggested a tight association between sulfoquinovosyldiacylglycerol (SQDG) and the protein-translocating CF_0-CF_1 (ATP synthase) complex. A nonspecific association between MGDG and the light harvesting complex of PSII was found by Quinn and Williams (1983). Thus, alterations in bulk membrane lipids, caused by dehydration or chilling, perturb the function of several intrinsic membrane protein complexes (Caldwell and Whitman, 1987; Koster et al., 1994) and also impair the activity of ATP synthase (Wright et al., 1982; Meyer et al., 1992).

In literature, stress-induced changes in expression and/or catalytic activity of proteins (including several enzymes) have been extensively studied, but very little attention has been paid towards the conformational changes of membrane proteins (particularly under water stress). Therefore, we have studied the over-all conformational changes in proteins of protoplasts exposed to osmotic and/or chilling stress, using FITC as a fluorescent probe. Conventionally, FITC has been used to study the conformational changes of proteins in plant as well as animal systems, e.g. the organization of the (Ca^{2+}-Mg^{2+})-ATPase studied in reconstituted systems (Munkonge et al., 1988) and conformational changes in the sodium-glucose cotransporter of rabbit intestine brush border (Peerce and Wright, 1984).

The fluorescence of FITC gets quenched on binding to proteins (Haugland, 1992). Osmotic stress had no effect on extent of binding of FITC to

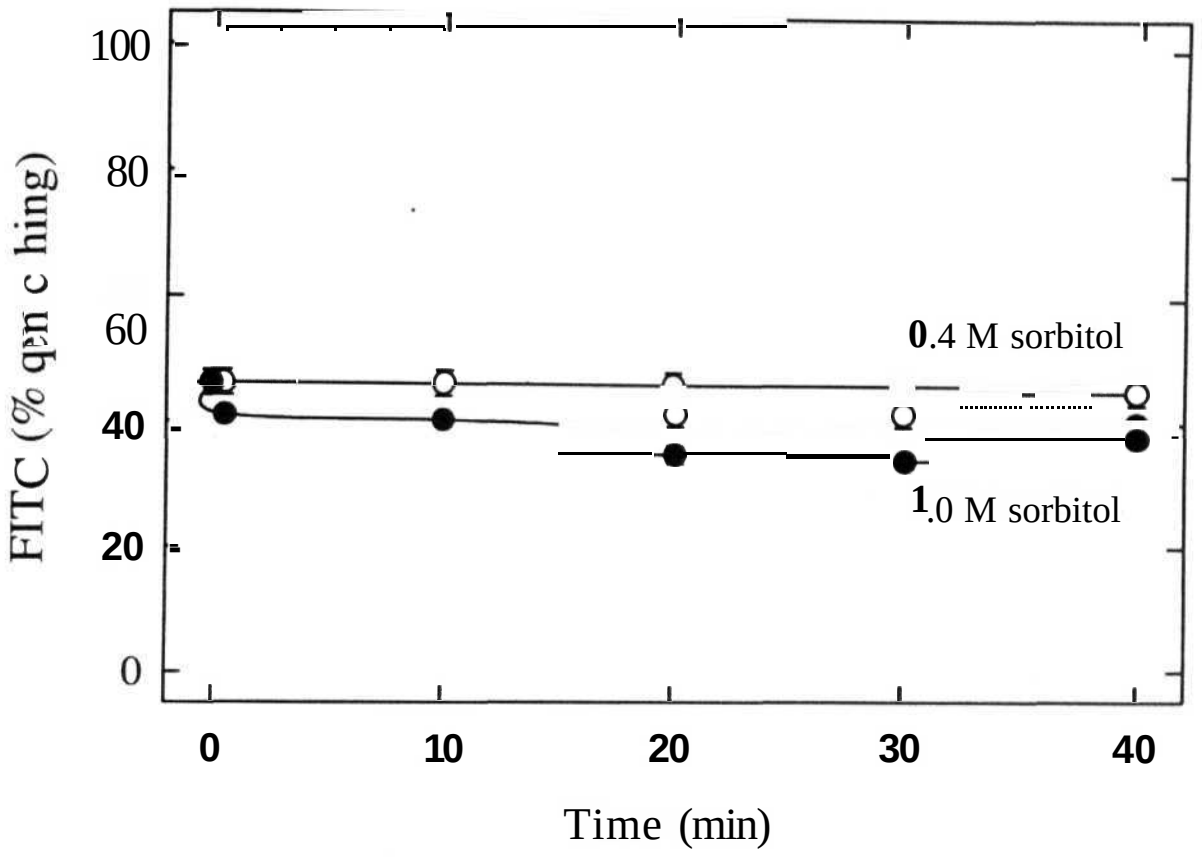

Figure 7.8. Negligible effect of osmotic stress on the extent of FITC (100 ng ml^{-1}) binding to membranes in pea mesophyll protoplasts as indicated by the quenching of FITC fluorescence. The fluorescence of FITC was quenched by 46 ± 1% on addition of protoplasts in 0.4 M medium. The quenching was immediate and the extent of quenching in 1.0 M sorbitol was almost similar to that in 0.4 M. The fluorescence emission of 100 ng ml^{-1} FITC in buffer alone was 1000 ± 28 arbitrary units (considered as control - 100%)

Table 7.2. *Fluorescence polarization of FITC incorporated into mesophyll protoplasts suspended in 0.4 M or 1.0 M sorbitol*

Measurements were made after 10 min of incubation in 0.4 or 1.0 M sorbitol at 0 °C or 25 °C.

Temperature	Polarization Value (P)		ΔP
	Sorbitol		
	0.4 M	1.0 M	
0°C	0.046 ±0.007	0.019 ±0.003	0.027
25 °C	0.025 ±0.003	0.016 ±0.001	0.009

protoplast proteins, since the quenching of FITC fluorescence decreased only by 5%, even after 40 min of exposure to osmotic stress (Fig. 7.8). However, there was a marked change in the conformation of proteins as reflected in their 'P' values (Table 7.2). These observations suggest that while there are no significant changes in the surface properties, the conformational status of proteins within membranes is markedly altered on exposure to water stress, particularly at 0 °C. The change in polarization values of FITC may also reflect the mobility of proteins. But it is difficult to draw final conclusions, due to the non-availability of relevant literature on this aspect. Biomembranes represent a higher organizational level than soluble proteins and their response to low water potentials and/or accompanying low temperature is complex (Heber et al., 1981).

Thus, the present results demonstrate marked changes due to osmotic stress in not only the lipid components, but also the proteins of plasma membrane in protoplasts. Changes in lipids are indicated by the changes in extent and polarization of TMA-DPH flourescence. These changes in plasma membrane lipids are much stronger than that in lipids of other compartments (as indicated by the use of DPH). The changes in the external

(superficial) properties of proteins in protoplast membrane as indicated by **the** quenching of FITC-fluorescence were quite subtle and were not much discernible during osmotic stress. However the changes in protein conformation within membranes during osmotic stress (as indicated by 'P' values) were as marked as those in lipids.

This is the first report documenting marked changes of protein conformation within protoplast membranes during osmotic stress. Our results also suggest that the system of protoplasts is quite convenient to handle, and can be used to study changes in membrane properties such as fluidity, in response to osmotic and/or chilling stress.

Chapter 8

Membrane Peroxidation, Thiols and Superoxide Scavenging Enzymes during Osmotic Stress and/or Photoinhibition

Chapter 8

Membrane Peroxidation, Thiols and Superoxide Scavenging Enzymes during Osmotic Stress and/or Photoinhibition

Peroxidation of membrane lipids and an increase in thiols are among the common responses of plant tissues to temperature stress or excess light (Asada and Takahashi, 1987; Mishra and Singhal, 1992; Sharma and Singhal, 1992; Tijskens et al., 1994). This chapter attempts to assess some of these responses to osmotic stress or photoinhibitory light or both. A major objective to assess and identify the exclusive response, if any, of these events to each type of stress in mesophyll protoplasts.

Lipid peroxidation, which involves oxidative degradation of polyunsaturated lipids occurs in plant tissues during adverse environmental conditions such as water stress (Dhindsa and Matowe, 1981; Leprince et al., 1990; Moran et al., 1994; Van Rensburg and Krüger, 1994) or photoinhibition of leaves (Asada and Takahashi, 1987; Robinson, 1988; Vaughn et al., 1988; Chauhan et al., 1992; Mishra and Singhal, 1992). Chloroplasts of leaf cells are at particular risk due to its high internal O_2 concentration and highly unsaturated fattyacid composition of thylakoids (Van Rensburg and Krüger, 1994).

Our results demonstrate peroxidation of protoplast membrane lipids, under stress, but the degree of response depended on the type of stress: osmotic stress or excess light (photoinhibition). There was an increase in the extent of lipid peroxidation under osmotic stress as well as photoinhibitory light, irrespective of temperature (0 °C or 25 °C). The extent of peroxidation of membrane lipids increased on exposure to osmotic stress but such increase was much more pronounced under photoinhibitory conditions. The lipid peroxidation increased by only 20% even after 40 min of incubation in hyper-osmotic medium while the peroxidation increased by >100% after 20 min of

exposure to photoinhibitory light (Fig. 8.1). When the osmotic stress was imposed at 0 °C in darkness, the extent of membrane lipid peroxidation increased marginally by 15%, even after 30 min of pre-incubation of protoplasts in 1.0 M sorbitol (data not shown). The enhancement of lipid peroxidation under photoinhibitory light was more pronounced in 1.0 M sorbitol than that in 0.4 M (Fig. 8.1).

The aggravation of lipid peroxidation under drought conditions in pea plants has recently been reported by Moran et al. (1994). Water stress restricts carbon fixation in leaf tissues but photosynthetic electron transport, however, is relatively unaffected (Kaiser, 1987; Saradadevi and Raghavendra, 1994). This imbalance between electron transport and carbon fixation rates may result in a over-reduction of electron transport chain. Under these conditions, the excess of excited electrons may move towards O_2, resulting the formation of superoxide, peroxide and hydroxyl radicals, which cause membrane damage (Moran et al., 1994).

There is evidence that in plant tissues drought stress can generate the $O_2^{\cdot-}$ which is converted to H_2O_2 by SOD. The increasing level of H_2O_2 in water stressed tissue has been found to be a function of increasing magnitude of water stress. An interaction between $O_2^{\cdot-}$ and H_2O_2 may also generate $^1O_2$ and $^{\cdot}OH$, which are cytotoxic (Van Rensburg and Krüger, 1994). Our results, however, demonstrate that osmotic stress alone does not cause much membrane lipid peroxidation, but it amplifies the susceptibility of plant tissue to oxidative damage under excess light.

A significant increase in the level of protein- and non-protein thiols was observed in protoplasts, when exposed to stress. However, there were discernible differences between protein- and non-protein thiols, in their responses to osmotic stress and/or photoinhibition. The level of protein thiols was raised by 40% (in 0.4 M sorbitol) or 50% (in 1.0 M sorbitol) within

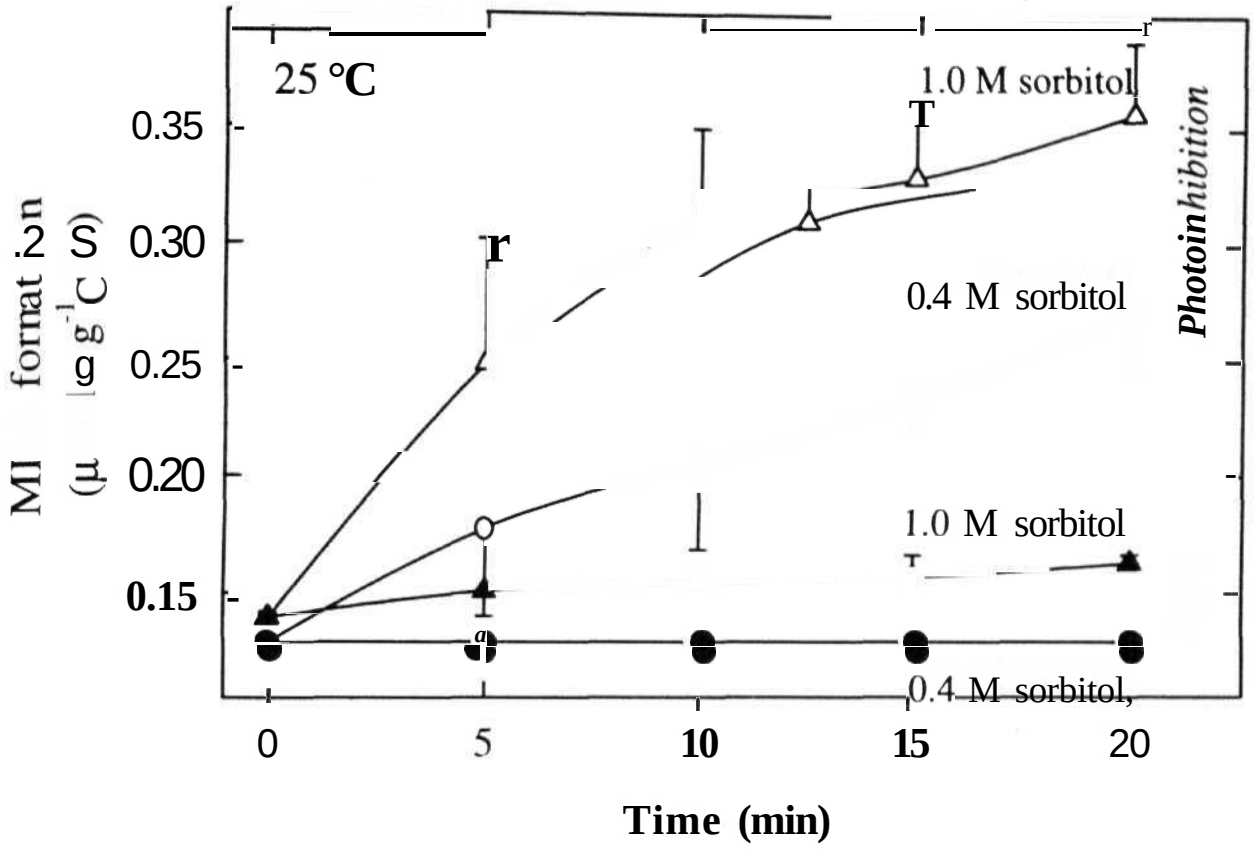

Figure 8.1. Lipid peroxidation of membranes in mesophyll protoplasts suspended in 0.4 M or 1.0 M sorbitol and either kept in darkness or exposed to photoinhibitory light. All the treatments were at 25 °C. Peroxidation of protoplast membranes increased slightly due to osmotic stress but rised remarkably under photoinhibitory light (3000 μE m^{-2} s^{-1}). Peroxidation increased further when protoplasts were exposed to photoinhibitory light in 1.0 M sorbitol.

10 min of protoplasts exposure to photoinhibitory light and was further increased to 70% when they were exposed for 20 min (Fig. 8.2).

We have also exposed protoplasts to osmotic stress in darkness, to avoid the interfering problem of light induced membrane damage. The increase in protein thiols level was negligible and was only 5-7%, when protoplasts were incubated in 1.0 M sorbitol at 25 °C (Fig. 8.2). Osmotic stress in darkness, (even in combination with chilling) could not elicit much increase in the level of protein thiols in mesophyll protoplasts of pea. Thus, the protein thiols increased only when mesophyll protoplasts were exposed to photoinhibitory light (either in 0.4 M or 1.0 M sorbitol), while such increase in response to osmotic stress was much limited.

Non-protein thiol content of protoplasts also increased, particularly under photoinhibitory conditions. On exposure to osmotic stress (i.e. in presence of 1.0 M sorbitol in stead of 0.4 M sorbitol), the non-protein thiols were increased by >30% within 5 min and 75% after 30 min, at 25 °C (Fig. 8.3). However, the increase in non-protein thiols was much more pronounced when protoplasts were exposed to excess-light in 0.4 M sorbitol, the increase being >50% after 5 min and 90% after 20 min illumination of protoplasts with photoinhibitory light. Further, the level of non-protein thiols in protoplasts rised by 130% (over dark controls) when they were photoinhibited (for 20 min) in hyper-osmotic (1.0 M) medium (Fig. 8.3).

We have also checked the effect of osmotic stress, in darkness at chilling temperature (0 °C) on content of both protein and non-protein thiols. There was very little increase in protein thiols under osmotic stress at 0 °C in darkness (Fig. 8.4A). On the other hand, the rise in non-protein thiols on exposure to osmotic stress (in darkness) was more pronounced at 0 °C (Fig. 8.4B) than that at 25 °C (Fig. 8.2). The non-protein thiols increased by 40% within 5 min and further increased to 85% after 30 min of incubation of protoplasts in 1.0 M sorbitol at 0 °C (Fig. 8.4B).

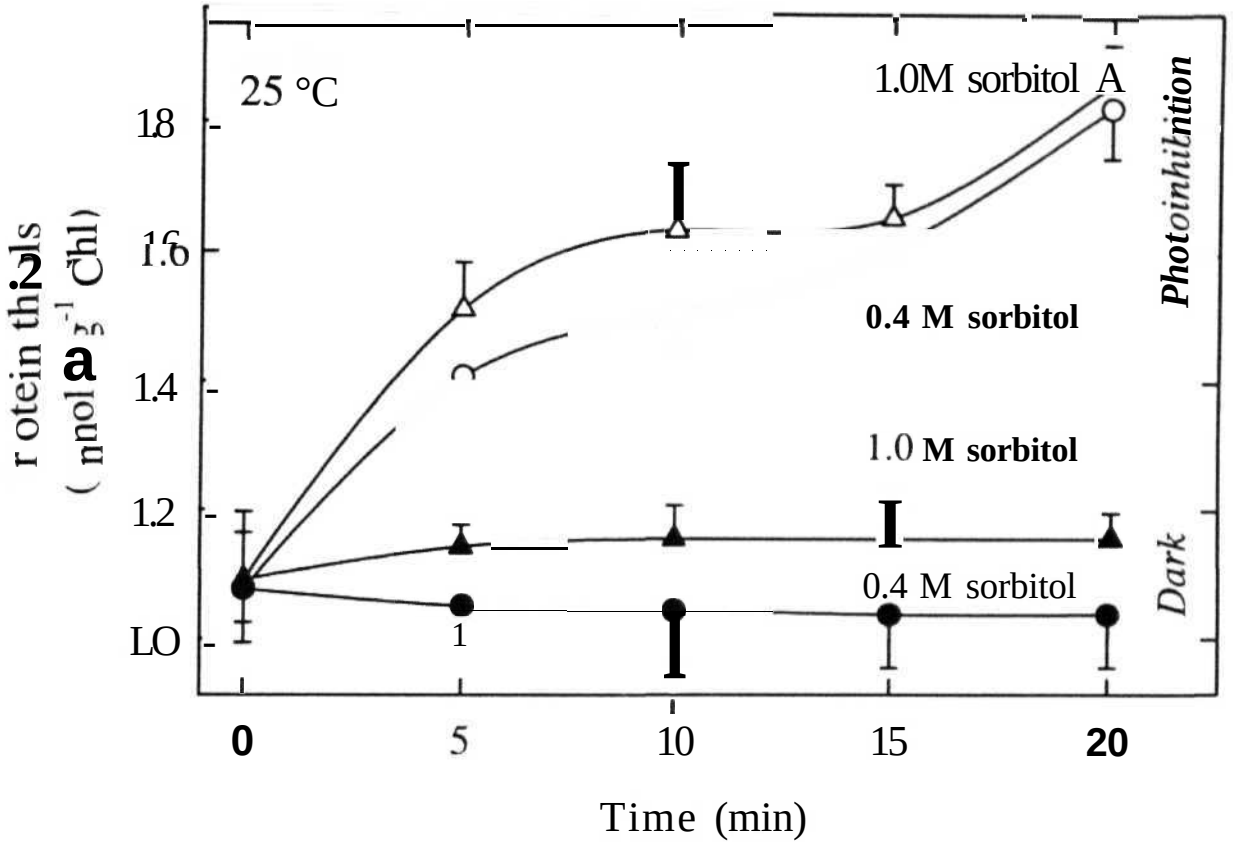

Figure 8.2. Effect of osmotic stress and/or photoinhibitory light (3000 μE m^{-2} s^{-1}) on the levels of protein thiols in mesophyll protoplasts, at 25 °C. Protein thiol content increased dramatically under photoinhibitory conditions, but responded marginally to osmotic stress (i.e. 1.0 M sorbitol).

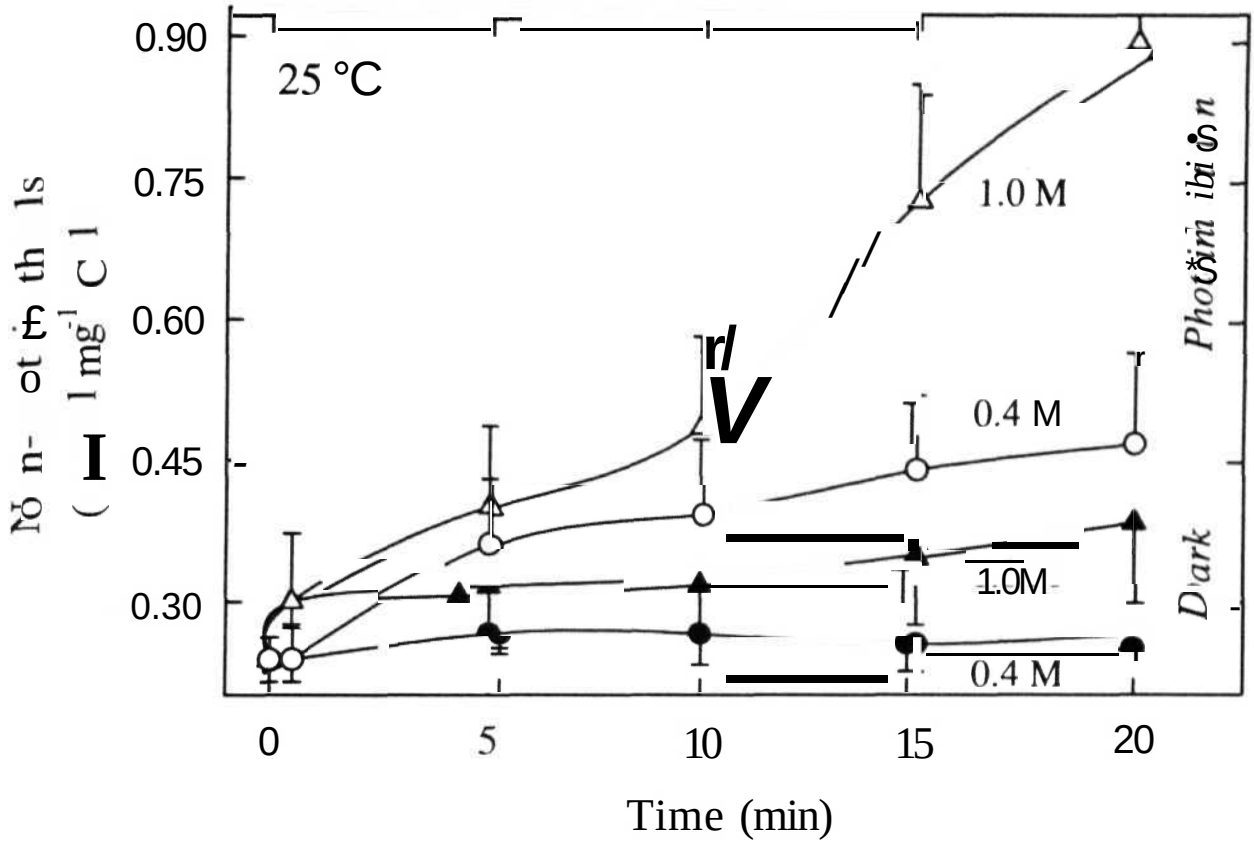

Figure 8.3. Effect of osmotic stress and/or photoinhibitory light (3000 μE m^{-2} s^{-1}) on non-protein thiols in mesophyll protoplasts, at 25 °C. Non-protein thiol content increased particularly under photoinhibitory conditions. There was a lag in the response up to 10 min. Only a marginal increase occurred in non-protein thiols when protoplasts were exposed to osmotic stress alone (i.e. 1.0 M sorbitol containing medium).

