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PROTECTION OF CUCUMIS LEAF PIGMENTS AGAINST PHOTO-OXIDATIVE DEGRADATION DURING CHILLING

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SUMMARY

The possible protection against photo-oxidative degradation of chlorophyll and carotene in *Cucumis* leaf discs at 1 °C by a wide variety of compounds was investigated. Dimethyl sulphoxide and glycerol as well as ferricyanide, ascorbate, KCN and hydroxylamine were ineffective. Photo-oxidation was, however, at least partly, prevented by benzoquinone, hydroquinone, naphtoquinone, guajacol, benzidine, diaminobenzidine, alizarinesulphonic-acid, triphenyltetrazoliumchloride and 3-(3,4-dichlorophenyl), 1,1-dimethylureea. Photo-oxidative damage was promoted by salicylaldoxine, disalicylidenediaminopropane, azide, ethylenediamine tetraacetic acid, trishydroxymethylaminomethane, diquat and benzyl-viologen. It was also increased at high pH values. It is suggested that the mechanism of protection of *Cucumis* leaf chloroplasts against low temperature photo-oxidative damage is a protection of the electron transport pathway from the reducing to the oxidizing side of the photosystems.

1. INTRODUCTION

NOACK (1925) observed that chlorophyll in *Fontinalis* leaves is protected against photo-oxidation by soaking the leaves in a 0.04% aqueous benzidine solution before irradiation. The leaves became brown because of oxidation of benzidine while the chlorophyll was left unaltered. Benzidine also protected chlorophyll from photo-oxidation in dried leaves, as well as in methanolic solution. The protective effect evidently is not restricted to living cells.

Surprisingly no further investigations on the effect of inhibitors of photo-oxidative damage in higher plants by chemicals seem to have been made. Application of glycerol and dimethyl sulphoxide (DMSO) inhibits frost damage to animal cells (LOVELOCK & BISHOP 1959). These compounds may also protect plant cells against freezing injury (QUATRANO 1968, TRUNOVA 1968). HEBER & ERNST (1967) showed that DMSO protects isolated chloroplast lamellae against freezing-induced uncoupling of phosphorylation.

The effect of application of glycerol and DMSO on the light induced chlorophyll degradation in *Cucumis* leaf discs during chilling was investigated. In addition the effect of pH was studied.

SETCHENKA et al. (1971) showed that the Hill reaction with DCPIP as an electron acceptor in isolated spinach chloroplasts is influenced by pH. Activity was maximal between pH 6.5 and 8.0 and decreased at pH lower than 6.5 and higher than 8.0. In addition, pH also affects photo-oxidation of chlorophyll in

vitro (EVSTIGNEEV 1972). This author found an enhanced photo-oxidation of chlorophyll-a by benzoquinone in ethanol at a pH lower than 6.0. In this study the effect of various inhibitors on the photooxidative degradation of pigments in *Cucumis* leaf discs during chilling is also described.

Two main groups of inhibitors were tested: firstly substances which may quench directly excitation of chlorophyll such as benzoquinone and secondly substances which affect the electron transport in the chloroplast such as DCMU and SAL.

2. MATERIAL AND METHODS

2.1 Material

Plants *(Cucumis sativus* (L) cv. Kleine Groene Scherpe) were grown in a greenhouse (minimum day temperature 25°C; minimum night temperature 20°C).

Discs of 7 mm diameter were punched between the largest veins of the first leaves of two weeks old plants.

Discs were placed in petri-dishes containing 25 ml of the test solutions and incubated at 1°C in the light (20,000 lux) or in darkness, as described previously (VAN HASSELT 1972). Before exposure to light at 1°C the leaf discs were pretreated on the test solutions in the dark for two hours at room temperature.

2.2 Measurement of the pigment concentration

Chlorophyll (a + b) concentration was measured spectrophotometrically in a 80% acetone extract of 40 leaf discs as described before (VAN HASSELT 1972) at 663 and 645 nm. In some of the experiments a more refined extraction method was used which enabled usage of samples of 10 discs (VAN HASSELT 1974). Carotene was separated from chlorophyll by cellulose column chromatography and its concentration was measured as described earlier (VAN HASSELT 1972).

2.3 Inhibitors

Chemicals were dissolved in 0.15 M phosphate buffer (pH 6.0) and stored in the dark in a refrigerator.