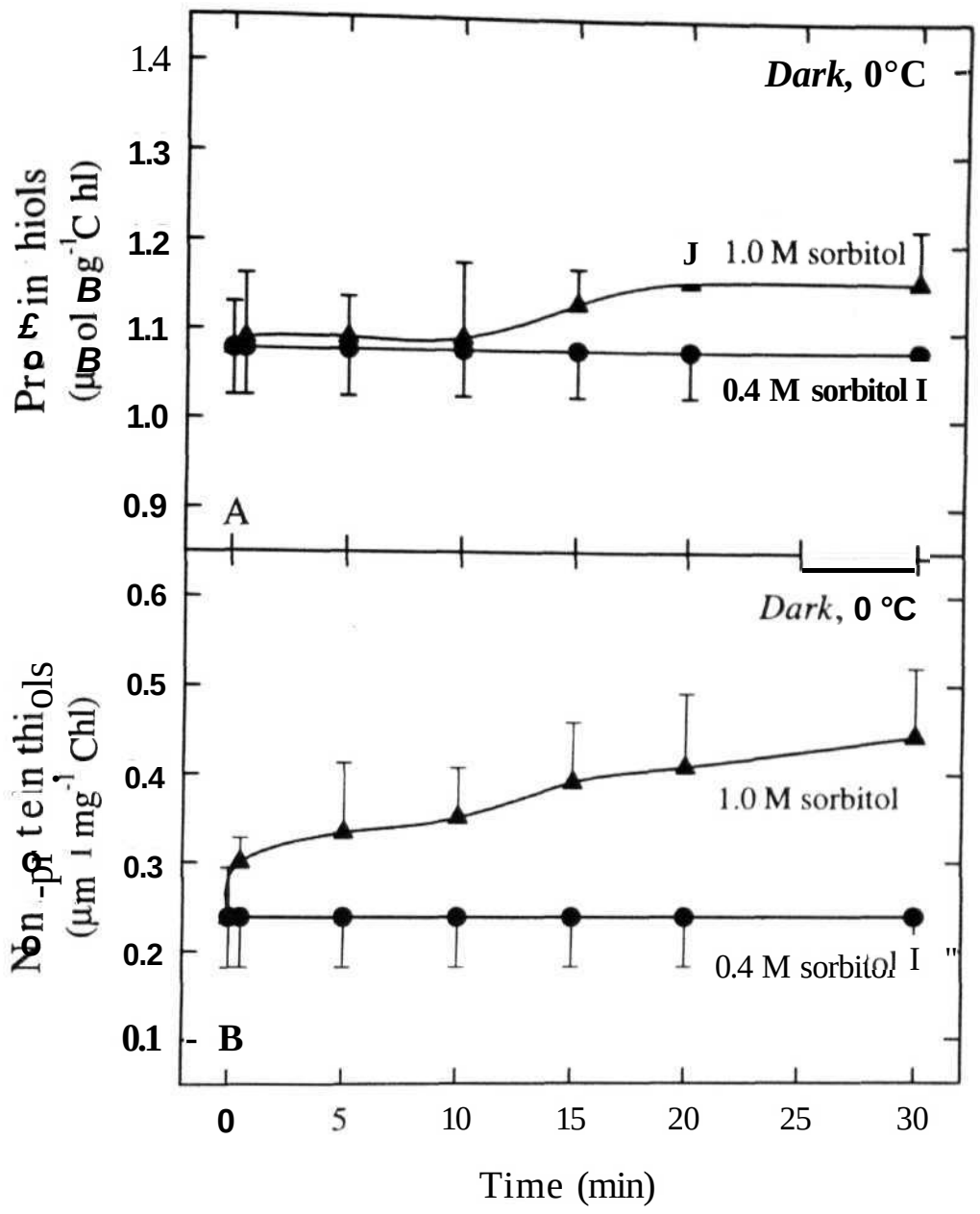

Figure 8.4. Effect of osmotic stress alone on protein or non-protein thiols in mesophyll protoplasts, kept in darkness at 0 °C. Incubation in 1.0 M sorbitol at 0 °C is expected to simulate osmotic stress at chilling temperature. A: Marginal increase (<10%) in protein thiol content due to osmotic stress at 0 °C, even after 30 min of incubation of protoplasts in 1.0 M sorbitol. B: There was a significant increase (>80%) in the content of non-protein thiols, when protoplasts incubated in 1.0 M sorbitol, at 0 °C.

The marked increase in the level of protein as well as non-protein thiols suggest that the thiols may play a significant role in defence system of a cell during stress. The major component of non-protein thiols is assumed to be glutathione (Cakmak and Marschner, 1992). GSH is an anti-oxidative agent which helps in removal of toxic oxygen species, produced during stress conditions (Asada and Takahashi 1987). Several enzymes lose activity due to the oxidation of essential sulfhydryl groups by activated O_2 species (Van Rensburg and Krüger, 1994). GSH can protect enzymes from such oxidative damage, being more accessible for reaction with O_2 than the thiol groups of enzyme. Further, GSH can activate/reactivate enzymes by reducing the oxidised sulfhydryl groups in the proteins (Burke et al., 1985).

Osmotic stress and supra-optimal light lead to an inevitable increase in superoxide and reactive oxygen radicals, particularly in chloroplasts. However, the plants are also adapted to minimise the damage which could occur during stress conditions (Mishra et al., 1993). Oxidative damage ensues only when the formation of free radicals due to unfavourable conditions exceeds the defensive mechanisms of the cell (Takahami and Nishimura, 1975; Wise and Naylor, 1987; Moran et al., 1994).

Active form of oxygen, superoxide anion radical ($O_2^{\cdot-}$) formed due to photoreduction of molecular oxygen in chloroplasts is dismuted by the enzyme SOD (Asada and Takahashi, 1987). Dismutation of $O_2^{\cdot-}$ results in the formation of H_2O_2, another toxic oxygen species, which inactivates several enzymes, although H_2O_2 is less reactive than $O_2^{\cdot-}$ (Asada and Takahashi, 1987). So the rapid removal of H_2O_2 is very important. H_2O_2 is scavenged by ascorbate-glutathione cycle which involves AsA, GSH, APX and GR (Foyer and Halliwell 1976; Foyer et al., 1994).

APX and GR are very important defensive (anti-oxidative) enzymes present in Chloroplast stroma and increase under stress conditions (Gillham and Dodge, 1986; Asada and Takahashi 1987; Cakmak and Marschner 1992). The

major peroxidase in leaf tissue is APX. APX is highly specific in its affinity to its electron donor AsA, where as GPX can oxidise both guaiacol and pyrogallol. The major peroxidase in leaf tissue is APX. In contrast, GPX activity is much higher in roots than that in leaf tissues and no GPX activity was detected in chloroplasts (Amako et al., 1994).

The activity of APX increased on exposure of protoplasts to photoinhibitory light, under both iso-osmotic and hyper-osmotic conditions,but not when protoplasts were in darkness. The enzyme activity increased by 4.5 fold on exposure of protoplasts to photoinhibitory light for 20 min. The activity of APX increased further, by six fold, when the protoplasts were in photoinhibitory light for 30 min in 0.4 M sorbitol (Fig. 8.5).

Activity of guaiacol peroxidase (GPX) increased during photoinhibition under iso-osmotic or hyper-osmotic conditions. The enzyme activity increased by nearly 50% after exposure of protoplasts to photoinhibitory light in 0.4 M sorbitol, but such increase was slightly subdued, being 35% in 1.0 M sorbitol (Table 8.1). Indeed, the activity of GPX slightly increased (by about 15%), when osmotic stress was imposed on protoplasts kept in darkness at 0 ^{o}C. However the increase in activity of GPX was not as high as APX. Nakano and Asada (1981) observed that the H_2O_2 scavenging peroxidase localised in chloroplasts is highly specific for ascorbate and has much less affinity for other electron donors such as guaiacol. By contrast, the non-chloroplastic peroxidases are less specific for ascorbate. Therefore our results suggest that the enhancement of H_2O_2 scavenging peroxidase activity under photoinhibitory conditions predominantly operates in chloroplasts.

The activity of GR also increased, only when protoplasts were exposed to photoinhibitory light either in 0.4 M sorbitol (by 2.8 fold) or 1.0 M sorbitol (2.2 fold) but not during osmotic stress alone in darkness (Table 8.1). However, when protoplasts were incubated 1.0 M sorbitol at 0 °C, GR activity increased by 25% (data not shown). Increase in activities of GR during stress is

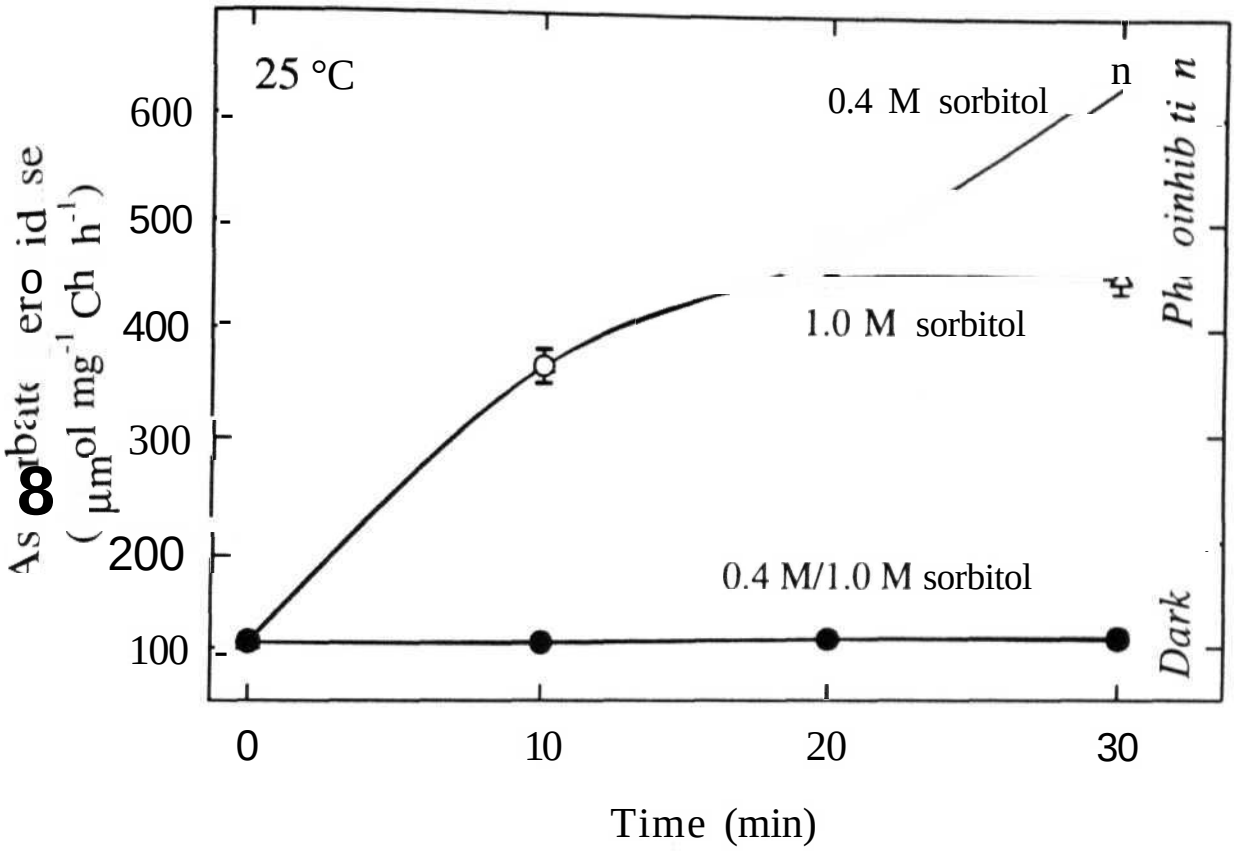

Figure 8.5. Effect of osmotic stress and/or photoinhibitory light on activity of ascorbate peroxidase (APX) in mesophyll protoplasts. Osmotic stress alone had no effect but the activity of APX increased markedly when protoplasts were exposed to photoinhibitory light (3000 μE m^{-2} s^{-1}) in either 0.4 M or 1.0 M sorbitol medium, at 25 °C.

Table 8.1. *Effect of osmotic stress and/or photoinhib it ion on the activities of guaiacol per oxidase and glutathione reductase in mesophyll protoplasts*

The en2yme activity in mesophyll protoplasts of pea was determined after imposition of osmotic stress in dark or after exposure of protoplasts, suspended in either iso-osmotic (0.4 M sorbitol) or hyper-osmotic (1.0 M sorbitol) medium to photoinhibitory light for 10 min at 25 °C.

Enzyme/ Osmotic medium	Enzyme activity		Activity after photoinhbitory treatment
	Dark	Photoinhibitory light*	
	mmol mg^{-1} Chl h^{-1}		*% respective dark treatment*
Guaiacol peroxidase (tetraguaiacol formation)			
0.4 M sorbitol	1488 ±75	2110 ± 84	149
1.0 M sorbitol	1390 ± 51	1869± 135	135
Glutathione Reductase (NADPH oxidation)			
0.4 M sorbitol	63 ±4	180 ± 18	286
1.0 M sorbitol	61 ± 5	132 ± 14	216

* At an intensity of 3000 $\mu E\ m^{-2}\ s^{-1}$.

often observed (Foyer et al., 1991; Burke et al., 1985).

It is difficult to relate directly the present observations made on protoplasts with earlier literature, mostly on leaves. The activities of APX and GR in leaves were reported to increase under water stress conditions (Burke et al., 1985; Van Rensburg and Krüger, 1994). In most of these studies, made under field conditions, the stress was imposed on intact plants. Therefore, the water stressed plants along with control plants, are usually exposed to light during day/night cycles. The illumination of the plants under water deficit

could probably **the** reason for increased activities of these scavenging enzymes. Baisak et al. (1994) have reported that water stress induces an increase in the activities of APX and GR in wheat leaf segments, with illumination during stress treatment. In our results, osmotic stress alone (i.e. protoplasts subjected to osmotic stress in darkness) did not cause any increase in activities of APX or GR but enzymes responded well when the protoplasts exposed to osmotic stress under photoinhibitory conditions (Fig. 8.5; Table 8.1).

Catalase is also one of the important enzymes which scavenge H_2O_2. Although the enzyme is absent in chloroplasts, H_2O_2 can diffuse across the membranes and gets reduced by catalase. Activity of catalase increased immediately by >85% within 5 min of protoplast exposure to photoinhibitory light in 0.4 M sorbitol (Fig. 8.6). But catalase activity declined with prolonged exposure of protoplasts to photoinhibitory light at 25 ^{o}C. Only 90% of the control activity was retained when protoplasts were exposed for 20 min. Such loss of enzyme activity with time of incubation, may be due to its well-known sensitivity of the enzyme to light. Catalase is light sensitive and suffers from photoinactivation (Feierabend et al., 1992; Mishra et al., 1993).

Catalase is a highly dynamic protein like D1 protein of PSII and undergoes rapid turnover, particularly in light. When degradation in light exceeds the capacity for repair, an apparent loss of protein is observed (Feierabend et al., 1992; Streb et al., 1993). Sensitivity of the enzyme is proportional to the light intensity applied (Streb et al., 1993). Decline in catalase activity can be induced at even moderate light intensity (to which the plants are generally adapted) when repair mechanism is suppressed by factors like osmotic- or temperature stress (Hertwig et al., 1992; Streb et al., 1993). Our results are in agreement and endorse the above observations.

Unlike the decrease in enzyme activity in protoplasts exposed to photoinhibitory light, the activity of catalase increased when the protoplasts were incubated in 1.0 M sorbitol at 0 °C (Fig. 8.7). The increase was quite

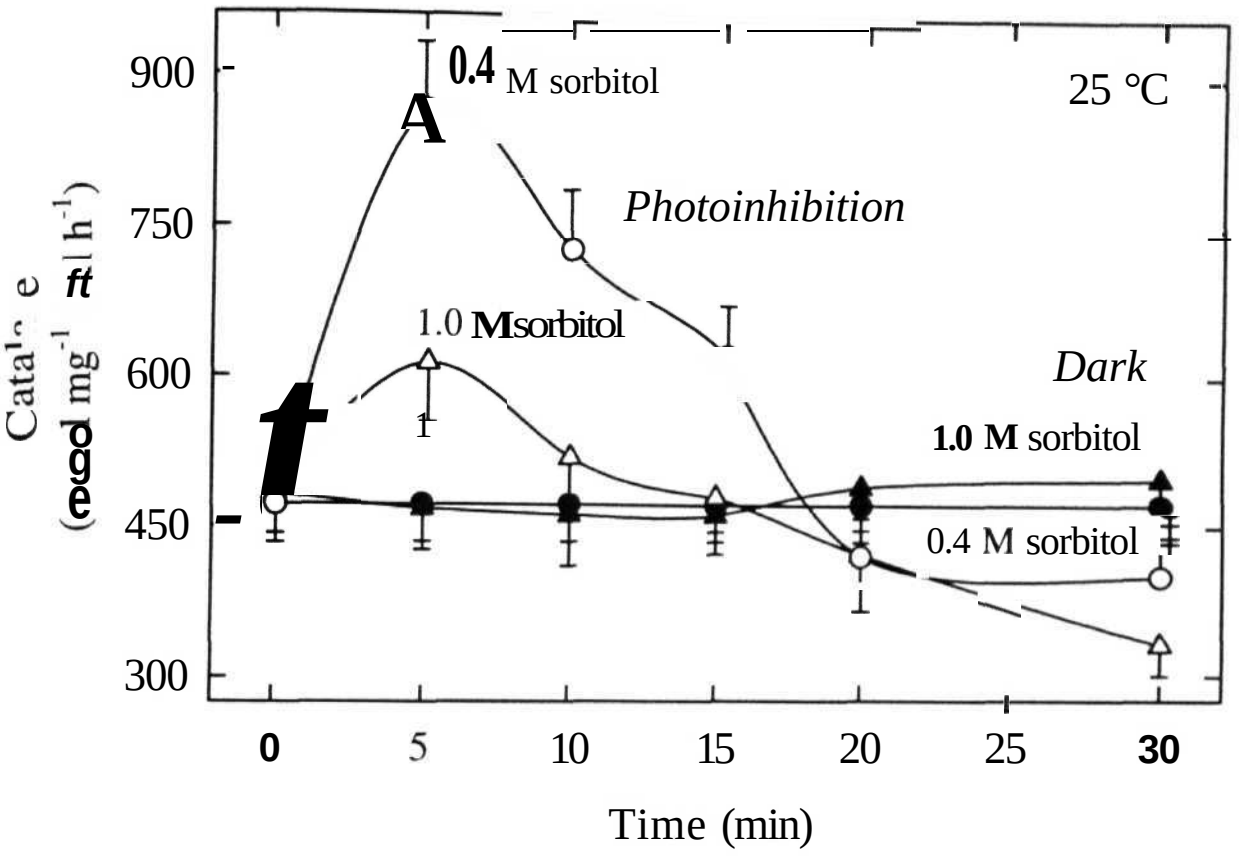

Figure 8.6. Effect of osmotic stress and/or photoinhibitory light on the activity of catalase in mesophyll protoplasts. There was not much change in the activity of catalase when protoplasts were incubated in 1.0 M sorbitol at 25 °C in darkness (closed symbols), but the activity increased remarkably within 5 min of exposure to photoinhibitory light (3000 μE m^{-2} s^{-1}) in 0.4 M or 1.0 M sorbitol (open symbols). However, the enzyme activity declined rapidly thereafter, and such decrease was pronounced in 1.0 M sorbitol.

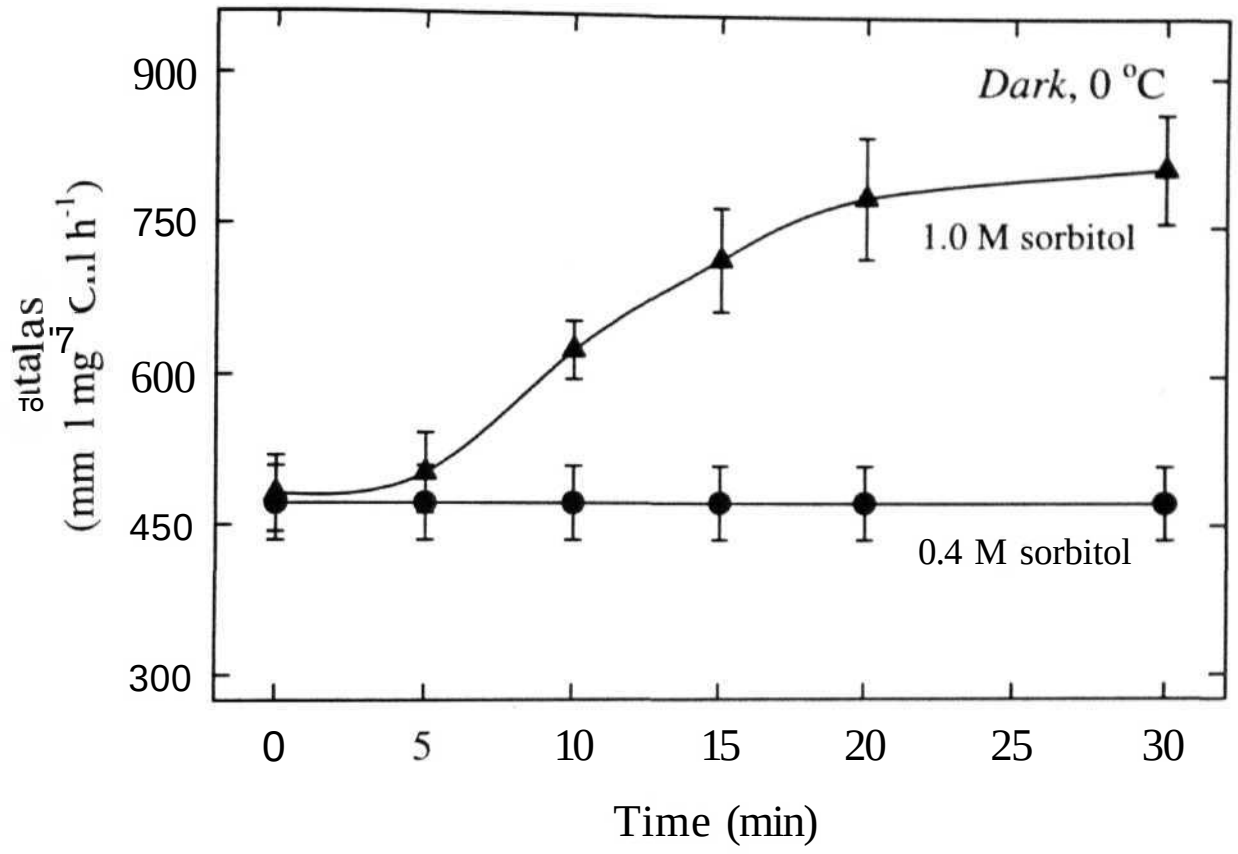

Figure 8.7. Effect of osmotic stress alone (in darkness) at 0 °C on catalase activity in mesophyll protoplasts. The activity of enzyme increased remarkably on exposure of protoplasts to osmotic stress (i.e. 1.0 M sorbitol).

marked (75% above the control), when protoplasts were incubated in 1.0 M sorbitol for 30 min at 0 °C, but not at 25 °C. These observations suggest that osmotic stress in combination with chilling temperature induces marked synthesis of catalase enzyme, even when osmotic stress was given in darkness.

From our results, we conclude that membrane damage is (as indicated by lipid peroxidation) much more severe under photoinhibitory conditions than that under osmotic stress alone (Fig. 8.1). This may be because oxygen radicals which are responsible for lipid peroxidation are generated particularly under excess light. To minimise such damage, the defence mechanisms which scavenge oxygen radicals are activated under conditions of photoinhibition. The pronounced increase in peroxide scavenging enzymes (such as ascorbate peroxidase, glutathione reductase) to photoinhibition may be partly because of the location of these enzymes in chloroplasts, the sites of light-perception. However, the extent of increase in the activity of these peroxide scavenging enzymes was subdued when protoplasts were exposed to photoinhibitory light in hyper-osmotic medium compared to the response in iso-osmotic medium. This may be one of the reasons for the high sensitivity of photosynthetic tissues (e.g. leaves) to photoinhibition in combination with osmotic stress rather than to photoinhibition alone.

At least two factors responded significantly to osmotic stress alone: non-protein thiols and catalase. However, both of them responded markedly to also photoinhibitory light. For e.g. catalase, a peroxisomal enzyme increased under both osmotic stress (particularly at chilling temperature) as well as photoinhibitory conditions. The subsequent decrease in the activity of catalase during further exposure of protoplasts to photoinhibitory light may be due to the inherent sensitivity of the enzyme to light.

Chapter 9

Summary and Conclusions

Chapter 9

Summary and Conclusions

Water stress is one of the most important stresses experienced by plants. It reduces leaf water potential and restricts photosynthesis and productivity, the problem being particularly severe in arid and semi-arid regions of the world (Jones and Corlett, 1992). The primary causes of inhibition of photosynthesis during water stress are under intense debate. The deleterious effect of water stress is frequently attributed to stomatal closure which results in depletion of CO_2 available to photosynthesising cells. But experiments with leaf slices, cells, protoplasts or chloroplasts indicated direct effects of water stress on biochemistry of photosynthesis.

The present investigation is an attempt to study the effect of osmotic stress, and its interaction with excess light or chilling temperature, particularly on the phenomena of photosynthesis (carbon assimilation/photochemical reactions), photoinhibition, dark respiration and membrane characteristics (chlorophyll fluorescence/fluidity) in mesophyll protoplasts of pea. Some of the experiments were conducted with detached leaf discs.

Osmotic stress was imposed by exposing protoplasts or leaf discs to hyper-osmotic concentrations of sorbitol, a non-permeating solute. Protoplasts were normally suspended in 0.4 M sorbitol (-1.3 MPa) containing iso-osmotic medium while their suspension in hyper-osmotic medium of 1.0 M sorbitol (-3.1 MPa) causes osmotic stress. The photosynthetic rate in mesophyll protoplasts decreased over 30%, soon after their exposure to 1.0 M sorbitol. On the other hand, PS II mediated activity was not much affected (the loss being <10%). The extent of photosynthetic inhibition in mesophyll protoplasts increased with the duration of incubation in hyper-osmotic medium. Chilling stress amplified the inhibitory effect of osmotic stress on photosynthesis.

The induction or lag phase of photosynthesis was prolnged due to 1.0 M sorbitol, which could be due to the interference with regeneration of Calvin cycle intermediates and/or decrease in light-activation of FBPase and SBPase.

Thus, our results endorse the observations made with leaf slices, isolated cells and chloroplasts which suggested that the biochemical reactions of photosynthesis are affected markedly by water stress and are non-stomatal mediated. Experiments with leaf discs also confirmed the same. Photosynthetic rates in leaf discs was quite sensitive to osmotic stress even under concentrations as high as 5% (v/v) of CO_2. The rate of photosynthesis decreased by nearly 70%, when leaf discs were incubated for 1 h in 1.0 M sorbitol.

The response of protoplast respiration to osmotic stress was intriguing. Respiration was stimulated if stress was induced at 25 °C, but was inhibited when protoplasts were subjected to osmotic stress at 0 °C.

The photosynthetic or respiratory activities of protoplasts recovered remarkably when protoplasts were transferred from hyper-osmotic medium back to iso-osmotic one. The slight loss of PS II mediated activity (as indicated by the *p*-benzoquinone depedent O_2 evolution) was completely restored after the transfer of stressed protoplasts from 1.0 M to 0.4 M sorbitol. These results demonstrate that the osmotic stress induced changes in protoplasts are reversible.

Another abiotic stress, that plants are often subjected to, is light stress. Supra-optimal light intensities cause inhibition of photosynthesis, a phenomenon known as 'photoinhibition'. It occurs *in vivo* when the photosystems absorb light in excess of their capacity of energy dissipation (Krause, 1988). The extent of photoinhibtion is amplified by water stress or chilling. For the first time, we have documented the phenomenon of photoinhibition in isolated mesophyll protoplasts.

When protoplasts were exposed to supra-optimal light of 3000 μE m^{-2} s^{-1}, for 10 min, the protoplasts lost 30 to 40% of their photosynthetic capacity with respect to dark-incubated samples at 25 °C, demonstrating that photoinhibition of photosynthesis occurred on exposure of protoplasts to strong light. The extent of photoinhibition was further amlified under osmotic/water stress conditions.

Among the components of photosynthesis, PS II reactions are the most sensitive to photoinhibition. D1 protein, an important constituent of the photosynthetic electron transport chain, is believed to be the primary target of photoinhibition (Kyle et al., 1984). This highly dynamic protein is synthesised as well as degraded continuously. Photoinhibition becomes apparent when the damage caused by light exceeds the extent of repair. Results obtained from our experiments, revealed that the non-phosphorylated D1, but not phsphorylated form of D1 protein, decreased markedly during photoinhibition. The extent of degradation of non-phosphorylated D1 protein was pronounced under photoinhibitory conditions, particularly under osmotic stress. Phosphorylation of D1 is likely to be an adaptation mechanism to protect thylakoids against photoinhibition (Rintamäki et al., 1995).

The rate of respiration was slightly stimulated when protoplasts were allowed to photosynthesize under normal light of 1250 μE m^{-2} s^{-1}, but their respiration was markedly reduced when exposed to photoinhibitory light.

Photoinhibition was remarkedly enhanced by the presence of specific respiratory inhibitors. Antimycin A, sodium azide (inhibit oxidative electron transport) and Oligomycin (oxidative phosphorylation). However, sodium fluoride (glycolysis) and sodium malonate (TCA cycle) did not have much effect on photoinhibition. These results reveal that both the oxidative electron transport and phosphorylation in mitochondria play an important role in protecting the protoplasts against photoinhibition of photosynthesis.

Recent reports establish that the oxidative metabolism in mitochondria has a beneficial and essential role in the photosynthetic process as well (Kr6mer et al., 1993; Raghavendra et al., 1994). Oxidative metabolism not only benefits photosynthesis, but also provides protection against photoinhibition in protoplasts by preventing the over-reduction of the photosynthetic electron transport chain in chloroplasts while providing an outlet for reduced equivalents to the cytosol or mitochondria.

Osmotic stress not only decreased photosynthesis of protoplasts/leaf discs, but also reduced the leaf area/protoplast volume. There was a high degree of positive correlation between the decrease of photosynthesis and the extent of reduction in volume of protoplasts/area of leaf discs. The extent of decrease in photosynthetic rates in leaf discs was positively correlated to reduction in leaf area, with a correlation coefficient (r^2) of 0.956. The correlation coefficient of photosynthetic inhibition versus reduction in volume of isolated protoplasts was as high as 0.987.

There was a marked decrease in the absorbance of protoplasts on exposure to hyper-osmotic media. The difference spectrum of osmotically stressed (suspended in 1.0 M sorbitol) against control ones (0.4 M sorbitol) presented a mirror-image of spectrum of 80% acetone extract of protoplasts, i.e. Chloroplast pigments, suggesting that most of the decrease in absorbance of protoplasts, due to osmotic stress, was accounted for by the chloroplasts. A recent study by Winter et al. (1994) revealed that besides vacuole, chloroplasts are the second largest compartment amounting to 16% of the total cellular volume in spinach leaf. As a result, the decrease in absorption of protoplasts, particularly at 440 nm (corrected for turbidity at 750 nm), indicating the contribution of mainly chloroplasts, and was highly correlated with the extent of inhibition in photosynthesis due to osmotic stress.

These results suggest that the measurement of protoplast volume (indicated by diameter or absorption at 440 nm) or the leaf disc area can be an useful criterion to assess the metabolic tolerance of cultivars to osmotic stress.

Chlorophyll fluorescence in leaves is another parameter which is known to be altered during osmotic (Ögren, 1990) or temperature stress (Burke, 1990). $(F_m-F_v)/F_v$, which indicates potential photosynthesis (Lichtenthaler, 1988) calculated from fluorescence induction curves decreased with the increase in the degree of osmotic stress imposed on leaf discs during pretreatment. The time taken to reach peak 'P' (F_m) of a dark adapted leaf disc on illumination increased along with sorbitol concentration during the pretreatment.

Changes in conformational status of the protoplast membranes due to osmotic stress were studied using different fluorescent probes, which interact specifically with either lipids: l,6-diphenyl-l,3,5-hexatriene (DPH); (1,4-trimethyl-ammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toulenesulfonate (TMA-DPH); or proteins: fluorescein isothiocyanate (FITC). TMA-DPH is used to study the changes in plasma membrane lipids while DPH reacts with all membrane lipids of protoplasts (Gantet et al., 1990). These two dyes exhibit negligible fluorescence in aqueous solution and fluoresce only when incorporated into membranes. On the other hand, the fluorescence ofFITC gets quenched, when the dye binds to the proteins in the membranes.

Our results from these experiments demonstrate that the extent of changes in lipids are more prominent in plasma membrane than those in chloroplasts or other cellular membranes. The relative insensitivity of Chloroplast lipids is also reflected in the limited effect of water stress on their PS II activity.

The extent of flourescence of TMA-DPH, which binds to plasma membrane, decreased during osmotic stress. The marked changes in the fluidity of plasma membrane lipids is further indicated by their polarization

('P') values. Osmotic stress particularly at 25 °C increased the fluidity of protoplast plasma membrane lipids as indicated by the decrease in 'P' value. Similar results were obtained during flow cytometry (Gantet et al., 1990).

Osmotic stress had no significant effect on the extent of FITC binding to protoplast proteins. However, there was a marked change in the conformation of proteins as reflected by the alteration in their 'P' values. This is the first report on a marked change in conformation of membrane proteins in mesophyll protoplasts under osmotic stress.

Under stress conditions, leaf tissue is subjected to oxidative damage. One of the typical responses of oxidative damage is lipid peroxidation (Dhindsa and Matowe, 1981, Sharma and Singhal, 1992). There was a change in the extent of peroxidation of membrane lipids of mesophyll protoplasts of pea both under osmotic stress and photoinhibitory condition. The extent of peroxidation of membrane lipids increased on exposure to osmotic stress. The extent of peroxidation was further enhanced, if the protoplasts were exposed to photoinhibitory light. The damage to lipids was thus quite pronounced under photoinhibitory conditions, possibly because of the formation of several toxic oxygen species.

There was a significant increase in the content of protein thiols and non-protein thiols under photoinhibitory conditions. Non-protein thiols increased even when osmotic stress was imposed in darkness. But protein thiols increased only under photoinhibitory conditions and not if the osmotic stress was given in darkness. Such increase in protein and non-protein thiol content in response to stress within the cell can be considered as one of the endogenous protective mechanisms against protein damage, because it may help in minimising the. consequences such as inactivation of enzymes under stress particularly under photoinhibitory conditions.

The photoreduction of molecular O_2 in chloroplasts is unavoidable during photoinhibition and leads to the formation of superoxide radicals ($O_2^{\cdot}$) and H_2O_2. As a protection against these toxic O_2 species, chloroplasts are equipped with several defensive enzymes (Cakmak and Marschner, 1992). Ascorbate peroxidase and glutathione reductase are among such enzymes, which help in scavenging H_2O_2. Otherwise H_2O_2 is quite toxic to the cell. The activity of the ascorbate peroxidase and glutathione reductase increased under photoinhibitory conditions but not with osmotic stress alone indicating that oxidative damage to lipids or proteins and production of protective enzymes is more under photoinhibitory stress than that under osmotic stress.

Catalase, an enzyme predominantly localised in peroxisomes of a cell, increased under both osmotic stress as well as photoinhibitory conditions. However, the activity of catalase rapidly declined with time of exposure of protoplasts to photoinhibitory light. The enzyme is known to be quite sensitive to light. Among the several components studied, catalase and non-protein thiols increased under even osmotic-stress alone, suggesting these components play an important role under water stress conditions.

In summary, major conclusions **from the present study are:**

1. Photosynthetic oxygen evolution of protoplasts was markedly inhibited (by 32%) soon after exposure to 1.0 M sorbitol. The extent of inhibition increased with duration of exposure (52% by 20 min) and was more at 0 °C than at 25 °C.

2. Osmotic stress prolonged the induction phase of photosynthesis, but had very little effect on PS II activity, i.e. benzoquinone dependent O_2 evolution.

3. Respiration showed intriguing responses to osmotic stress. Respiratory O_2 uptake increased if osmotic stress was imposed at 25 °C, but was inhibited at 0 °C.

4. The inhibitory effect of osmotic stress on photosynthesis or respiration was reversible.

5. Osmotic stress increased the fluidity of membranes and changed the conformation of both lipids and proteins in protoplast membranes.

6. Exposure of protoplasts to supra-optimal light intensities resulted in photoinhibition. The extent of photoinhibition was amplified by osmotic stress and/or chilling.

7. Only the non-phosphorylated D1 protein was lost during photoinhibition, while there was no marked loss in phosphorylated (inactive) Dl protein in protoplasts during photoinhibition.

8. Mitochondrial respiration, particularly oxidative electron transport/phosphorylation, was important in protecting photosynthesis against photoinhibition.

9. The marked decrease in the leaf disc area or protoplast volume (indicated by volume or absorbance at 440 nm corrected for turbidity at 750 nm) due to osmotic stress was positively correlated with the extent of photosynthetic inhibition.

10. There were marked changes due to osmotic stress in chlorophyll fluorescence induction kinetics in leaves. Osmotic stress prolonged the time to reach peak value (Fm) and decreased the (Fm - Fv)/Fv (indicates potential photosynthesis). However, the changes in chlorophyll fluorescence were not as marked as those in leaf disc area/protoplast volume.

11. Lipid peroxidation, activity of oxidative enzymes (e.g. ascorbate peroxidase and glutathione reductase) and protein thiols increased under stress. Such increase was more pronounced during photoinhibition than that under osmotic stress alone.

12. Non-protein thiols and catalase increased even under osmotic stress alone besides their marked increase under photoinhibition.

A major scientific contribution of the present work is the demonstration of the use of mesophyll protoplasts as an experimental system for several lines of study: osmotic/chilling stress, photoinhibition, dynamics of Dl protein and membrane fluidity. Another interesting point is the possible application of decrease in leaf disc area as a simple diagnostic criterion to assess drought tolerance of cultivars.

Chapter 10

Literature Cited

Chapter 10

Literature Cited

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Appendix

Appendix

List of Publications in Science Journals/Books

*❶K. **Saradadevi** and A.S. Raghavendra (1992) Dark respiration protects photosynthesis against photoinhibition in mesophyll protoplasts of **pea** (*Pisum sativum*). ***Plant Physiol*** **99:** 1232-1237

©**K. Saradadevi, K.** Padmasree and A.S. Raghavendra (1992) Interaction between respiration, photosynthesis and photoinhibition in mesophyll protoplasts of pea (*Pisum sativum).* ***Research in Photosynthesis,*** *Vol.* **4.** (Ed. N. Murata), Kluwer Academic Publishers, Dordrecht. pp. 725-728

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*©K. **Saradadevi** and A.S. Raghavendra (1994) Inhibition of photosynthesis by osmotic stress in pea *{Pisum sativum)* mesophyll protoplasts is intensified by chilling or photoinhibitory light: intriguing responses of respiration. ***Plant Cell Environ*** **17:** 739-745

*©**A.S.** Raghavendra, K. Padmasree and **K. Saradadevi** (1994) Interdependence of photosynthesis and respiration in plant cells: Interactions between chloroplasts and mitochondria. ***Plant Sci*** **97: 1-14**

*❼**K. Saradadevi,** K. Padmasree and A.S. Raghavendra (1995) Correlation between the inhibition of photosynthesis and the decrease in area of detached leaf discs or volume/absorbance of protoplasts under osmotic stress in pea *(Pisum sativum).* ***Physiol Plant*** (in press)

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Dark Respiration Protects Photosynthesis Against Photoinhibition in Mesophyll Protoplasts of Pea (*Pisum sativum*)[1]

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ABSTRACT

The optimal light intensity required for photosynthesis in mesophyll protoplasts of pea (*Pisum sativum*) is about 1250 microeinsteins per square meter per second. On exposure to supra-optimal light intensity (2500 microeinsteins per square meter per second) for 10 min, the protoplasts lost 30 to 40% of their photosynthetic capacity. Illumination with normal light intensity (1250 microeinsteins per square meter per second) for 10 min enhanced the rate of dark respiration in protoplasts. On the other hand, when protoplasts were exposed to photoinhibitory light, their dark respiration also was markedly reduced along with photosynthesis. The extent of photoinhibition was increased when protoplasts were incubated with even low concentrations of classic respiratory inhibitors: 1 micromolar antimycin A, 1 micromolar sodium azide, and 1 microgram per milliliter oligomycin. At these concentrations, the test inhibitors had very little or no effect directly on the process of photosynthetic oxygen evolution. The promotion of photoinhibition by inhibitors of oxidative electron transport (antimycin A, sodium azide) and phosphorylation (Oligomycin) was much more pronounced than that by inhibitors of glycolysis and tricarboxylic acid cycle (sodium fluoride and sodium malonate, respectively). We suggest that the oxidative electron transport and phosphorylation in mitochondria play an important role in protecting the protoplasts against photoinhibition of photosynthesis, Our results also demonstrate that protoplasts offer an additional experimental system for studies on photoinhibition.

Photoinhibition is the phenomenon of a severe reduction in photosynthetic performance under supra-optimal light intensities, particularly in the absence of CO_2 and O_2 (10, 15, 22), Photoinhibition occurs *in vivo* when the photosystems absorb light in excess of their capacity of energy dissipation (10). The susceptibility to photoinhibition is usually enhanced under environmental stresses like drought, high temperature, and chilling/freezing, or under conditions of depleted intercellular CO_2 concentration (*e.g.* see ref. 8).

The nature of photoinhibition is similar irrespective of the causal factor (14). Among the components of photosynthesis, PSII reactions are the most sensitive to photoinhibition in green algae as well as in higher plants (14, 23). Q_B protein,[2] an important constituent of the photosynthetic electron transport chain, is believed to be the primary target of photoinhibition. This highly dynamic protein is synthesized as well as degraded continuously. Photoinhibition becomes apparent when the damage caused by light exceeds the extent of repair (16, 20).

The phenomenon of photoinhibition has so far been demonstrated in leaves, algal cells, chloroplasts, and thylakoid membranes (reviewed in refs. 10, 14, 15; see also refs. 17, 30). The present report describes the phenomenon of photoinhibition in another system, namely mesophyll protoplasts of pea. Protoplasts are useful tools to examine plant metabolism because they do not have any barrier against diffusion of O_2, pose no problem of recycling of gases within the intercellular spaces, and could further allow an evaluation of the externally added compounds. The interaction between photosynthesis and respiration within a leaf tissue has been a matter of intense debate (6, 7). However, recent reports establish that there is a rapid and strong interaction between respiration and photosynthesis in plant tissue. Respiratory metabolism is beneficial for photosynthesis in the plant cell, as shown in leaves and protoplasts (12, 13, 28). The rate of dark respiration in leaves is increased after prolonged illumination and is presumed to be one of the long term effects of photosynthesis. This phenomenon, which was recently termed LEDR, is demonstrated in leaves (26) as well as in protoplasts (24), even during short periods.