The following abbrevations are used for the various chemical agents.

Aliz., alizarin sulphonic acid, 1,2-dehydroxy anthrachinon sulphonic acid sodium salt; Az., sodiumazide; Asc., sodiumascorbate; Benz., benzidinedihydrochloride; BQ, 1,4-benzoquinone; BV, benzylviologen; DAB, 3,3'-diaminobenzidine tetrahydrochloride; Diquat, 1,1-ethylene-2,2'-bipyridylium ($2Br^-$); DCMU, 3-(3,3-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichloro-phenol-indophenol; DMSO, dimethyl sulphoxide; DSPD, NN'-disalicylidene-1,3'-diaminopropane; EDTA, ethylenediamine tetraacetic acid; ferricyanide, potassiumferricyanide; Gua., guajacol-o-methoxyphenol; HAM, hydroxylammonium chloride; HQ, 1,4-hydroxy benzoquinone; NQ, 1,4-naphtoquinone; SAL, salicylaldoxime; TTC triphenyltetrazoliumchloride; TRIS, Tris

(hydroxy-methyl) aminomethane; KCN, potassium cyanide. Further abbrevations; PS I = photosystem I; PS II = photosystem II.

3. RESULTS

3.1 Effect of glycerol and dimethyl sulphoxide

No effect of glycerol or DMSO was observed within a wide range of concentrations *(table 1)*, indicating that the protection of these compounds against freezing differs from that against chilling.

3.2 Effect of pH

PH influences the chlorophyll concentration irrespective of the light conditions *(fig. 1)*. In the dark the chlorophyll concentration was maximal at pH 6 and was only slightly different at pH 7 and 8. At a pH lower than 6 and higher than 8 slight degradation of chlorophyll occurred. Dark degradation was maximal at pH 2. Since at this pH pigments leaked from the discs into the medium the leaf cells were evidently damaged.

After 26 hours in the light photo-oxidative degradation of chlorophyll had occurred. As in darkness degradation was minimal at pH 6.0. In the acid region chlorophyll degradation was higher at pH 4 as compared with the dark value, again increasing towards lower pH till a maximal effect occurred at pH 2. At this pH again chlorophyll was lost to the medium.

In the alcaline region chlorophyll degradation was more pronounced in the light than in the dark. Photo-oxidative degradation increased gradually to a maximal effect at pH 11. It is concluded that especially at high pH photo-oxidative chlorophyll degradation in *Cucumis* leaf discs during chilling was accelerated. Therefore, further studies were carried out at pH 6.0.

3.3 Effect of inhibiting and stimulating chemicals

The effect of various substances which affect electron transport in the chloroplast on leaf pigment degradation is shown in *tables 2* and *3*.

Photo-oxidative degradation of chlorophyll and carotene in *Cucumis* leaf discs during chilling was accelerated by application of Diquat, SAL and DSPD.

Table 1. The effect of glycerol and dimethyl sulphoxide on photo-oxidative degradation of chlorophyll in *Cucumis* leaf discs after a treatment of 40 hours at 20,000 lux at 1 °C. The data are expressed as percentage of the initial pigment concentration.

Concentration (M)	Glycerol	Dimethyl sulphoxide
0	54	54
0.05	58	54
0.1	53	52
0.5	57	56
1	56	52
2	53	–