The present article demonstrates that dark respiration could protect the protoplasts against photoinhibition of photosynthesis. Our results suggest that the oxidative electron transport and phosphorylation in mitochondria play a much more important role than the reactions of glycolysis or tricarboxylic acid cycle in protecting the protoplasts against photoinhibition,

MATERIALS AND METHODS

Plant Material

Plants of pea (*Pisum sativum* L. cv *Arkel*) were raised in plastic trays filled with soil and organic manure. The plants were grown outdoors under a natural photoperiod of approximately 12 h and average daily temperature of 30°C during the day and 20°C at night.

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[2] Abbreviations: Q_B protein, 32-kD herbicide binding protein of P5II that binds Q_B; LEDR light enhanced dark respiration; PCR, photosynthetic carbon reduction.

Protoplast Isolation

The mesophyll protoplasts were isolated from first and second fully unfolded leaves of 8- to 10-d-old plants, as already described (4, 28), with a few modifications. The leaf pieces stripped of their abaxial (lower) epidermis were left for 30 min in a Petri dish containing prvplasmolysis medium of 0.3 M sorbitol and 1 mM $CaCl_2$ in 10 mM Mes-KOH, pH 6.0. The strips were then subjected to digestion in a medium composed of 2% (w/v) Cellulase Onozuka R-10, 0.2% (w/v) Macerozyme R-10 (Yakult Honsha Co. Ltd., Nishinomiya, Japan), 0.25% (w/v) BSA, 10 mM sodium ascorbate, 0.4 M sorbitol, 1 mM $CaCl_2$, and 0.25 mM EDTA, in 10 mM Mes-KOH, pH 5.5. Digestion was done at 30°C for 30 min under an illumination of 50 $\mu E\ m^{-2}\ s^{-1}$.

After digestion, the medium was gently removed and a few milliliters of washing medium containing 0.4 M sorbitol and 1 mM $CaCl_2$ in 10 mM Mes-KOH, pH 6.0, were added. Tapping and swirling of the Petri dish released a large number of protoplasts into the medium. The crude protoplast suspension was filtered successively through nylon filters of pore size 300 and 60 μm and centrifuged at 50*g* for 5 min. The pellet was washed twice with the washing medium and once with suspension medium of 0.4 M sorbitol, 1 mM $CaCl_2$, and 0.5 mM MgCl.- in 10 mM Hepes-KOH, pH 7.0. The protoplast pellet was finally suspended in the above medium to give 200 $\mu g\ Chl\ mL^{-1}$ and kept on ice.

O_2 Uptake/Evolution

Respiratory O_2 uptake in the dark and photosynthetic O_2 evolution in the light by protoplasts were monitored at 30°C using a Clark type O_2 electrode (model DW'2, Hansatech Ltd., King's Lynn, UK). The reaction medium of 1 mL contained 0.4 M sorbitol, 1 mM $CaCl_2$, 1 mM MgCl., and 1 mM $NaHCO_3$ in 10 mM Hepes-KOH, pH 7.5, and protoplasts equivalent to 20 μg of Chl. Water at a constant temperature of 30°C was circulated through the outer jacket of the reaction chamber by using a refrigerated circulatory water-bath. Illumination (1250 $\mu E\ m^{-2}\ s^{-1}$) wasprovided by a 35 mm slide projector (Atul Electronics Corporation, India; lamp: Xenophot [halogen], 24 V/150 W).

Photoinhibitory Treatment

Protoplasts (in the suspension medium and containing 200 $\mu g\ Chl\ mL^{-1}$) were exposed to supra-optimal light intensity (2500 $\mu E\ m^{-2}\ s^{-1}$) provided by two tungsten bulbs (Philips, Calcutta, India: Comptalux, 225 V/75 W), one on either side of the thermo-jacketed preincubation chamber at 30°C. Additional water filters between the bulbs and preincubation chamber prevented heating. For comparison, protoplasts were exposed to normal light (1250 $\mu E\ m^{-2}\ s^{-1}$) or kept in darkness at 30°C. The protoplast suspension was gently stirred dunng incubation in photoinhibitory/normal light or darkness.

Preincubation with Respiratory Inhibitors

The respiratory inhibitors were included during preincubation at the concentrations indicated in the text. The preincubation was done in the chamber (described above), kept either in darkness or under photoinhibitory light (2500 $\mu E\ m^{-2}\ s^{-1}$). The incubation was for 10 min unless otherwise specified. An aliquot (100 μL) was taken out and examined for metabolic activities with reference to control samples. A minimum *oi* a 10-fold dilution of the preincubated samples avoided the direct effect, if any, ot the inhibitors during assays.

Peroxide Scavenging En/ymes

Catalase (EC 1.11.1.6) activity was assayed by following the disappearance of H_2O_2 at 240 nm, using an extinction coefficient of 25 $mM^{-1}\ cm^{-1}$ for H_2O_2 (27). Peroxidase (EC 1.11.1.7) was assayed using guaiacol as the substrate. The formation of tetraguaiacol was measured at 470 nm using an extinction coefficient *oi* 26.6 $mM^{-1}\ cm^{-1}$ (29). Both the enzymes were monitored in a Shimadzu UV-VIS Spectrophotometer (model UV-160A).

Other Procedures

Chl was determined after extraction into 80% (v/v) acetone (1). The experiments were repeated on different days. The data represent the averages (±SE) of results from at least three measurements.

RESULTS

The optimal light intensity required for maximal rates of photosynthetic O_2 evolution by mesophyll protoplasts of pea was 1250 $\mu E\ m^{-2}\ s^{-1}$. The maximum rates of CO_2-dependent O_2 evolution at 30°C ranged from 116 to 170 $\mu mol\ mg^{-1}\ Chl\ h^{-1}$.

The pattern ot photosynthesis by mesophyll protoplasts after exposure to photoinhibitory light was compared to those kept in either darkness or the optimal light intensity of 1250 $\mu E\ m^{-2}\ s^{-1}$. There was a slight decrease in the photosynthetic rate of protoplasts when kept in dark or normal light. On the other hand, when protoplasts were exposed to supra-optimal light of 2500 $\mu E\ m^{-2}\ s^{-1}$, their photosynthetic rates declined rapidly with time (Fig. 1). Protoplasts lost nearly 35% of their photosynthetic activity after 10 min and approximately 60% of the activity by 20 nun, with respect to dark-incubated samples, demonstrating that photoinhibition of photosynthesis occurred when protoplasts were exposed to strong light.

The rate of respiration was slightly stimulated when protoplasts were allowed to photosynthesize under normal light of 1250 $\mu E\ m^{-2}\ s^{-1}$ (Fig. 2), but their respiration was markedly reduced when exposed to photoinhibitory light. The protoplasts lost about 40% of their respiratory activity after exposure to photoinhibitory light.

The response of protoplasts to photoinhibitory light was examined in the presence of classic inhibitors known to suppress different components of respiration: antimycin A, sodium azide, Oligomycin (oxidative electron transport/phosphorylation), sodium fluoride, and sodium malonate (glycolysis/tricarboxylic acid cycle). Figure 3 presents the results of a typical experiment done with antimycin A. There was a

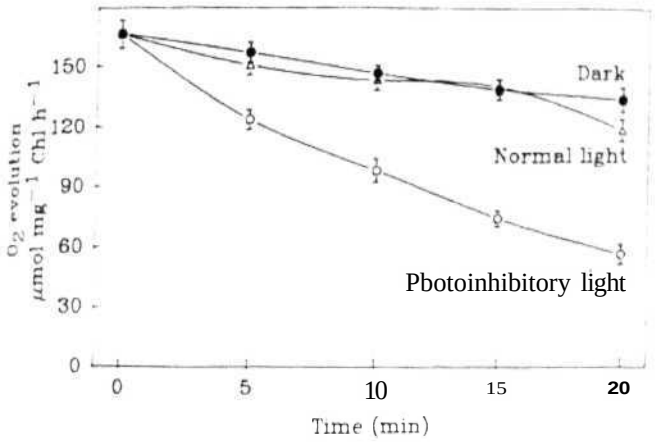

Figure 1. The rates of photosynthesis in mesophyll protoplasts at different intervals of time after incubation at 30°C in darkness or in light. When protoplasts ttcic kept in normal light intensity (1250 μE m^{-2} s^{-1}), the loss was negligible (up to 15 min) or marginal (20 min). Photosynthetic rate declined steeply on exposure to supra optimal or photoinhibitory light (2500 μE m^{-2} s^{-1}). The Chl concentration during preincubation was 200 μg Chl ml^{-1}.

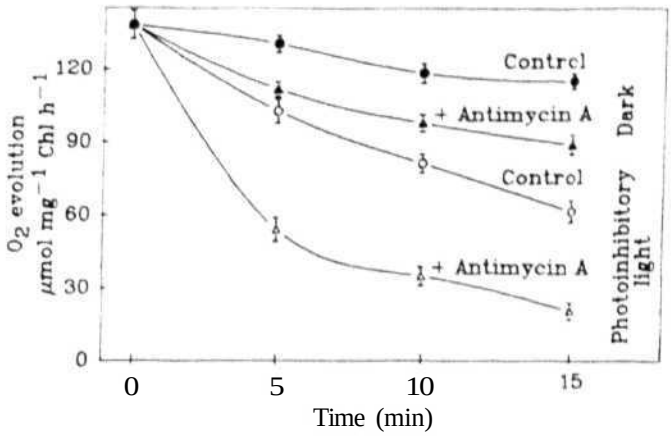

Figure 3. Effect of 1 μM antimycin A on the extent of photoinhibition of photosynthesis. Shown is the rate of photosynthesis at different intervals of time when protoplasts were preincubated without (control) or with antimycin A in either darkness or photoinhibitory light. Other details were as in Figure 1.

slight decrease in photosynthesis of protoplasts in the presence of 1 μM antimycin A after preincubation, even under darkness, but the inhibition of photosynthesis was much more marked under illumination with photoinhibitory light (Fig. 3). When the activities were expressed in relation to their respective dark controls, pronounced photoinhibition in presence of antimycin A became very clear. After even 10 min of incubation under photoinhibitory light, the protoplasts retained only about 70% of photosynthetic activity compared to the dark control, whereas the activity dropped further to 35% in presence of antimycin A. The marginal loss of activity during incubation in darkness probably resulted because the stability of protoplasts was affected after 15 min at 30°C. All the subsequent experiments were therefore limited to 10 min of exposure/incubation.

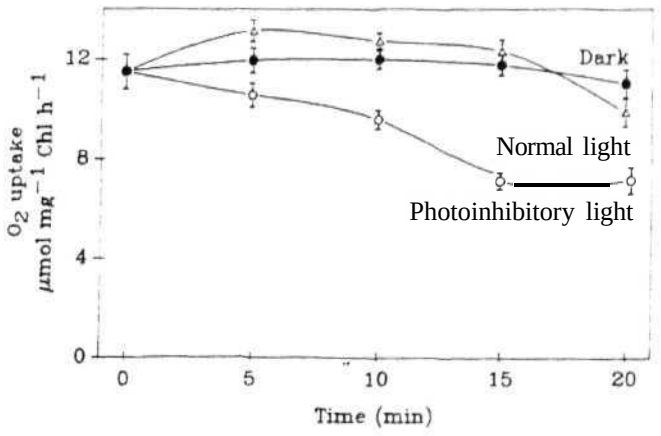

Figure 2. Inhibition of respirators oxygen uptake in mesophyll protoplasts on exposure to photoinhibitory light, compared to the samples kept in darkness or normal light. Further details were as in Figure 1.

There was already a marked effect of antimycin A by 10 min (Fig. 3). The effective concentrations of three classic respiratory inhibitors were then determined in the next set of experiments by exposing the protoplasts to photoinhibitory light or darkness for 10 min. Photoinhibition was remarkably enhanced by the presence of even 1 μM antimycin A (Fig. 4A), 1 μM sodium azide (Fig. 4B), and 1 μg ml^{-1} of Oligomycin (Fig. 4C).

These metabolic inhibitors could exert a direct inhibitory effect on photosynthesis. This aspect was checked by including the inhibitor in the assay medium while measuring photosynthesis. However, antimycin A, sodium a/ide, or Oligomycin did not affect photosynthetic O_2 evolution by protoplasts at these low concentrations (data not shown, but see Table 1 for a similar experiment).

Antimycin A, sodium a/ide, and Oligomycin inhibit oxidative electron transport and phosphorylation. The effect of two more inhibitors, sodium fluoride and sodium malonate, which interfere with glycolysis and the tricarboxylic acid cycle, respectively, were examined. However, sodium fluoride and sodium malonate did not have much effect on photoinhibition (Table I). On the other hand, antimycin A, sodium azide, or Oligomycin markedly promoted photoinhibition of photosynthesis. At these concentrations and experimental incubatory conditions, the inhibition by test compounds of the respiratory activity was quite marked and ranged from 29 to 46% (Table II).

It is possible that these respiratory inhibitors (antimycin A, sodium azide, and Oligomycin) may affect enzymes capable of scavenging H_2O_2, such as catalase or peroxidase. However, antimycin A or Oligomycin did not have any effect on catalase or peroxidase when assayed in protoplasts, even at concentrations as high as 100 μM or 100 μg mL^{-1} (data not shown). Sodium az-ide did not inhibit peroxidase, but suppressed about 15 and 40% of catalase activity at concentrations of 1 and 10 μM, respectively.

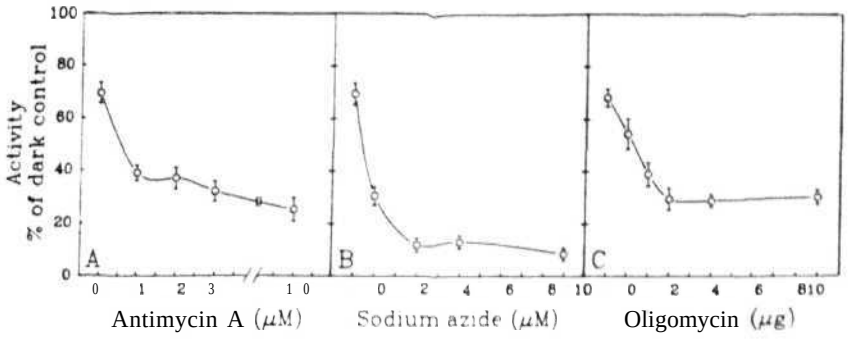

figure 4. Further promotion of photoinhibition of photosynthesis in mesopln II protoplasts In < lassic respiratory inhibitors. Protoplasts were either kept in darkness or exposed to photoinhibitory light for 10 min at 30°C. The test inhibitors (A, antimycin A; B, sodium azide; C, oligomycin) were included at the indicated concentrations. The figures represent the activity after photoinhibition compared to the corresponding sample kept in darkness. The photosynthetic activity after photoinhibition in the absence of inhibitors (indicated at zero concentration in A, B, C) ranged from 65 to 74% of that in dark incubated sample. Other details were as in Figure 1.

DISCUSSION

The present article establishes that the protoplasts can be employed to study the process of photoinhibition. There is a brief report on photoinhibition in protoplasts describing changes in fluorescence induction curves (3). Our observations further indicate that the oxidative metabolism in mitochondria plays an important role in protecting protoplasts against photoinhibition of photosynthesis.

Each experimental system, however, has its own advantages and disadvantages. For example, leaves have a nonuniform light profile between adaxial and abaxial surfaces and exhibit variation in partial pressures of CO_2/O_2 levels within intercellular spaces (14). The interaction between different organelles of a cell cannot be assessed while using chloroplasts. The system of protoplasts offers an additional tool to study the phenomenon of photoinhibition. An advantage is the possibility of assessing the interaction between organelles and testing the effect of exogenously added metabolites/inhibitors. The major disadvantage, however, is the limited stability of protoplasts, particularly at room temperature. Most of the experiments in the present report were limited to 10 min. Within this period, the loss in photosynthetic activity of pea mesophyll protoplasts was negligible (Fig. 1).

Plant systems have many defense mechanisms to minimize the damage of photoinhibition. Carbon dioxide and O_2, which are the basic substrates for photosynthesis and photorespiration, respectively, protect the plant cell against photoinhibition (11). The operation of PCR cycle facilitates a continuous supply of terminal electron acceptor of photochemical reactions (NADP) and permits a steady rate of photochemical deexcitation of reaction centers (11). In the absence of CO_2, the protection against photoinhibition is provided by photorespiratory carbon metabolism (9, 21).

Table I. *A Comparison of the Effect of Five Metabolic Inhibitors on Photosynthesis and Photoinhibition in Protoplasts*

These compounds affect different components of respiration -• sodium fluoride (inhibits glycolysis), sodium malonate (tricarboxylic acid cycle), antimycin A, sodium azide (oxidative electron transport), and oligomycin (oxidative phosphorylation). The protoplasts were examined for their photosynthetic activity after a preincubation (with or without inhibitors) for 10 min at 30°C in either darkness or photoinhibitory light.

Respiratory Inhibitor	Photosynthetic Rate after Preincubation		Effect on Photosynthesis*	Photosynthesis after Photoinhibition
	Dark	Photoinhibitory light		
	μmol O_2 evolved mg^{-1} Chl h^{-1}		% of control	% of respective dark treatment
None **(control)**	122 ± 8	78 ± 5	100	64
10 mM Sodium fluoride	117 ± 5	70 ± 4	96	60
10 mM Sodium malonate	120 ± 5	69 ± 3	98	58
1 μM Antimycin A	100 ± 4	38 ± 5	**82**	38
1 μM Sodium azide	113 ± *b*	38 ± 4	**93**	34
1 μg mL~' Oligomycin	105 ± 4	48 ± 5	80	46

•' Possible direct effect seen on incubation in darkness.

Table II. *Extent of Inhibition of Dark Respiration by Test Compounds under the Present Experimental Conditions*

The protoplasts were preincubated (with or without inhibitor) for 10 min at 30°C in darkness and were examined for their rate of respiratory oxygen uptake.

Metabolic Inhibitor	Respiratory Activity	
	Rate	Inhibition
	μmol O_2 uptake mg^{-1} (Chl) h^{-1}	% of control
None (control	8.4 ± 0.3	100
10 mM Sodium fluoride	5.9 ±1.1	70
10 mM Sodium malonate	5.0 ± 1.3	60
1 μM Antimycin A	6.0 ± 0.5	71
1 μM Sodium azide	4.5 ± 1.3	54
1 μg mL^{-1} Oligomycin	5.1 ± 1.0	61

Some of the other mechanisms that alleviate the effects of photoinhibition include the violaxanthin cycle (*e.g.* see ref. 18) and the rapid responses in turnover of D1 protein (30).

The present report reveals that dark respiration forms an additional defense mechanism to protect the leaf cells against photoinhibition. Our suggestion is based on three observations: restriction of respiration by test compounds under present experimental conditions (Table II), decrease in respiratory rates due to photoinhibitory light (Fig. 2), and marked promotion of photoinhibition, even at very low concentrations of classic inhibitors of mitochondrial metabolism (Figs. 3 and 4; Table I).

The effect of O_2 on photoinhibition is intriguing. In an excess of light, O_2 forms superoxide radicals, which are harmful to the photosynthetic apparatus (5). Leaf cells are vulnerable to photooxidative damage because they are exposed to bright light while they produce dioxygen. The leaves are equipped to suppress the production and/or to remove immediately the superoxide radicals/H_2O_2. Superoxide dismutase and catalase are among the important factors that help the plant cells in scavenging of superoxide radicals, thus avoiding further photoinhibition (reviewed in ref. 2).

Some of these inhibitors could affect the scavenging of superoxide radicals by suppressing the activities of catalase or peroxidase to make the photosynthetic system more vulnerable. Antimycin A, sodium azide, or Oligomycin do not affect catalase or peroxidase at the concentrations used during photoinhibitory treatment. Catalase is known to be inhibited by sodium azide (19), but at a much higher concentration than that required to promote photoinhibition (Fig. 4B; Table I). We do not know of any report on the inhibition of catalase/peroxidase by antimycin A or Oligomycin. Antimycin also may affect photosynthetic carbon metabolism, but again at a high concentration and at certain conditions (25). Therefore, we suggest that the promotion of photoinhibition by low concentrations of antimycin A, sodium azide, or Oligomycin is basically due to their interference with respiratory metabolism.

There are three possible factors that could facilitate respiration to protect the mesophyll protoplasts against photoinhibition. Respiratory metabolism could either (a) elevate the level of intracellular of CO_2, particularly at CO_2-limiting conditions, (b) provide extra energy toward the turnover of D1 protein required to prevent photoinhibition, or (c) help to maintain an optimal redox state in chloroplasts or cytosol of the cells. Sodium fluoride and malonate, which inhibit glycolysis and tricarboxylic acid cycle, respectively, would decrease the decarboxylation of carbon compounds but could not affect the extent of photoinhibition (Table I). Apparently, respiratory CO_2 evolution was not a major factor in modulating photoinhibition. Similarly, Oligomycin, which inhibits oxidative phosphorylation, was not as effective as sodium azide or antimycin A (inhibitors of oxidative electron transport) in enhancing the extent of photoinhibition. Therefore, we believe that oxidative electron transport in mitochondria plays a much stronger role than the oxidative phosphorylation during protection of protoplasts against photoinhibition.

Active photosynthetic carbon metabolism in light promotes respiration during the subsequent dark period. This phenomenon of LEDR is already demonstrated in leaves and protoplasts (24, 26). The decrease in respiratory oxygen uptake after photoinhibitory treatment could, therefore, be a consequence of restriction in the photosynthetic capacity during the preceding light period.

Recent reports established that the oxidative metabolism in mitochondria has a beneficial and essential role in the photosynthetic process as well (12, 13, 28). It is possible that the marginal interference by respiratory inhibitors of photosynthetic metabolism (Table II) makes protoplasts highly susceptible to photoinhibitory light. Respiration has been proposed to prevent the over-reduction of the photosynthetic electron transport chain in chloroplasts by providing an outlet for reduced equivalents to the cytosol or mitochondria (12). We suggest that oxidative metabolism protects the plant cell against photoinhibition possibly by preventing the over-reduction of electron transport chain in chloroplasts.

CONCLUSIONS

Oxidative metabolism not only benefits photosynthesis, but also provides protection against photoinhibition in protoplasts. The system of protoplasts offers an additional tool to study the phenomenon of photoinhibition.

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Inhibition of photosynthesis by osmotic stress in pea (*Pisum sativum)* mesophyll protoplasts is intensified by chilling or photoinhibitory light; intriguing responses of respiration

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ABSTRACT

The effects of reduced osmotic potential on photosynthesis and respiration were studied in mesophyll protoplasts of pea *(Pisum sativum).* Osmotic stress was induced by increasing the sorbitol concentration in the medium from 0·4 kmol m^{-3} (−1·3 MPa) to 1·0 kmol m^{-3} (-31 MPa). Protoplasts lost up to 35% of the maximum capacity of photosynthetic carbon assimilation (but not PS II mediated activity) soon after exposure to 1·0 kmol m^{-3} sorbitol. The response of protoplast respiration to osmotic stress was intriguing. Respiration was stimulated if stress was induced at 25 °C, but was inhibited when protoplasts were subjected to osmotic stress at 0 °C. Photosynthesis was also much more sensitive to osmotic stress at 0 °C than at 25 "C. The inhibitory effects of osmotic stress on photosynthesis as well as respiration were amplified by not only chilling but also photoinhibitory light. The photosynthetic or respiratory activities of protoplasts recovered remarkably when they were transferred from hyperosmotic (1·0 kmol m^{-3} sorbitol) back to iso-osmotic medium (0·4 kmol m^{-3} sorbitol), demonstrating the reversibility of osmotic-stress-induced changes in protoplasts. Respiration was more resistant to osmotic stress and was quicker to recover than photosynthesis. We suggest that the experimental system of protoplasts can be useful in studying the effects of osmotic stress on plant tissues.

Key-words: Pisum sativum: chilling; osmotic stress; pea, photoinhibition; photosynthesis; protoplasts; respiration.

Abbreviations: FBPase, fructose-1,6-bisphosphatase; SBPase, sedoheptulose-1,7-bisphosphatase.

INTRODUCTION

Water stress reduces leaf water potential and restricts plant photosynthesis and productivity, the problem being particularly severe in arid and semi-arid regions of the world (Boyer 1982; Jones & Corlett 1992). The primary cause of photosynthetic inhibition during water stress is debatable (Boyer 1976; Kaiser 1984; Leegood, Walker & Foyer 1985). Water stress causes stomatal closure, which is sometimes 'patchy', leading to non-uniform photosynthesis in the leaves of several plants (e.g. Ni & Pallardy 1992; Wise. Ortiz-Lopez & Ort 1992) The deleterious effects of low water potential on photosynthesis are therefore frequently attributed to the indirect effects of stomatal limitation (Jones 1985; Cornic, Papageorgiou & Louason 1987). Nevertheless, water stress is known to affect photosynthesis directly by inhibition of electron transport/photophosphorylation, inactivation of key assimilatory enzymes (Kaiser 1984; Leegood *et al.* 1985; Bunce 1988; Sharkey & Sccmann 1989) or alterations in carbon partitioning (Harn & Daie 1992).

We attempted to study the effects of osmotic stress on protoplasts. The use of protoplasts rather than leaves allows one to avoid the limitations of not only non-uniform photosynthesis or stomatal patchiness but also the recycling of CO_2/O_2 within intercellular spaces, Further, protoplasts offer a closer system to the *in vivo* situation than do the isolated organelles, since the responses of a leaf to factors like water stress or photoinhibition can often be quite different from those of isolated chloroplasts. The present article evaluates the effects of osmotic stress on photosynthesis as well as respiration in isolated mesophyll protoplasts of pea *(Pisum sativum).*

The effect of drought is amplified by other environmental factors such as supra-optimal light (e.g. Kirschbaum 1987; Masojídek *et al.* 1991 and references therein). We have therefore also examined the interaction, if any, between osmotic stress and chilling or photoinhibition during the responses of photosynthesis and respiration in protoplasts. The phenomenon of photoinhibition was recently demonstrated in pea mesophyll protoplasts (Saradadevi & Raghavendra 1992).

The present article describes the marked and reversible inhibition of photosynthetic carbon metabolism and respiration in pea mesophyll protoplasts on exposure to osmotic stress, using sorbitol as the osmoticum. Sorbitol is known to be a non-permeating osmoticum and has been used by earlier workers to impose osmotic stress on leaf slices, protoplasts or chloroplasts (e.g. Kaiser *et al.* 1981a,b; Kaiser 1982; Sen Gupta & Berkowitz 1987; Sundari & Raghavendra 1990). The response of protoplast respiration to osmotic stress was intriguing. Chilling or photoinhibitory light aggravated further the inhibition of not only photosynthesis but also respiration due to osmotic stress.

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MATERIALS AND METHODS

Pea plants (*Pisum sativum* L. cv. Bonneville) were raised from seeds in plastic trays filled with soil and organic manure. Seeds were procured from Pocha Seeds Company, Pune, India. Plants were grown outdoors, under a natural photoperiod of approximately 12 h and average daily temperatures of 30 °C during the day and 20 °C at night.

Protoplast isolation

Mesophyll protoplasts were isolated from first and second fully unfolded leaves of 8- to 10-d-old plants as already described (Devi *et al.* 1992; Saradadevi & Raghavendra 1992).

O_2 uptake/evolution

Respiratory O_2 uptake in the dark and photosynthetic O_2 evolution in the light of protoplasts were monitored at 25 °C using a Clark-type O_2 electrode (Model DW2, Hansatech Ltd, King's Lynn, UK). The reaction medium (1·0 cm^3) contained 0·4 kmol m^{-3} sorbitol (unless otherwise stated), 1 mol m^{-3} $CaCl_2$, 1 mol m^{-3} $MgCl_2$, 1 mol m^{-3} $NaHCO_3$ in 10 mol m^{-3} Hepes-KOH pH 7·5 and protoplasts equivalent to 20 µg chl. In some of the experiments, 1 mol m^{-3} *p*-benzoquinone was used instead of bicarbonate. Illumination (1250 µmol m^{-2} s^{-1}) was provided by a 35 mm slide projector (Atul Electronics Corporation, India; lamp: Xenophot [Halogen] 24 V/150 W).

Exposure of protoplasts to stress

Protoplasts were subjected to one or a combination of stresses, namely osmotic stress, chilling and high irradiance (photoinhibitory light).

To impose water or osmotic stress, the concentration of sorbitol in the suspension medium of protoplasts was altered from 0·4 (−1·3 MPa) to 1·0 kmol m^{-3} (−3·1 MPa). Hence the same osmoticum was maintained in the reaction medium. Except for sorbitol, the components of the incubation/reaction medium were unaltered. In some of the experiments, protoplasts were pre-incubated for 10/20 min at either 25 °C or 0 °C (on ice, to evaluate the effect of chilling).

Photoinhibitory treatment of protoplasts was accomplished as described previously (Saradadevi & Raghavendra 1992). Protoplasts (200 µg chl cm^{-3}) suspended in the incubation medium containing 0·4 kmol m^{-3} or 1·0 kmol m^{-3} sorbitol were exposed for 10 min to supra-optimal light (3000 µmol m^{-2} s^{-1}) provided by four tungsten lamps (Philips, India: Comptalux 225 V/75 W), two on either side of the thermo-jacketed pre-incubation chamber which was maintained at 25 °C. Additional water filters and a flow of cool air between the lamps and the pre-incubation chamber ensured that the chamber was not heated by the lamps. The protoplast suspension was gently stirred during incubation in photoinhibitory light or darkness.

An aliquot of protoplast suspension was examined for photosynthesis or respiration soon after the photoinhibitory treatment.

Other procedures

Chlorophyll content was determined after extraction into 80% (v/v) acetone (Arnon 1949). The data presented in this paper are the average values (±SE) of results from at least three experiments conducted on different days.

RESULTS

Inhibition of photosynthesis

The photosynthetic rate in mesophyll protoplasts decreased on exposure to solutions of increasing osmolarity (Fig. 1). Protoplasts lost over 30% of their photosynthetic capacity, when assayed in a medium containing 1·0 kmol m^{-3} sorbitol. On the other hand, *p*-benzoquinone-dependent O_2 evolution was not much affected (the loss being <10%, even after exposure to 1·0 kmol m^{-3} sorbitol).

The extent of photosynthetic inhibition in mesophyll protoplasts increased with the duration of incubation in hyperosmotic medium (1·0 kmol m^{-3} sorbitol). The reduction in photosynthesis after exposure to 1·0 kmol m^{-3} sorbitol for 10 min was 25% at 25 °C and 45% at 0 °C (Fig. 2). The slight reduction in photosynthesis in 0·4 kmol m^{-3} sorbitol at 25 °C was due to the inherent instability of protoplasts at warm temperatures. Most of the experiments were therefore limited to 10 min. Only a few were extended to 20 min or more.

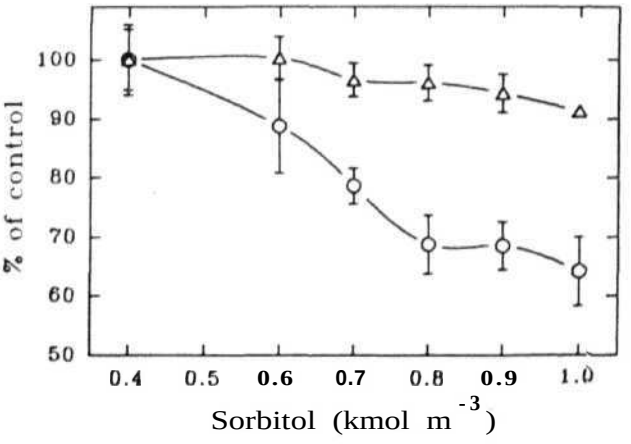

Figure 1. Effect of osmotic stress on carbon fixation (bicarbonate-dependent oxygen evolution) or PS II mediated photochemical reactions. The stress was imposed by increasing the concentration of sorbitol in the assay medium from 0·4 to 1·0 kmol m^{-3}, as indicated. Photosynthesis was monitored as O_2 evolution with the help of an O_2 electrode, as described in the text. The rates of p-benzoquinone and bicarbonate-dependent oxygen evolution in 0·4 kmol m^{-3} sorbitol were 290 ± 2 and 150 ± 11 mmol g^{-1} chl h^{-1}, respectively, and were taken as the controls (100%). (A), *p*-benzoquinone; (○), bicarbonate.

Changes in respiration

The respiratory rate decreased on exposure to solutions of increasing osmolarity at OT, but was stimulated at 25 T (Fig. 3). After a pre-incubation of 20 min in 1·0 kmol m^{-3} sorbitol at 0 °C, about 25% of respiratory activity was lost. When protoplasts were exposed to 1·0 kmol m^{-3} sorbitol at 25 T for 20 min, their respiratory rate was increased by 35% (Fig. 4).

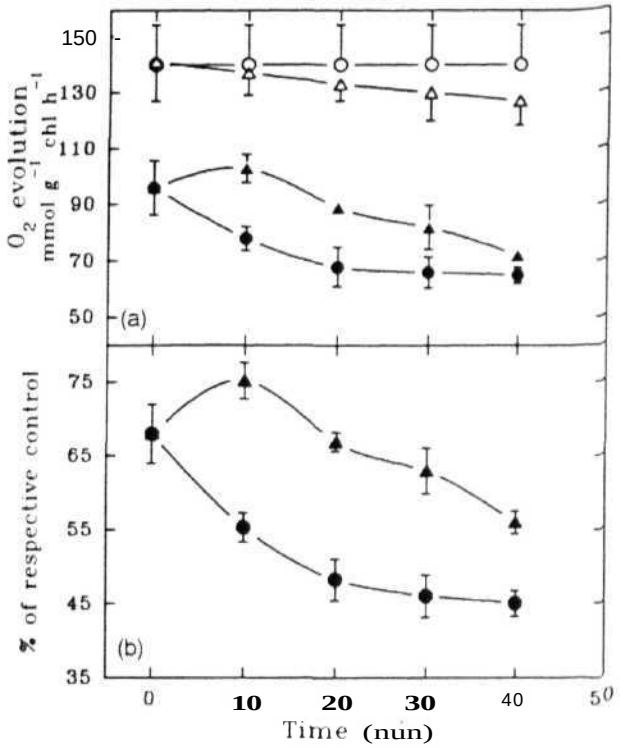

Figure 2. Progressive inhibition of photosynthesis with time in pea mesophyll protoplasts on exposure to osmotic stress at either 25 °C or 0 °C. Protoplasts were pre-incubated in 0·4 or 1·0 kmol m^{-3} sorbitol for varying periods as indicated (a) Rates of photosynthetic oxygen evolution; (b) photosynthesis at 1·0 kmol m^{-3} sorbitol as % of the respective control (0·4 kmol m^{-3}). (○),0·4 kmol m^{-3} sorbitol at 0 °C; (A), 0·4 kmol m^{-3} sorbitol at 25 °C;(●), 1·0 kmol m^{-3} sorbitol at 0 °C; (A), 1·0 kmol m^{-3} sorbitol at 25 °C.

Prolongation of photosynthetic induction

An immediate effect of osmotic stress in protoplasts is the prolongation of the period of photosynthetic induction. Fig. 5 demonstrates that the duration of the induction or lag phase of photosynthesis was increased soon after the exposure to 1·0 kmol m^{-3} sorbitol in the reaction medium. The prolongation of the photosynthetic induction period in protoplasts depended on the concentration of sorbitol (0·4 to 1·0 kmol m^{-3}) in the assay medium (Fig. 6).

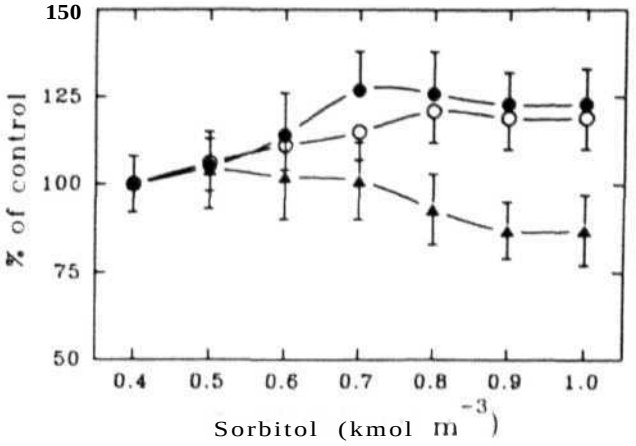

Figure 3. Inhibition of respiratory O_2 uptake in pea mesophyll protoplasts on exposure to osmotic stress in either assay medium (○) or after pre-incubation for 10 min (●, 25 V; *A*, 0 °C). Osmotic inhibition of respiration was evident, particularly at 0 °C. There was slight stimulation of respiration when protoplasts were exposed to hyperosmotic media at 25 °C. The respiratory rate at 25 °C of an aliquot of protoplast suspension after osmotic pre-treatment at 0 °C or 25 °C was measured The respirator) rate in 0·4 kmol m^{-3} sorbitol at 25 °C was 16 ± 1·7 mmol of O_2 uptake g^{-1} chl h^{-1} (taken as control, 100%).

Reversibility of the effects of osmotic stress and chilling

Since the inhibition of photosynthesis or respiration was marked at ()'(", the reversibility of the effect of osmotic stress was also examined at (IT. The inhibition of photosynthesis in protoplasts due to osmotic stress was reversible, as indicated by the marked recovery in the activity when the osniolarity of the medium was decreased from 1·0 kmol m^{-3} to 0·4 kmol m^{-3} (Fig. 7). The slight loss of PS II mediated activity in response to a *p*-benzoquinone

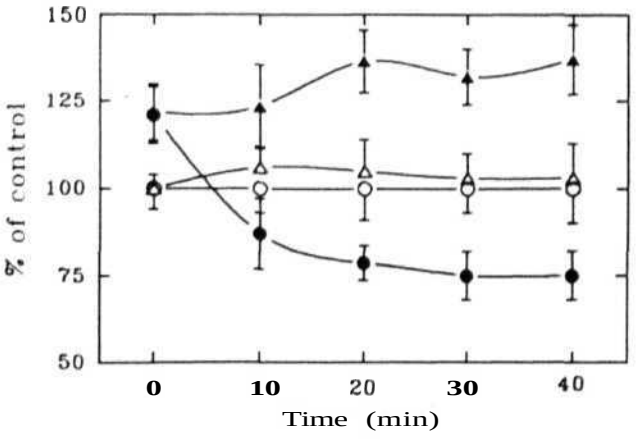

Figure 4. Progressive inhibition of respiration with time as **a** result of osmotic stress in mesophyll protoplasts at either 25 °C or 0 T. Protoplasts were incubated at 25 T or 0 °C in 0·4 kmol m^{-3} (△ and ○, respectively) or 1·0 kmol m^{-3} (• and ●, respectively) sorbitol for varying periods as indicated and assayed in the dark at 25 T. The respiratory rate in 0·4 kmol m^{-3} sorbitol was 18 ± 0·9 mmol O_2 uptake g^{-1} chl h^{-1} **(taken** as control, 100%).

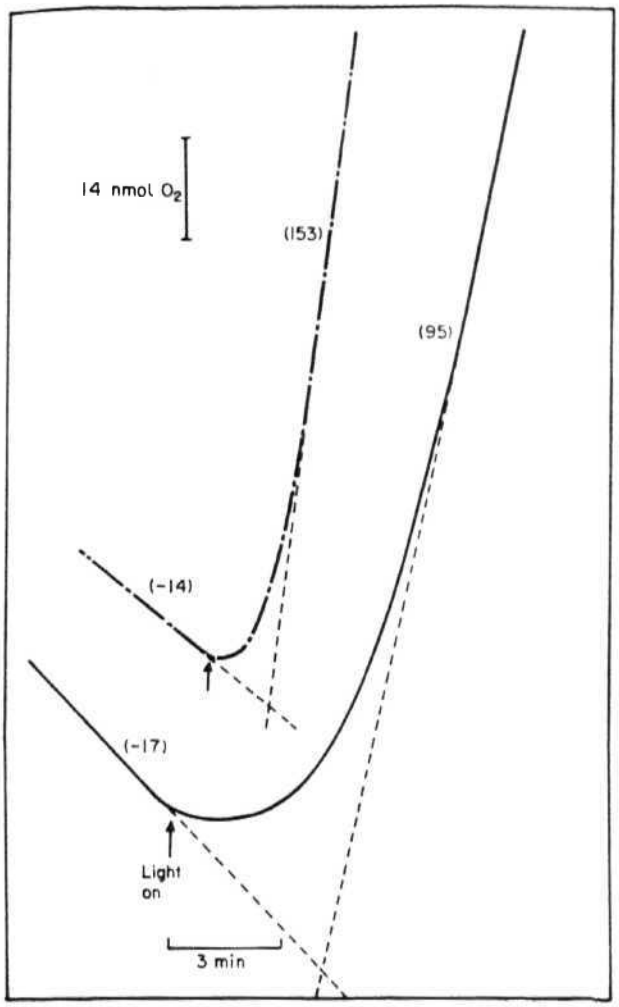

Figure 5. Prolongation of photosynthetic induction on exposure to osmotic stress, as indicated by the recorder traces of O_2 evolution by protoplasts. The induction period was about 1·5 min when protoplasts were in 0·4 kmol m^{-3} sorbitol (dot-dashed line) but extended to >4 min in 1·0 kmol m^{-3} sorbitol (solid line). Maximal rates of photosynthesis were reduced by 35% in 1·0 kmol m^{-3} sorbitol.

reduction was completely restored on the re-exposure of stressed protoplasts to 0·4 kmol m^{-3} sorbitol (data not shown).

The inhibition of respiration due to osmotic stress was also reversible when the protoplasts were transferred from 1·0 kmol m^{-3} to 0·4 kmol m^{-3}. However, the inhibition of respiration was less and the recovery faster than those of photosynthesis (Fig. 8).

The protoplasts on exposure to 1·0 kmol m^{-3} sorbitol for 20 min at 25 °C lost <35% of their photosynthetic activity, but the loss of photosynthetic activity was over 50% at 0°C (Table 1). The amplifying effect of chilling on the osmotic inhibition of photosynthesis in protoplasts was completely reversible. Transfer of protoplasts from 0 °C to 25 °C after 10 min resulted in a <25% reduction in photosynthetic activity.

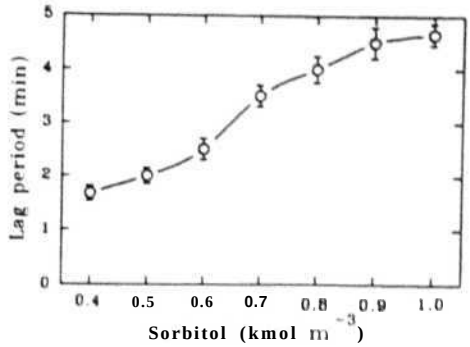

Figure 6. The period of photosynthetic induction in protoplasts as a (unction of sorbitol concentration in the medium. The lag-period pattern is illustrated in Fig. 5.

Photoinhibition of photosynthesis

In the iso-osmotic medium of 0·4 kmol m^{-3} sorbitol, protoplasts lost about 35% of their photosynthetic activity, when exposed to supra-optimal light (Table 2). On the other hand, over 50% of photosynthetic activity was lost if protoplasts were subjected to photoinhibitory light in 1·0 kmol m^{-3} sorbitol. The loss soared to nearly 70% if protoplasts were pre-incubated at 0 °C for 10 min before exposure to photoinhibitory light.

Changes in respiration under photoinhibitory light

Protoplasts lost 18% of their respiratory activity on exposure to photoinhibitory light (for 10 min at 25 °C) in the

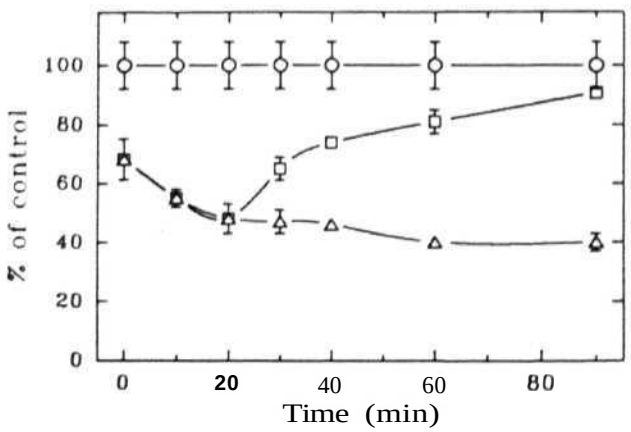

Figure 7. The reversal of the osmotic inhibition of photosynthesis on re-exposure of mesophyll protoplasts to 0·4 kmol m^{-3} sorbitol at 0 °C. Protoplasts were exposed to 1·0 kmol m^{-3} sorbitol and then were transferred back to 0·4 kmol m^{-3} after 20 min at 0 °C (□). The rate of photosynthetic O_2 evolution at 25 °C in an aliquot of such a pre-stressed/stress-reversed protoplast preparation was measured. Protoplasts were also diluted in 04 and 1·0 kmol m^{-3} sorbitol (○ and A, respectively), incubated and assayed as respective controls to assess the problem of a possible change in activity as a result of dilution and incubation for different periods. The photosynthetic rate in 0·4 kmol m^{-3} sorbitol at zero time (at 25 °C) was 160±15 mmol of O_2 evolved g^{-1} chl h^{-1} (taken as control, 100%).

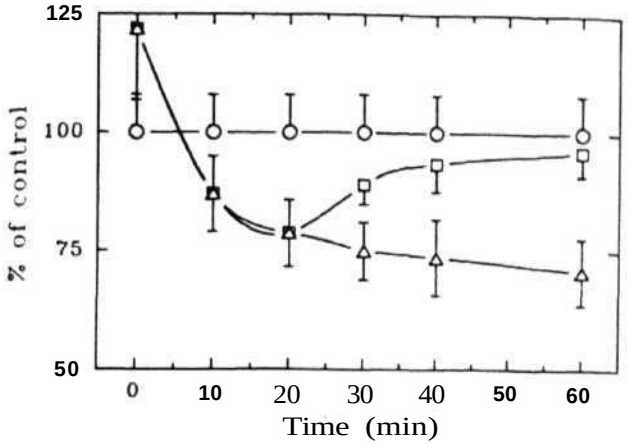

Figure 8. The recovery of respiration after osmotic stress inhibition in pea mesophyll protoplasts on re-exposure to 0·4 kmol m^{-3} sorbitol (at 0 °C). The rate of respiratory O_2 uptake at 25 °C in an aliquot of pre-stressed/stress-reversed protoplast preparation was measured. The respiratory rate in 0·4 kmol m^{-3} sorbitol at zero time was 17 ± 2 mmol of O_2 uptake g^{-1} chl h^{-1}. Further details are as in Fig. 7.

iso-osmotic medium of 0·4 kmol m^{-3} sorbitol. The loss of respiratory activity after exposure to photoinhibitory light was almost doubled (32%) in the hyperosomotic medium of 1·0 kmol m^{-3} sorbitol.

DISCUSSION

The present study demonstrates the reversible effects of osmotic stress on photosynthetic carbon assimilation (Figs 1 & 7) as well as on respiration (Figs 3 and 8) in protoplasts. The inhibition of photosynthesis due to osmotic stress was not only immediate but also persistent, indicating that the osmotic inhibition was not primarily due to a transitory loss of permeability as observed in chloroplasts (Kaiser 1984). Osmotic inhibition of respiration was evident at 0 °C, but not at 25 °C, and was also reversible.

Surprisingly, protoplasts did not rupture on exposure to even high concentrations of sorbitol. Protoplasts shrank (decreased in size) but remained intact, as indicated by routine microscope observations. Kaiser *et al.* (1981b) also observed that protoplasts are very good at retaining their

Table 1. The reversible effect of temperature during pre-incubation on the inhibition of photosynthesis by osmotic stress in protoplasts. The inhibition was aggravated on exposure of the protoplasts to chilling at 0 °C, but the activity recovered on re-exposure to 25 °C

Temperature treatment	Photosynthesis (mmol O_2 evolution g^{-1} chl h^{-1}) Sorbitol (kmol m^{-3}) 0·4 (control)	1·0	Inhibition due to 1·0 kmol m^{-3} sorbitol (%)
Direct assay	141 ± 14	96 ± 7	32
Preincubation before assay			
25 °C - 10 min	137 ± 8	103 ± 6	25
25 °C – 20 min	133 ± 6	89 ± 2	33
0 °C - 10 min	141 ± 14	78 ± 4	45
0 °C – 20 min	141 ± 14	68 ± 7	52
0 °C – 10 min/25 °C – 10 min	137 ± 8	105 ± 9	23

Table 2. Photomhibition of photosynthesis in mesophyll protoplasts in relation to osmotic stress and chilling at 0 °C

Preincubation/ osmotic treatment*	Photosynthesis (mmol O_2 evolution g^{-1} chl h^{-1}) Darkness (control)	After exposure to photoinhibitory light**	Photoinhibition of photosynthesis (%)
No preincubation			
0·4 kmol m^{-3} sorbitol	137 ± 8	8917	35
1·0 kmol m^{-3} sorbitol	IO3±6	4715	54
Preincubation for 10 min at 0 °C			
1·0 kmol m^{-3} sorbitol	105 ± 9	3514	67

• At 25 °C.
** At an intensity of 3000 µmol m^{-2} s^{-1} for 10 min at 25 °C.

integrity under osmotic stress. The possibility of physical shock being an important factor in protoplasts subjected to osmotic stress is therefore quite remote. Although it is difficult to envisage changes within a protoplast as a result of osmotic stress, changes in any component, for example, the tonoplast membrane, could lead to an altered microenvironment within the cell.

A small proportion of protoplasts were found to be broken on re-exposure to 0·4 kmol m^{-3} sorbitol (data not shown). This may be the reason why the rates of photosynthesis and respiration in stressed protoplasts did not completely recover to equal the control values (without osmotic stress). Nevertheless, the reversibility suggests that the effects of osmotic stress are primarily a result of either the concentration of solutes within the protoplasts (Kaiser 1984) or conformational changes in the intracellular proteins/membranes (Gantet, Hubac & Brown 1990), or both.

The inhibition of photosynthesis by 1·0 kmol m^{-3} sorbitol (-31 MPa) in mesophyll protoplasts (Figs 1 & 2) was not as severe as that caused by similar water potentials in an intact plant. Similar observations were made by Kaiser *et al.* (1981a), who suggested that one of the reasons for this difference may be that protoplast photosynthesis is not limited by stomatal or diffusional resistances.

Our results endorse observations made using leaf slices, cells and chloroplasts (Sharkey & Badger 1982; Berkowitz & Gibbs 1984; Kaiser 1984) which suggested that the biochemical reactions of photosynthesis are affected directly by water stress and are therefore non-stomatal-mediated (Sharkey & Badger 1982; Berkowitz & Gibbs 1984; Kaiser 1984). However, photochemical activity such as *p*-benzoquinone-dependent O_2 evolution was not very sensitive to osmotic stress (Fig. 1), as observed by earlier workers (Sharkey & Badger 1982; Berkowitz & Gibbs 1984; Kaiser 1984; Martin & Ruiz-Torres 1992).

The marked increase in the duration of the photosynthetic induction phase (Figs 5 & 6) is similar to that found in observations on chloroplasts, and is possibly due to a disturbance in the regeneration of Calvin cycle intermediates (Berkowitz & Gibbs 1984; Boag & Portis 1985; Leegood *et al.* 1985). The light activation of FBPase and SBPase is affected as a result of the decrease in the extent of stromal alkalization in osmotically stressed chloroplasts (Sharkey & Badger 1982; Berkowitz & Gibbs 1984; Leegood *et al.* 1985). A leak of Calvin cycle metabolites may also occur from such stressed protoplasts as a result of a transient loss of permeability (Kaiser *et al.* 1981b). However, such leakage may not be crucial, since the recovery of photosynthesis on transfer to isotonic sorbitol (0·4 kmol m^{-3}) is remarkable and nearly total (Figs 7 & 8).

The literature on the effect of osmotic or water stress on respiration in plant tissue is contradictory, with reports indicating either stimulation or inhibition or no change (cf. Hsiao 1973; Hanson & Hitz 1982). In our experiments, the change in respiration as a result of osmotic stress was dependent on temperature. The inhibition of respiration as a result of osmotic stress at 0 °C was quite similar to the response of photosynthesis (Fig. 4). We suggest that osmotic stress affects the function of mitochondria as well as that of chloroplasts. The reversibility of inhibition, in the case of not only photosynthesis but also respiration (Figs 7 & 8), suggests that reversible changes, presumably in membrane structure, could form the basis of osmotic stress effects (Gantet *et al.* 1990).

Our results demonstrate that protoplasts can be additional tools to study photosynthetic responses to osmotic stress. Conventionally, studies on photosynthesis under water stress are carried out using either leaves, chloroplasts or cells (Sharkey & Badger 1982; Berkowitz & Gibbs 1984; Kaiser 1984, 1987). Although there have been a few reports on volume changes in protoplasts under water stress (Dowgert, Wolfe & Steponkus 1987; Sen Gupta, Berkowit7 & Pier 1989), photosynthetic measurements using protoplasts are limited (cf. Kaiser 1984, 1987).

One of the disadvantages of protoplasts is their instability at room temperature over long periods of time. We have limited most of our experiments at 25 °C to 10 or 20 min. Protoplasts are turgorless and may be slightly dehydrated compared to intact plants. However, their high rates of photosynthesis/respiration (Fig. 5) and the reversibility of stress responses (Figs 7 & 8) illustrate clearly that the protoplasts retain their metabolic integrity to a great extent. In spite of their fragility and limited stability, protoplasts are useful for experiments involving rapid fractionation of organelles, metabolite analysis, and membrane fluidity during osmotic stress.

The sensitivity of photosynthesis in protoplasts was further aggravated on exposure to chilling or high irradiance or both (Table 1). The synergistic effect of water stress and photoinhibition in leaves is a known phenomenon (e.g, Masojídek *et al.* 1991). We suggest that this properly can be used for amplifying the photosynthetic responses in protoplasts to photoinhibition and vice versa (i.e. to amplify osmotic inhibition of photosynthesis by exposure to supra-optimal light).

The effect of low temperature/chilling was reversible, since the photosynthesis of protoplasts inhibited at 0 °C recovered to a great extent when they were transferred to room temperature (Table 2). The antagonistic effects of warm temperature on photoinhibition and of water stress on PS II of leaves of several plants have recently been reported (Havaux 1992). Our results suggest that low-temperature-induced reversible changes in protoplasts are partly responsible for the inhibition of their photosynthesis. Reversible changes in intracellular membranes are a likely factor, since temperature change causes dramatic changes in lipid structure and membrane fluidity (Shinitzky 1984; Kasamo *et al.* 1992).

A consistent feature of mesophyll protoplasts was that the inhibition of photosynthesis as a result of osmotic-stress was always much more marked than that of respiration. After a 10 min pre-incubation at 0 °C under osmotic stress (i.e. in 1·0 kmol m^{-3} sorbitol), there was a reduction of only about 13% in respiratory activity, while there was a

45% loss of photosynthetic activity. Similarly, when protoplasts were relieved of osmotic stress, the recovery of respiration (Fig. 8) was faster than that of photosynthesis (Fig. 7). Schwab, Schreiber & Heber (1989) reported that respiration was less sensitive to dehydration than photosynthesis and recovered faster during rehydration in resurrection plants like *Craterostigma plantagineum*.

Concluding remarks

Osmotic stress prolonged the induction phase, inhibited photosynthetic carbon metabolism and stimulated respiration in protoplasts at room temperature (25 °C). Chilling at 0 °C aggravated the osmotic inhibition of photosynthesis and caused a marked inhibition of respiration. The effects of osmotic stress or chilling were reversible by re-exposure to an iso-osmotic medium or to room temperature, respectively. Because of the ease of manipulation, the experimental system of protoplasts is useful to monitor changes under water stress, such as changes in the intracellular metabolite distribution and the lipid composition of the plasma membrane. The recovery of respiration was quicker than that of photosynthesis on removal of osmotic stress around protoplasts. In view of the very limited literature, further experiments are necessary to examine respiratory metabolism under stress in detail.

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Review article

Interdependence of photosynthesis and respiration in plant cells: interactions between chloroplasts and mitochondria

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Abstract

Photosynthesis and respiration in an illuminated plant cell are not only interdependent but also mutually beneficial. Respiratory rates increase after hours of illumination due to carbohydrate (substrate) accumulation. Besides such long-term effects, photosynthesis and respiration interact even during short illumination periods of a few minutes. The rate of respiration in isolated leaf protoplasts increases severalfold after 10–15 min of illumination. Such light-enhanced dark respiration (LEDR) has been demonstrated in protoplasts as well as in leaves. The stimulation of LEDR by bicarbonate and its sensitivity to inhibitors of photosynthesis (DCMU) or the Calvin cycle (D,L-glyceraldehyde) point out the importance of photosynthetic carbon metabolism for respiration. From metabolite analyses of protoplasts, the majority of LEDR is due to mitochondrial oxidation of malate produced by chloroplasts. Simultaneous measurements of photosynthesis and respiration, using mass spectrometry, demonstrate that mitochondrial TCA cycle-based CO_2 evolution is inhibited by illumination while O_2 uptake is either unaffected or stimulated. The marked sensitivity of photosynthesis in leaves or protoplasts to classic mitochondrial inhibitors such as oligomycin, sodium azide or antimycin A implies that mitochondrial metabolism is essential for photosynthesis. Respiration not only benefits photosynthesis but also protects illuminated leaf protoplasts against photoinhibition. Oxidative electron transport and phosphorylation play a much more important role than the reactions of glycolysis and the TCA cycle in this beneficial interaction. The metabolite shuttles involving PGA-DHAP and/or OAA-malate across the Chloroplast and mitochondrial membranes could form the biochemical basis of the interaction between photosynthesis and respiration. Alternatively, cytosolic NAD(P)H, derived from photosynthetic products, can be directly acted upon by the mitochondrial external NAD(P)H dehydrogenase and oxidised through the mitochondrial electron transport system. Mitochondrial oxidation of NAD(P)H (even if indirect) helps to prevent the over-reduction of the cytosol and, consequently, the Chloroplast in illuminated leaf cells. Besides the direct interaction with chloroplasts, mitochondria can supply reducing equivalents through malate to peroxisomes during photorespiration and provide citrate as the precursor of oxoglutarate, necessary for glutamine and glutamate formation. These two phenomena further complement the strong interdependence of photosynthesis and mitochondrial metabolism.

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Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethyl urea; DHAP, dihydroxyacetone phosphate; LEDR, light-enhanced dark respiration; OAA, oxalacetate; PDH, pyruvate dehydrogenase; PGA, 3-phosphoglyceric acid; Rubisco, ribulose 1,5-bisphosphate Carboxylase/oxygenase; TCA, tricarboxylic acid.

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Key words: Carbon metabolism; Chloroplasts; Cytosol; Mitochondria; Oxidative electron transport; Photosynthesis; Respiration; TCA cycle

1. Introduction

High photosynthetic efficiency helps to achieve maximal plant growth and plant productivity [1,2]. Similarly, high rates of respiration are characteristic of rapidly growing tissues [3]. Both photosynthesis and respiration, therefore, form essential components of plant growth. A recent survey demonstrated the strikingly positive correlation between photosynthetic and respiratory rates of several tree species [4].

Photosynthesis results in O_2 evolution and the generation *of* ATP and NADPH which are used for the reduction of CO_2 (or other compounds like NO_2^- or SO_4^{2-}). On the other hand, respiration accomplishes oxidation of carbon compounds and evolution of CO_2. NADH produced in these reactions is utilised for ATP production and oxygen consumption. ATP is generated in both processes, while pyridine nucleotides are reduced during photosynthesis but are oxidised in respiration. The biochemical nature of photosynthesis and the respiratory reactions implies that these two processes are complementary to each other. However, studies on the extent and pattern of mitochondrial respiration during photosynthesis have remained ambiguous and conflicting. Several excellent reviews summarize the early literature [5-10]. Readers are also referred to a few related reviews on the interaction between respiration, photorespiration, photosynthesis and nitrogen metabolism [11–13]; for a recent reference, see [14].

The present review focuses on the recent work and emphasizes the marked interdependence and beneficial interaction between photosynthesis and respiration. We also try to analyse and identify the biochemical basis of such an interaction between these two processes. Although most of the literature covered in this review deals with higher plants, occasional reference is made to algal cells to emphasize the arguments.

2. Long- and short-term interactions

A few hours of illumination stimulate the rate *of* leaf respiration. It has been proposed that this is primarily due to the accumulation of carbohydrates [10,15,16]. Such an increase in dark respiration is often proportional to the period of illumination and depends on temperature [10]. The enhanced rate of post-illumination mitochondrial respiration is suggested to represent the rate in the preceding light period and may partly be due to an enhanced activity *of* the alternative pathway of electron transport [17]. However, the high rate of respiration declines slowly to reach a steady state characteristic of the dark level. This effect of photosynthetic activity on respiration should be treated as a long-term effect since the interaction becomes pronounced only after hour(s) of illumination.

There are several other processes of CO_2 release or O_2 consumption in leaves/green cells apart from dark respiration (Table 1). Although each of these processes is important in influencing photosynthesis, this article concentrates only on the interaction between photosynthesis and dark respiration (particularly mitochondrial). Readers further interested in other short-term interactions are referred to the recent articles/reviews on the subject: photorespiration [18–22], Kok effect (for a recent reference, see [23]), Mehler reaction [24-26] and chlororespiration [27–29].

3. Respiratory reactions under photosynthetic conditions

The status of dark respiration in illuminated green cells/leaves has been a topic of interest. However, there is considerable disagreement in the literature on the effect of light on respiration [5,6,30,31]. In some of the studies, dark respiration was hardly affected or even stimulated, while in others there was an inhibition of up to 100%

Table 1
Respiration and other metabolic phenomena which interact with photosynthesis

Phenomenon	Reaction event	Biochemical basis
Long-term		
Dark respiration	CO_2 efflux/O_2 uptake	**Accumulation of carbohydrates due to** prolonged **illumination**
Short-term		
Mitochondrial respiration	O_2 uptake/CO_2 efflux	**Not precisely identified (possibly** oxidative **election transport** phosphorylation)
Photorespiration	CO_2 efflux/O_2 uptake	**Production** and **metabolism of** glycine **through** C$_2$ cycle, **in light**
Chlororespiration	CO_2 efflux	**Partial reactions of** pentose **phosphate pathway**
Mehler reaction	O_2 uptake	**Formation of superoxide radicals upon** illumination, **due mainly to PS I**
Kok effect	Decrease in photosynthetic quantum efficiency	**Reduction** in **photosynthetic rate due to dark respiration at low** light

(Table 2). Apart from those listed in Table 2, there are several other reports on the light modulation of respiratory reactions in plant tissues or algal cells, but the extent of inhibition/stimulation due to light varies widely [32–38]. Such a large variation is due to several factors: the component of dark respiration being monitored, the experimental technique being used, and finally the type of

Table 2
Marked variation in the reports on the effect of light on dark respiration in leaves[a]

Plant source	**Respiratory reaction event**	Effect **of** light **(%) stimulation (+) or inhibition (–) over** the **dark rate**	Reference
Zea mays	**CO; release**	–96	98
Phaseolus vulgaris	$^{14}CO_2$ **metabolism**	-KI	
Spinacea oleracea	CO; release	-82	100
Phaseolus mungo	Turnover **of** ^{14}C-labelled **citrate and** fumarate	-73	101
Triticum aestivum	CO; release	**-70**	102
Vicia sepium	**Assimilation of** ^{14}C-labelled succinate	-45	101
Pisum sativum	O_2 uptake	-42	103
Spinacea oleraceu	O_2 uptake	–39[b]	103
Glycine max	O_2 uptake	–40[b]	103
Triticum aestivum	Metabolism of ^{14}C-labelled TCA cycle compounds	-25	40
Chenopodium bonus henricus	**Assimilation of** ^{14}C-labelled succmate	–15	101
Eucalyptus pauciflora	CO; release	-3	23
Nicotiana tabacum	CO_2 **release**	+32	98

[a]A selection of plant sources is given in order to show the wide variation. **Further literature is referred to** in the text.
[b]Determined at a very low light intensity of 10 μE M^{-2} s^{-1}.

plant tissue being studied. For example, it is difficult to assess the operation of photosynthesis or respiration in leaves based on net uptake/evolution of either O_2 or CO_2 since the measurements are compromised by inter- and intracellular recycling of the gases (e.g. 39,40]. Another problem is the technical difficulty of monitoring precisely the different types of oxidative reactions besides respiration (e.g. photorespiration. Mehler reaction, pseudocyclic electron transport; see Table 1). All these processes result in O_2 uptake and operate concurrently in light. Nevertheless, a promising solution appears to be the technique of mass spectrometry which distinguishes between uptake and efflux of CO_2 or O_2, occurring simultaneously, as discussed further in the following paragraph and Table 3.

Dark respiration is comprised of three components: (i) glycolytic reactions: (ii) decarboxylation of carbon compounds to produce CO_2 and reduced nucleotides (NADH and FADH); and (iii) oxidation of NADH/FADH leading to O_2 consumption and ATP production. A strong indication that the processes of CO_2 efflux and O_2 uptake are not as tightly coupled in light as in darkness came from observations on carnation cells [41]. An overwhelming majority of the earlier literature also supports the view that TCA cycle activity and substrate decarboxylation, in particular, are inhibited in the light, although to varying extents [3,30,31]. Mass spectrometric studies using $^{13}C^{12}CO_2$ and $^{18}O^{16}O$ demonstrate that respiratory CO_2 efflux is inhibited upon illumination, while O_2 uptake is either relatively unaffected or even stimulated (Table 3). Thus, TCA cycle activity (decarboxylation of TCA cycle compounds) is inhibited but oxidative electron transport is unaffected by illumination. The intermediates of the TCA cycle are possibly diverted upon illumination into chloroplasts for amino acid metabolism [42,43].

The inhibition of the TCA cycle in light is

Table 3
The extent of respiration during illumination in homogeneous systems of cells or protoplasts determined by mass Spectrometry[a]

Plant material/system	Respiratory reaction (% stimulation/inhibition)		Reference
	CO_2 evolution	O_2 uptake	
Higher plants			
Dianthus caryophyllus			
Photoautotrophic cells	**-56**	-6	41
Euphorbia characias			
Photoautotrophic cells	-60	NE[b]	KM
Photoautotrophic cells	-33	-7	57
Dividing cells	NE[b]	+5	105
Mature leaves	NE[b]	+63	105
Nicotiana plumbaginifolia			
Mesophyll protoplasts	NE[b]	+150	106
Commelina communis			
Mesophyll protoplasts	-31	+62	74
Guard-cell protoplasts	-37	-14	74
Green algal cells			
Selenastrum minutrum	-59	-15	107
Chlamydomonas reinhardtii (wild type)			
Photoautotrophic cells	NE	+11	108
C. reinhardtii (mutant devoid of Rubisco)			
Heterotrophic cells	-73	+157	77

[a] This technique distinguishes respiration from photosynthetic reactions and provides fairly accurate estimates of respiration. The figures indicate the percentage stimulation (+). or inhibition (-) over the dark rate.
[b] NE, not examined.

possibly due to the inactivation of pyruvate dehydrogenase (PDH), the entry step into the cycle [44]. Illumination increases the level of ATP in the cytosol or mitochondria [45-47]. and protein phosphorylation inactivates the PDH enzyme [48,49]. Not only photosynthesis, but also photorespiratory metabolism can stimulate PDH inactivation [50.51].

4. Light-enhanced dark respiration

The rate of respiratory O_2 uptake is often stimulated after even short periods of illumination (see Table 3). This phenomenon, termed light-enhanced dark respiration (LEDR), has been demonstrated in spinach leaves [52] and mesophyll protoplasts of pea [53,54] and barley [55,56]. The rate of respiration in mesophyll protoplasts increased several fold after 15 min of illumination. The extent of LEDR increased with the duration of illumination and exhibited two phases. The initial high rate of respiration decreased in about 10 min to a low steady value similar to that before illumination [53].

The presence of bicarbonate/CO_2 promoted LEDR in mesophyll protoplasts as well as in spinach leaves. The sensitivity of LEDR to DCMU or D,L-glyceraldehyde (a Calvin cycle inhibitor) in protoplasts and leaves further confirmed that the upsurge of respiratory O_2 uptake was dependent on the products of photosynthetic carbon assimilation/electron transport [52–55]. Hill and Bryce [55]. however, observed that carbon fixation by Rubisco was not essential for LEDR in barley mesophyll protoplasts. Although LEDR could be observed when barley protoplasts were illuminated with low light intensities [56], maximal photosynthetic rates (in the preceding light period) were necessary for high rates of LEDR. The high rate of LEDR in the presence of saturating CO_2 [53,54] and its insensitivity to aminoacetonitrile, an inhibitor of mitochondrial glycine metabolism [56]. demonstrate that this process is quite distinct from a photorespiratory post-illumination burst of CO_2.

It is possible that LEDR is not directly dependent, but rather indirectly stimulated by Calvin cycle activity. Metabotite analysis of barley protoplasts indicated that malate was oxidized to pyruvate during LEDR [55]. Thus, photosynthetically derived compounds in chloroplasts, like malatc. form the substrates for subsequent LEDR in mitochondria.

In most of the reports, the processes of photosynthesis and respiration are not monitored simultaneously due to technical limitations. During the usual measurement of net O_2 exchange, either photosynthesis or respiration alone can be followed. However, it is possible to assess simultaneously respiratory O_2 uptake and photosynthetic O_2 evolution by monitoring $^{18}O_2$ and $^{16}O_2$, respectively. Using this technique, when photoautotrophic cells of carnation or *Euphorbia characias* were darkened after a few minutes of illumination, the rate of respiratory O_2 uptake in darkness (soon after switching off the light) was enhanced [41,57], once again confirming the phenomenon of LEDR.

5. Essentiality of mitochondrial respiration for photosynthesis

Although the contribution of mitochondrial oxidative phosphorylation to the ATP demand of a photosynthesizing cell has earlier been doubted [5,6], recent reports establish that mitochondrial activity is essential for the optimal performance of photosynthesis in green cells.

Oligomycin, an inhibitor of mitochondrial oxidative phosphorylation (but not photophosphorylation), suppressed photosynthesis by 30-40% in illuminated barley *(Hordeum vulgare* L.) leaf protoplasts [58]. This inhibition was reversed and the full rate of photosynthesis was restored when the protoplasts were ruptured leaving the chloroplasts intact. When fed via the transpiration stream, Oligomycin inhibited photosynthesis in barley leaves by up to 60% [59]. In protoplasts as well as intact leaf tissue, the ratio of ATP to ADP decreased while the levels of glucose and fructose 6-phosphatc increased, due to Oligomycin [59]. A significant part of the ATP requirement for cytosolic sucrose synthesis appears to be met by mitochondrial oxidative metabolism.

Further experiments with Oligomycin and barley

leaf protoplasts revealed that the importance of mitochondrial metabolism to photosynthesis may depend on light intensity [60,61]. Mitochondrial ATP synthesis was active under limiting as well as saturating light intensities and under both photorespiratory and non-photorespiratory conditions [60]. A major function of the mitochondrion in a photosynthesizing cell, particularly under low light intensities, seems to be the supply of ATP for cytosolic carbon metabolism, i.e, sucrose synthesis. In high light, mitochondria take on the additional role of oxidising the excess reducing equivalents generated by photosynthesis, preventing over-reduction of chloroplastic redox carriers and. thus, maintaining high rates of photosynthesis.

Studies with a starchless mutant of *Nicotiana sylvestris* suggest that a respiratory supply of ATP could affect assimilate partitioning into sucrose and, thereby, modulate photosynthesis [62]. The mutant NS458 contains a defective plastid phosphoglucomutase and accumulates only trace amounts of starch. Treating mutant leaf protoplasts and young leaves with Oligomycin reduced photosynthesis by as much as 25 and 40%, respectively. The wild-type failed to show inhibition by Oligomycin, i.e. its effect was masked when starch and sucrose synthesis could interact [62]. Maximal CO_2 assimilation in the mutant thus appears to be fine-tuned by mitochondrial metabolism such that any interruption of either sucrose synthesis or mitochondrial processes would generate oscillations in photosynthesis.

Mitochondrial respiration not only benefits photosynthesis, but also provides protection against photoinhibition in protoplasts [54,63]. The suggestion that dark respiration forms an additional defense mechanism to protect illuminated leaf cells against photoinhibition is based on two major observations: a decrease in respiratory rates due to photoinhibitory light, and a marked promotion of photoinhibition even at very low concentrations of classic inhibitors of mitochondrial metabolism (e.g. NaN_3).

Three possible factors could cause respiration to protect illuminated mesophyll protoplasts against photoinhibition. Respiratory metabolism could either (a) elevate the level of intracellular CO_2, particularly at CO_2-limiting conditions, (b) provide extra energy for the turnover of the PS II-associated D_1 protein within the chloroplast, by meeting cytosolic demands for ATP or (c) help to maintain an optimal redox state in the chloroplasts or cytosol of the cells. NaF and sodium malonate (which inhibit glycolysis, among other processes, and the TCA cycle, respectively) did not a fleet the extent of photoinhibition (63]. Apparently, respiratory CO_2 evolution was not a major factor in modulating photoinhibition. Similarly, Oligomycin was not as effective as NaN_3 or antimycin A (inhibitors *of* oxidative electron transport) in enhancing the extent of photoinhibition. Therefore, oxidativc electron transport in mitochondria appears to play a more prominent role than oxidative phosphorylation during protection of illuminated protoplasts against photoinhibition [63]. Even a marginal interference of photosynthetic metabolism by respiratory inhibitors made protoplasts highly susceptible to photoinhibitory light.

A slightly different situation was observed in the cyanobacterium *Anacystis nidulans*, where dark respiration modulated the process of photoinhibition of photosynthesis and its reactivation [64]. Photoinhibition is a result of the interference with the degradation and repair of the D_1 protein which requires energy (for a recent reference, sec (65]). Any limitation in ATP supply would accelerate photoinhibition. Decreased ATP production due to restricted respiration is proposed as the primary cause for stimulation of photoinhibition in *Anacystis* [64].

6. Mutually beneficial interaction between photosynthesis, respiration and related processes

The significance of the interaction between photosynthesis and respiration was not completely realised since most of the earlier studies were made with intact leaves. It is difficult to establish the extent and mechanism(s) of interaction between photosynthesis and respiration based on gas-exchange characteristics of leaves (for a relevant discussion, see [40]). The marked interaction between photosynthesis and respiration is clearly illustrated by experiments with leaf mesophyll pro-

toplasts [54]. Protoplasts are useful tools to examine plant metabolism since they do not have any barrier against diffusion of CO_2/O_2, pose no problem of recycling the gases within the intercellular spaces, and further allow an evaluation of the effect of externally added compounds. The processes of photosynthetic oxygen evolution and respiratory oxygen uptake in mesophyll protoplasts influenced each other significantly, even during short cycles of light and darkness [66]. The rate of dark respiration was nearly doubled by preillumination, while there was a marginal but significant increase in photosynthetic rate of protoplasts after a short period(s) of darkness.

The interdependence of photosynthesis and respiration was even more striking in the presence of metabolic inhibitors [66]. Inhibitors of photosynthesis decreased the extent of light-enhanced dark respiration while respiratory inhibitors also suppressed photosynthesis. For example, DCMU or D,L-glyceraldehyde, restricted respiratory oxygen uptake in the dark. Similarly, respiratory inhibitors like antimycin A, NaN_3 or Oligomycin markedly reduced the rates of photosynthetic oxygen evolution. The concentration of Oligomycin used in these experiments does not affect Chloroplast function [58]. The statistical significance of the interaction between photosynthesis and respiration only in the presence of CO? (but not in its absence) suggested that carbon assimilation was a prerequisite.

Glycine and malate, both of which are formed under conditions of active photosynthesis (i.e. light and CO_2), form the main substrates for leaf-mitochondrial oxidation in vivo [67]. At least 25% of the NADH formed during oxidation of these metabolites is expected to be used for extra-mitochondrial requirements, particularly hydroxypyruvate reduction in peroxisomes and nitrate reduction in cytosol. The requirement of reducing power for hydroxypyruvate reduction in peroxisomes is proposed to be met in the form of malate from chloroplasts as well as mitochondria [68,69]. In addition, mitochondria provide the carbon source for nitrogen assimilation in the light, as citrate formed during malate oxidation acts as the precursor for oxoglutarate required for nitrogen metabolism [67,70]. Thus, mitochondrial metabolism becomes a major link between photosynthesis, photorespiration and nitrogen assimilation.

7. Interaction in specialized cells

The interaction between respiration and photosynthesis is quite pronounced in cells which are deficient in Rubisco Calvin cycle activity, such as stomatal guard cells and mutants of *Chlamydomonas*. Guard cells have high rates of respiratory activity [71] but contain very low levels of Rubisco and consequently limited carbon metabolism through the Calvin cycle [72,73]. The pattern of changes in typical respiratory reactions (i.e. O_2 uptake and CO? efflux) upon illumination of guard cells is quite different from that in leaf mesophyll cells. In guard-cell protoplasts *of Commelina communis*, light caused only a small decrease in O_2 uptake but a steep drop in CO_2 evolution [74], possibly due to the inhibition of TCA cycle reactions, but not oxidative electron transport.

Experiments with *Vicia faba* guard-cell protoplasts indicated that despite limited CO? fixation, the reducing equivalents produced by their chloroplasts were exported to the cytosol through OAA/malate or PGA/DHAP shuttles [75]. The reduced pyridine nucleotides (NAD[P]H) formed in the cytosol from the oxidation of malate or DHAP may act as the respiratory substrates for mitochondrial ATP production, needed for K^+ uptake. A very strong interaction between respiration and photosynthesis has recently been shown in guard cell protoplasts of *V. faba* and *Brassica napus* at varying O_2 concentrations [76]. A strong cooperation between chloroplasts and mitochondria is likely to be essential for the maintenance of guard-cell bioenergetic processes.

A similar situation appears to operate in two mutants of *Chlamydomonas reinhardtii*, one devoid of Rubisco [77] and the other lacking the β-subunit of Chloroplast ATP synthase and therefore the ability to produce ATP during photophosphorylation. Photosynthesis in the FUD50su mutant, deficient in the Chloroplast coupling factor, was extremely sensitive to antimycin A. a specific inhibitor of mitochondrial electron transport. Lemaire et al. [78] suggested that photosynthesis in the FUD50su strain was achieved by an

unusual interaction between mitochondria and chloroplasts. Export of light-generated reduced compounds from the chloroplasts to mitochondria elicited ATP formation in the latter, and ATP was subsequently imported into the Chloroplast.

8. Biochemical basis for interaction: metabolite exchange between chloroplasts, cytosol and mitochondria

The biochemical basis of the mutually beneficial interaction between the processes of photosynthesis and dark respiration is the rapid exchange of metabolites between chloroplasts, cytosol and mitochondria. Photosynthesis can influence respiratory metabolism through the modulation of not only respiratory substrates, but also the levels of adenine/pyridine nucleotides in the cytosol. The transport of PGA-DHAP and OAA-malate between chloroplasts and the cytosol is a well-known phenomenon [79-81]. Not only a rapid transport of ATP. ADP and Pi, but also metabolite shuttles of malate or OAA occur between mitochondria and the cytosol [82,83]. Such metabolite transport systems can achieve a favourable balance of adenine and pyridine nucleotides in these compartments.

The adenylate ratio (relative level of ATP to ADP) is an important regulatory factor of mitochondrial electron transport [30]. Therefore, the relative levels of ATP/ADP could modulate respiration as ATP levels in both the Chloroplast and cytosol increase during illumination [84,85]. However, the adenylate levels do not appear to be crucial since the cytosolic ATP/ADP ratios are not much affected by light/dark transitions (compared to the NAD(P)H to NAD(P) ratio, for example) and they are usually not high enough to inhibit mitochondrial oxidative metabolism [45-47,86. 87]. Furthermore, the CN-resistant alternative pathway which operates in plant mitochondria helps to maintain oxidative electron transport, independent of ATP formation [3,88]. Similarly. the enhancement of TCA cycle activity due to the availability of more carbon skeletons in the light is unlikely because the reactions of the TCA cycle are inhibited, resulting in a decreased CO_2 efflux during illumination [6,40,89] (also see Table 3).

An attractive possible means of regulation is the mitochondrial oxidation of photosynthetically reduced pyridine nucleotides. Illuminated chloroplasts are expected to have excess NADPH or related metabolites since their electron transport activity exceeds the capacity of carbon fixation [90]. The excess reducing equivalents are transported from the chloroplasts (in the form of DHAP or malate) to the cytosol to generate NAD(P)H [91]. Mitochondria are capable of oxidising external NADH or NADPH under certain conditions [30,92,93]. The oxidation of external NADH, the rates of which are very high, could be a significant factor mediating the interaction between chloroplasts and mitochondria. Alternatively, the reducing equivalents can be exported into mitochondria through a malate-OAA shuttle [82]. Unfortunately, there is no evidence for direct mitochondrial oxidation of either NADH or malate under normal conditions since the cytosolic NADH concentration is much lower than the K_m value of NADH dehydrogenase [67,94,95]. The oxidation could be indirect through the shuttles of related metabolites formed during photosynthesis.

Heineke et al. [91] have demonstrated a large difference in redox potentials between the stroma (NADPH/NADP) and cytosol (NADH/NAD) while attempting to identify the process(es) responsible for the maintenance of this redox gradient. Redox equivalents can be transferred from the Chloroplast stroma to the cytosol by two different metabolic shuttles: the triose-P/PGA shuttle mediated by the phosphate translocator and the malate-OAA shuttle facilitated by the dicarboxylate translocator [81]. The operation of a malate-OAA shuttle by mitochondria facilitates the exchange of reducing equivalents between mitochondria and the cytosol or peroxisomes [82]. Since these metabolite shuttles have the capacity to equalize the redox states of the stromal and cytosolic compartments, regulation of these processes is required to maintain the specific redox states of the two metabolic compartments. The triose-P/PGA shuttle is controlled by Pi availability for counter-exchange by the phosphate translocator, chloroplastic PGA reduction and cytosolic triose-P oxidation. The malate-OAA shuttle is regulated by stromal NADP-malate

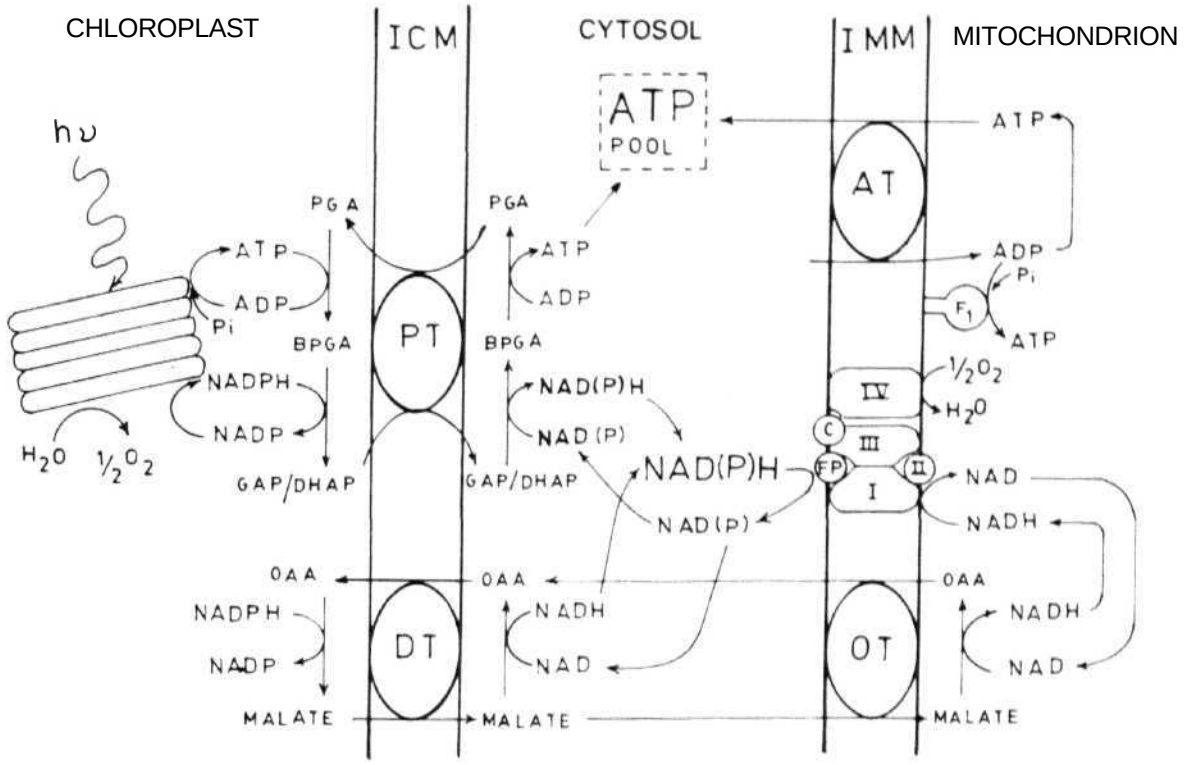

Fig. 1. Metabolite shuttles between chloroplasts and mitochondria via the cytosol are proposed to be the biochemical basis for the interaction between photosynthesis and respiration. Chloroplasts export ATP and reduced pyridine nucleotide equivalents to the cytosol via the phosphate translocator (PT) and or dicarboxylate translocator (DT) located in the inner chloroplast envelope membrane (ICM). Mitochondria may either import reduced pyridine nucleotide equivalents via the OAA translocator (OT) or oxidise cytosolic NAD(P)H directly by the external NA[](P)H dehydrogenase (FP) and components of the electron transport system (complexes I — IV and cytochrome *c*), located in the inner mitochondrial membrane (IMM). The direct export of ATP by the mitochondrial adenylate translocator (AT) contributes to the intracellular ATP pool, along with the supply from the chloroplasts. The net result is that mitochondria help to prevent the over-reduction of chloroplasts and cytosol. Similarly, chloroplasts could prevent the over-oxidation of mitochondria and cytosol. c, cytochrome *c*; F_1, coupling factor; GAP, glyceraldehyde 3-phosphate; BPGA, glycerate 1,3-bisphosphate.

dehydrogenase and the [NADPH]'[NADP]. and also by the translocating step across the inner Chloroplast envelope membrane [91].

Respiration can prevent the over-reduction of the photosynthetic electron transport chain in illuminated chloroplasts by providing an outlet for excess reducing equivalents to the cytosol or mitochondria [91]. Oxidative electron transport and phosphorylation in mitochondria appear to play a much more important role than the reactions of glycolysis or the TCA cycle in benefiting photosynthesis and protecting isolated leaf protoplasts against photoinhibition [63,66].

On the basis of the metabolite movements described above, we propose that the photosynthetic and respiratory activity in chloroplasts and mitochondria, respectively, is modulated by one or both of the following factors: (a) the redox slate due to NAD(P)/NAD(P)H levels, and (b) inter-organelle movement of key metabolites such as PGA, DHAP, malate and OAA (Fig. 1).

Adenine nucleotides (ATP, ADP, AMP) and/or

cytosolic pH could also play an indirect regulatory role in the mitochondrial and/or chloroplastic reactions. For example, energy is necessary to stimulate transport of metabolites such as pyruvate or malate across mitochondrial membranes [30]. Further studies on the pattern of carbon assimilation, partitioning of carbon and the levels of adenine/pyridine nucleotides are necessary to establish the exact mechanism(s) of the increase in mitochondrial O_2 uptake after even short periods of illumination.

9. Concluding remarks

Although a rapid and mutually beneficial interaction between Chloroplast photosynthesis and mitochondrial respiration is now well documented, the exact molecular mechanism(s) of this interaction is yet to be established. The role of key metabolites involved in rapid transport between various leaf-cell compartments (DHAP/PGA or OAA/malate) should be examined in detail. The redox states of chloroplasts, cytosol or mitochondria at different rates of photosynthesis or respiration should be assessed. Furthermore, the relative importance of the CN-sensitive and CN-resistant/alternative pathways of oxidative electron transport [96.97] in the interaction between respiration and photosynthesis should be ascertained.

The 'coarse' (long-term) control of the interaction between photosynthesis and respiration appears to be through the levels of soluble sugars, while the 'fine' (short-term) control is exerted by intracellular redox states or adenine pyridine nucleotide levels. The major purpose of this review is to emphasize the importance of mitochondrial metabolism to photosynthesis and vice versa. Nevertheless, extra-mitochondrial oxidative processes such as the Mehler reaction [24,25] or photorespiration [19-21] are also essential to sustain high rates of photosynthesis. The dependence of glutamine and glutamate biosynthesis on chloroplasts (for ATP and reducing power) and mitochondria (for oxoglutarate) makes the triangular interaction between photosynthesis, nitrogen assimilation and respiration extremely interesting. However, the available information on such an interaction in higher plants is much less than that in algal cells [9,13].

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PHYSIOLOGIA PLANTARUM 95: xx–xx 1995

Correlation between the inhibition of photosynthesis and the decrease in area of detached leaf discs or volume/absorbance of protoplasts under osmotic stress in pea *(Pisum sativum)*

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Saradadevi, K., Padmasree, K. and Raghavendra, A. S. 1995. Correlation between the inhibition of photosynthesis and the decrease in area of detached leaf discs or volume/absorbance of protoplasts under osmotic stress in pet *(Pisum sativum)* - Physiol. Plant 95: xx-xx .

Exposure to osmotic stress reduces leaf area and protoplast volume while decreasing photosynthesis. But the measurement of protoplast volume is tedious while rapid determinations of leaf area in the Held are difficult. We evaluated the quantitative relationship between the extent of decrease in area of detached leaf discs or the volume of protoplast of pea *(Pisum sativum)* and reduction in their photosynthetic capacity under osmotic stress. Osmotic stress was induced by increasing sorbitol concentration in the surrounding medium of the leaf discs from zero to 1.0 *M* (–3.1MPa, isotonicity) to 1.0 *M*(–3.1 MPa, hypertonicity). There was a high degree of positive correlation between the extent of reduction in the area of detached leaf discs or the volume of protoplasts (indicated by diameter or absorption at 440 nm) and the decrease in photosynthesis. The correlation coefficients between inhibition of photosynthesis and the decrease in leaf disc area or protoplast volume were 0 96 and 0 99, respectively. We therefore suggest that the decrease in absorbance at 440 nm (corrected for turbidity at 750 nm) can be used as a simple measure to predict the inhibition due to osmotic stress of photosynthesis in mesophyll protoplasts. Similarly, the reduction in area of detached leaf discs could also be a very simple and useful criterion to assess osmotic tolerance of photosynthesis.

***Key words* - Drought tolerance, leaf disc area, osmotic stress, pea, photosynthesis, *Pisum sativum saiivum.* protoplasts, protoplast volume.**

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Introduction

The inhibition of photosynthesis during water stress is due to both nonstomatal (direct) and stomatal (indirect) factors (Kaiser 1987, Graan and Boyer 1990). Direct inhibition of photosynthetic carbon fixation by water stress is confirmed by the use of experimental systems that have no limitation imposed by stomatal conductivity, e.g., thin leaf slices, cells, isolated protoplasts and isolated chloroplasts (Jones 1973, Plaut and Bravdo 1973, Kaiser et al. 1981a,b, Sharkey and Badger 1982, Saradadevi and Raghavendra 1994). The sensitivity of photosynthesis to water stress was also demonstrated in leaves, at very high partial pressures of CO_2 (around the leaf to avoid stomatal limitation), employing the techniques of monitoring either gas-exchange (Graan and Boyer 1990) or chlorophyll fluorescence (Ögren 1990).

Osmotic adjustment is an important step during the acclimation of a plant cell to low ψ_w (Berkowitz and Kroll 1988, Sen Gupta and Berkowitz 1988, Evans et al, 1992). Water stress leads to marked reduction in leaf expansion and leaf area (Burke et al. 1985). A reduction in size of the protoplast/chloroplast has frequently been observed along with the inhibition of photosynthesis in plant tissues during osmotic stress (Acevedo et al. 1979, Kaiser 1982, Santakumari and Berkowitz 1989, 1990).

We have been studying the effects of light- and osmotic stress on photosynthesis in protoplasts of pea,

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Pisum sativum (Saradadevi and Raghavendra 1992, 1994). The present article is an attempt to evaluate the quantitative relation between the inhibition of photosynthesis and the decrease in volume of mesophyll protoplasts on exposure to osmotic stress. Our objective was also to develop simple methods based on leaf disc area as well as protoplast volume to monitor and/or predict the inhibition of photosynthesis due to osmotic stress. In this direction, we used detached leaf discs for an assessment of their response to osmotic stress in the laboratory.

Abbreviations - ψ_s, osmotic potential; ψ_w, water potential.

Materials and methods

Plant material

Plants of pea (*Pisum sativum* L. cv. Bonneville) were raised from seeds (Pocha Seeds Company, Pune, India) in plastic trays filled with soil and organic manure. Plants were grown outdoors, under a natural photoperiod of approximately 12 h and average temperatures of 30 °C during the day and 20 °C at night.

Experiments were performed either with detached leaf discs or mesophyll protoplasts. Leaf discs of ca 0.25 cm^2 were punched under water. Mesophyll protoplasts were isolated by enzymatic digestion from first and second fully unfolded leaves of 8- to 10-day-old plants as described by Saradadevi and Raghavendra (1992).

O_2 evolution by leaf discs/protoplasts

Photosynthesis by leaf discs was measured by a leaf-disc O_2-electrode system (LD-2, Hansatech Ltd, King's Lynn, UK). Nineteen small discs (each of ca 0.25 cm^2) were arranged symmetrically in three successive rings (of 1, 6 and 12 discs) within the chamber. Photosynthesis was measured at 25°C under high CO_2(5%, v/v) to avoid stomatal limitation. Leaf discs were pre-illuminated with red light of 200 µmol m^{-2} s^{-1} (supplied by ultra-bright LEDS, peak wavelength 635nm, from Model LH 36U, Hansatech, King's Lynn, UK) and were kept in darkness for 5 min, before illumination with a series of progressively increasing light intensities (Walker 1988).

Photosynthetic oxygen evolution by protoplasts was monitored at at 25°C using a Clark type O_2 electrode (Model DW2, Hansatech Ltd, King's Lynn, UK) as described earlier (Saradadevi and Raghavendra 1994).

Exposure of leaf discs/protoplasts to stress

Leaf discs were incubated in varying concentrations of sorbitol from 0.1 - 1.0 *M* (–0.1 - -3.1 MPa) for 1 h.

Protoplasts were subjected to osmotic stress by varying the concentration of sorbitol in the suspension medium from 0.4 – 1.0 *M* (–1.3 - -3.1 MPa) and incubated for 10 min at 0°C (on ice). Accordingly, the same osmoticum was maintained in the reaction medium. Except for sorbitol, the remaining components of incubation/reaction media were unaltered.

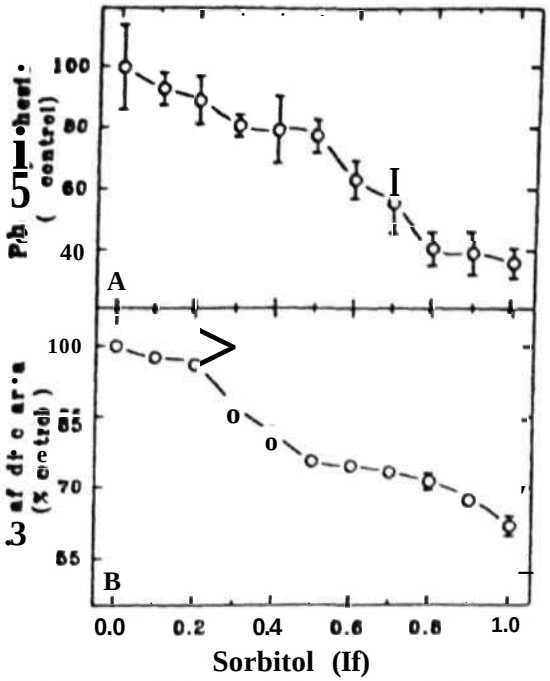

Fig. 1. Inhibition of photosynthetic oxygen evolution (A) and the decrease in the area (B) of detached leaf discs of pea after pre-incubation for 1 h in increasing concentration of sorbitol from 0.1-1.0 *M* (–0.1 - –3.1 MPa). Photosynthesis was measured by monitoring O_2 evolution at ca 5% CO, (v/v) in a leaf disc electrode at 25°C. The photosynthetic rate of fully-turgid leaf discs (pre-incubated in water for 1 h) was 46.4 ±4.9 µmol O_2 evolved m^{-2} s^{-1} (taken as 100% control). The average area of a fully turgid leaf disc was 0 27 ± 0 01 cm^2 (taken as 100% control). Standard errors, if not seen, are within the symbol.

Measurement of leaf disc area/protoplast volume

The diameter of leaf discs was measured under a stereo dissecting microscope by placing the discs on a glass plate below which a cm-graph paper was attached. The area of each leaf disc was calculated by nr^3.

The diameter of the palisade and spongy protoplasts after incubation in varying concentration of sorbitol (0.4 - 1.0 A/), was measured under a microscope using a pre-calibrated occular micrometer. Volume was calculated by assuming spherical protoplasts. Forty protoplasts were measured during each observation and the experiment was repeated at least four times on different days. Each value of protoplast volume therefore represents an average of at least 160 measurements.

Other procedures

The absorbance of protoplasts was scanned between 250 and 800 nm in a spectrophotometer (Shimadzu UV-160A). Chlorophyll was determined after extraction into 80% (v/v) acetone (Arnon 1949). The data pre-

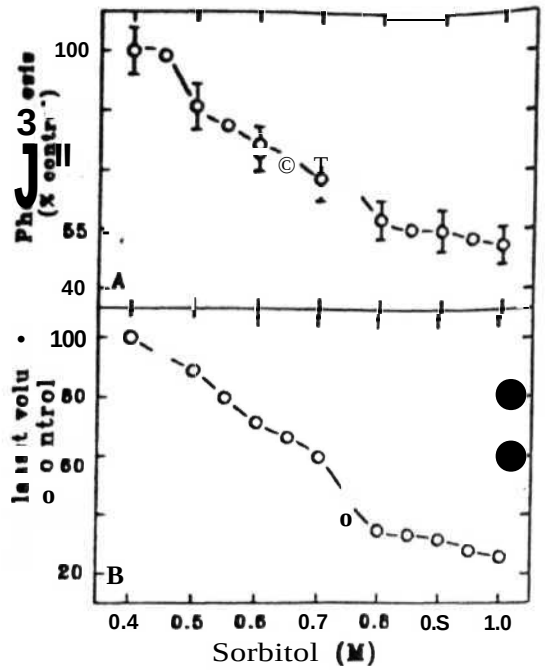

Fig. 2. Inhibition of photosynthetic oxygen evolution (A) and reducbon in volume (B) of pea mesophyll protoplasts on exposure to osmotic stress by increasing sorbitol concentration in suspension (protoplasts were preincubated for 10 min in suspension medium) as well as reaction media from 0.4 - 1.0 *M* (-1.3 MPa, isotonic - - 3.1 MPa hypertonic). The rate of bicarbonate dependent oxygen evolution by protoplasts suspended in 0 4 *M* sorbitol was 170 ± 8 μmol mg^{-1} Chl h^{-1}, and taken as control (100%). The degree of shrinkage was similar in both palisade and spongy protoplasts (data not shown separately). The average values Of palisade and spongy mesophyll protoplasts in 0 4 *M* sorbitol (control) are 2.6 x 10^{-5} ± 0.5 x 10"' mm^{1} and 0.8 x 10^{-5} 0.2 x 10^{-5} mm^{1}, respectively. Average values of mixed popularion (palisade and spongy) are represented.

sented are the average values (± SE) of results from 3 to 6 experiments conducted on different days.

Results

Photosynthetic rales in detached leaf discs of pea were quite sensitive to osmotic stress. The rate of photosynthesis decreased by nearly 70% at LOW sorbitol (Fig. 1A). There was a marked reduction also in the area of these leaf discs under hyperosmotic concentrations of sorbitol (Fig. 1B). There was reduction of up to 409b in leaf area when exposed to 1.0 W sorbitol for I h as compared to fully turgid leaf discs.

Protoplasts lost >50% of their photosynthetic capacity when they were incubated in 1.0 W sorbitol containing medium for 10 min (Fig. 2A). They shrank markedly on exposure to hypertonic medium, i.e., sorbitol exceeding 0.4 W as indicated by the decrease in their size (Fig. 2B). The volume of the protoplasts decreased by >70%, when they were exposed to 1.0 *M* sorbitol for 10 min at 0°C in comparison with protoplasts in 0.4 W sorbitol. The degree of shrinkage was similar in both palisade and spongy mesophyll protoplasts (data not shown).

The extent of decrease in photosynthetic rates (even under very high CO_2 concentration, i.e. 5%) in leaf discs on exposure to osmotic stress was positively correlated to reduction in leaf area (Fig. 3A). The extent of shrinkage in protoplast volume (in response to the increasing concentrations of sorbitol) was positively correlated with the inhibition of photosynthetic activity. The correlation coefficient of photosynthetic inhibition versus reduction in volume was 0.987 (Fig. 3B).

There was a marked decrease in the absorbance of protoplasts on exposure to 1.0 *M* sorbitol for 10 min (Fig. 4B). The difference spectrum of osmotically stressed protoplasts (suspended in LO *M* sorbitol) against control (0 4 W sorbiiol) presented a mirror-image of the spectrum of 80% acetone extract of protoplasts, i.e., Chloroplast pigments (Fig. *AC).* There was a positive correlation between the inhibition of photosynthesis and decrease in A_{440} of protoplasts on exposure to osmotic stress (Fig. SA). However, the correlation coefficient between the photosynthetic inhibition and the decrease in absorbance of protoplasts corrected for turbidity (i.e. A_{440} - A_{750}) and its statistical significance (Fig. 5B) were much greater than those in case of A_{440} alone.

Discussion

Protoplasts are quite useful for studies on photosynthesis as they avoid the limitations of leaves, including uneven stomatal distribution and recycling of CO_2/O_2 within in-

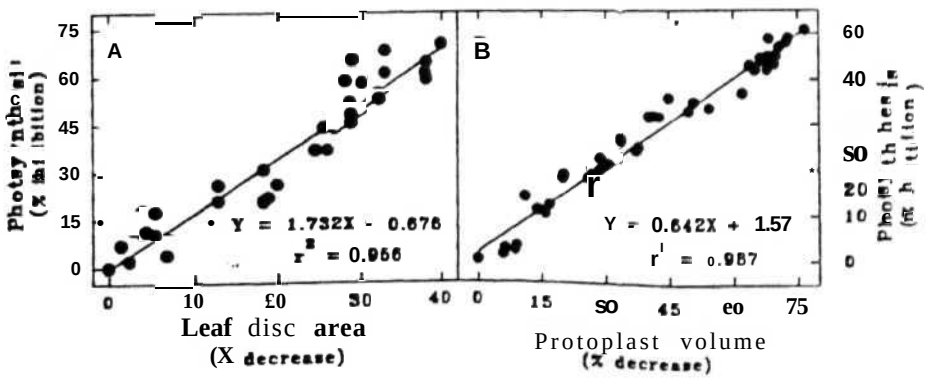

Fig. 3. Very high positive correlations between decrease in leaf disc area (A) or protoplast volume (B) and inhibition of their photosynthetic capacity on exposure to osmotic stress. Both the correlation coefficients (r^2) were statistically significant ($P<0.001$). Other details were as in Figs 1 and 2.

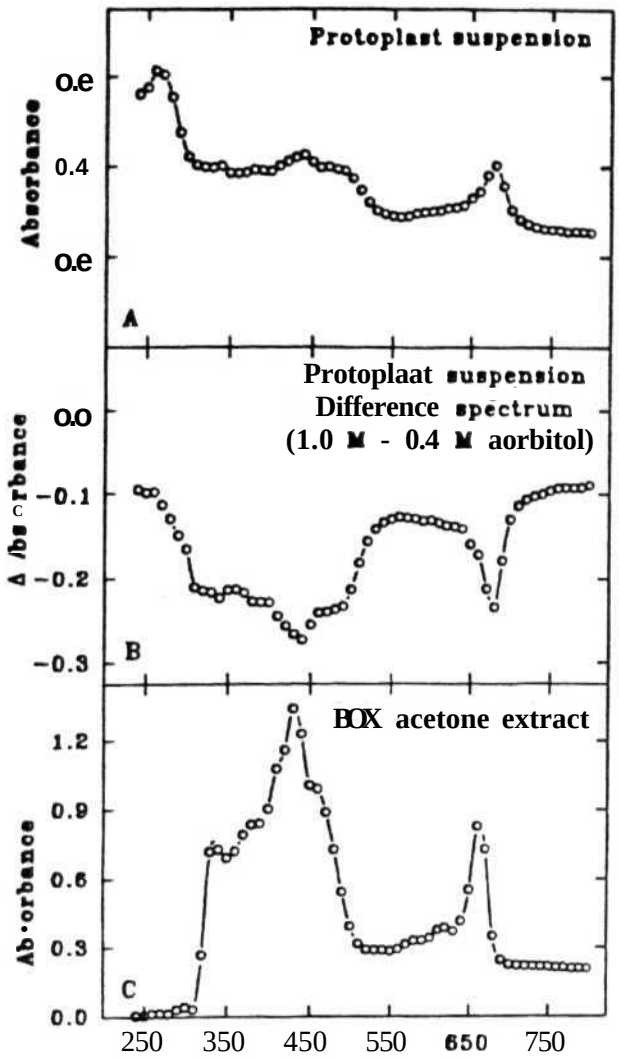

Fig. 4. Absorption spectrum of a protoplast suspension (A), or its acetone extract (C), containing protoplasts equivalent to 10 µg Chl ml^{-1}. Difference spectrum of protoplasts (B) at hyper - tonic concentration of 1.0 *M* sorbitol (kept in sample cuvette) and those at isotonic concentration of 0.4 *M* sorbitol (in refer. ence cuvette). The decrease in absorbance was a reflection of the spectrum of Chloroplast pigments.

tercellular spaces. However, they have certain disadvantages such as limited stability at warm temperature, fragile nature and tendency to sediment. The present report, while confirming that the system of isolated protoplasts can be used for studies on photosynthesis under water stress, demonstrates a highly significant correlation between the decrease in protoplast volume as well as leaf disc area and inhibition of photosynthesis in isolated protoplasts or detached leaf discs under different degrees of osmotic stress (Fig. 3). The highlight of the present work is the identification of two simple criteria, based on protoplast volume/leaf disc area, which can be easily used to monitor or predict the inhibition of photosynthesis due to osmotic stress: spectrophotometric measurement of the absorbance of protoplasts and determination of the area of detached leaf discs under a stereo dissecting microscope.

The earlier literature also suggests that a decrease in photosynthesis is associated with a reduction in protoplast and/or Chloroplast volume (Kaiser 1982, Santakumari and Berkowitz 1989, 1990). Drought resistant varieties undergo osmotic adjustment to maintain their photosynthetic efficiency as well as to minimize the reduction in volume of protoplast or Chloroplast, at low water potentials (Acevedo et al. 1979, Morgan 1984, Sen Gupta and Berkowitz 1987, Berkowitz and Kroll 1988, Santakumari and Berkowitz 1990).

There are two widely accepted experimental techniques to monitor the relationship between protoplast volume and water potential. One of them, the pressure/volume curve analysis, depends on an accurate estimate of a theoretically derived value. But several hours are needed to obtain the data for a single pressure/volume curve. The second technique is the use of dual label infiltration, developed by Kaiser (1982). However, a disadvantage with this technique is the scope for large error because of the equilibration of ^{14}C-sorbitol into the symplast due to vacuum infiltration-induced loss of membrane integrity (Santakumari and Berkowitz 1989). As per the present observations, a decrease in absorbance of protoplasts at 440 nm. corrected for turbidity at 750 nm, can be used as a convenient measure of protoplast volume.

The difference spectrum of osmotically-stressed protoplasts (1.0 *M*) compared to that of control (0.4 *M*) resembled closely the chlorophyll spectrum (Fig. 4), indicating that most of the decrease in protoplast size was accounted for by the chloroplasts. A recent study by Winter et al. (1994) also revealed that besides the vacuoles, chloroplasts constitute the second largest compartment, amounting to 16% of the total cellular volume in spinach leaf. As a result, the decrease in absorption of protoplasts at 440 nm (corrected for non-specific turbidity at 750 nm), indicating the contribution of mainly chloroplasts, was highly correlated with the extent of inhibition in photosynthesis due to osmotic stress (Fig. 5B).

The shrinkage of protoplasts is due to the loss of water as well as the decrease in the size of cellular organelles. As shown in Fig. 4B nearly two-thirds of the decrease in absorbance of protoplasts appears to be due to chloroplasts, while the remaining third is due to other components. Obviously, the contribution of chloroplasts to the absorbance of protoplasts is much more dominant than that of vacuoles. However, this is not surprising, as chloroplasts are green while vacuoles are not brightly coloured.

An important reason for inhibition of photosynthesis in chloroplasts under osmotic stress has been proposed to be the reduction in stromal volume (Kaiser 1982, Sen

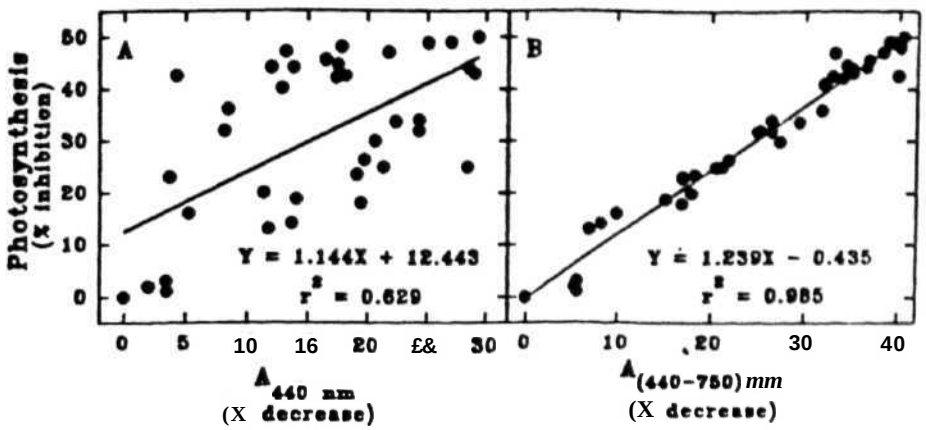

Fig. 5. A. Positive (but limned) correlation between the decrease in absorbance of protoplasts at 440 nm and inhibition of their photosynthetic capacity on exposure to osmotic stress. The average absorbance of protoplasts (equivalent to 20 MS Chl m1' at 440 nm was 0.91±0.03.B. Very high positive correlation between the decrease in absorbance of protoplasts al 440 nm (corrected for turbidity at 750 nm) and inhibition of their photosynthesis on exposure to osmotic stress. The average absorbance ($A_{440,750}$) of protoplasts (equivalent to 20 μg Chl ml^{-1}) was 0.35 ± 0.01. Both the correlation coefficients were statistically significant (P <0.001).

Gupta and Berkowitz 1987). The volume reduction occurs when stromal ψ_w becomes equilibrated with external r. by dehydration and the resulting biophysical changes inhibit photosynthesis (Santakumari and Berkowitz 1990). During osmotic dehydration, intracellular solutes are concentrated. The accumulation of solutes in the chloroplasts may affect the activity of stromal enzymes, ultimately resulting in a decrease in the rate of photosynthesis (Kaiser and Heber 1981, Kaiser 1982).

The measurement of protoplast size or volume under a microscope is more tedious than determining the absorption of a protoplast suspension. We suggest that the decrease in absorbance at 440 nm (corrected for turbidity at 750 nm) indicating the protoplast size, can be used as a simple criterion to monitor or predict the inhibition of photosynthesis under osmotic stress in mesophyll protoplasts.

Most of the earlier studies on leaf area were with intact plants and in the field. We now demonstrate a rapid decrease in leaf disc area along with the photosynthetic rate in detached leaf discs when used in the laboratory (Figs 1 and 5A). This technique is simple, easy and quick. The area of leaf disc also is a convenient parameter for monitoring and evaluating inhibition of photosynthesis by osmotic stress. It would be of interest to evaluate this concept with agronomic cultivars of crop species.

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