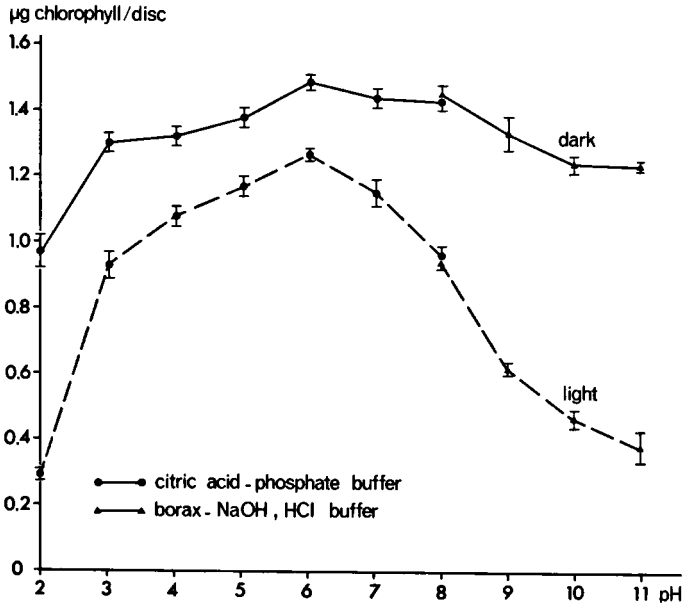

Fig. 1. Effect of pH on chlorophyll content of *Cucumis* leaf discs after incubation for 26 hours in light (20,000 lux) or in darkness at 1 °C. Each point represents the mean value of 6 samples of 10 discs together with their standard deviation.

Table 2. The effect of several photosynthetic inhibitors on the chlorophyll and carotene concentration in *Cucumis* leaf discs after a treatment of 40 hours at 20,000 lux or darkness at 1 °C.

The chemicals are dissolved in 0.15 M phosphate buffer at pH 6.0. The data are expressed as percentage of the initial pigment concentration, obtained from samples of 40 discs for each treatment.

Treatment	Chlorophyll		Carotene	
	Light	Dark	Light	Dark
control	57	102	17	106
DSPD, 10^{-2} M	39	94	11	106
SAL, 10^{-2} M	1	95	0	96
Diquat, 10^{-4} M	9	96	3	99
DCMU, 10^{-5} M	76	104	42	100
DAB, 10^{-2} M	77[1]	103	41[1]	107
TTC, 10^{-2} M	82[2]	98	56[2]	110

[1] discs and solution blue.
[2] solution red.

Table 3. The effect of various reagentia on chlorophyll degradation in *Cucumis* leaf discs after a treatment of 30 hours at 20,000 lux or darkness at 1 °C. The reagentia were dissolved in 0.15 M phosphate buffer at pH 6.0. Data are expressed as percentage of the initial chlorophyll concentration and represent the mean of 3 samples of 10 discs together with their standard deviation.

Treatment	Light	Dark
control	77 ± 1	97 ± 3
TRIS, 0.5 M	16 ± 1	94 ± 1
EDTA, 0.01 M	57 ± 3	104 ± 2
Az., 0.01 M	28 ± 2	96 ± 1
HAM, 0.01 M	78 ± 2	98 ± 2
Ferricyanide, 0.01 M	72 ± 1	101 ± 2
KCN, 0.01 M	79 ± 2	99 ± 2
BV, 0.01 M	24 ± 2	99 ± 1

DCMU, TTC and DAB inhibited pigment degradation *(table 2)*. No pigment degradation by any of the above chemicals was observed in darkness.

Table 3 shows that TRIS, EDTA, Az., and BV increased photo-oxidative degradation of chlorophyll. Ferricyanide, HAM and KCN were without effect. The effect of application of quinones and related compounds on photo-oxidative degradation of chlorophyll and carotene is presented in *table 4*. All substances tested in this experiment inhibited photo-oxidation. BQ, HQ, and Gua. were most effective, followed by Benz.; Aliz. and NQ were less effective. Again no pigment degradation occurred in the dark. Carotene and chlorophyll degradation was inhibited to the same degree by NQ, BQ, and HQ.

Table 4. The effect of quinones and related compounds on the degradation of chlorophyll and carotene in Cucumis leaf discs after a treatment of 40 hours at 20,000 lux or darkness at 1 °C. Each figures presents the pigment concentration of a sample of 40 discs, expressed as percentage of the initial pigment concentration. The chemicals were dissolved in 0.15 M phosphate buffer at pH 6.0.

Treatment (10^{-2} M)	Chlorophyll		Carotene	
	Light	Dark	Light	Dark
control	19	101	3	98
NQ	51	101	17	91
HQ	88[1]	98	45[1]	91
BQ	97[1]	102	46[1]	89
GUA	86	97	–	–
Benz.	72[2]	99	–	–
Aliz.	58[1]	98	–	–

[1] discs and solution brown.
[2] discs and solution blue.

Table 5. The combined effect of photosynthetic inhibitors on chlorophyll degradation after a treatment of 26 hours in the light (20,000 lux) or darkness at 1 °C. Light values represent the mean of 6 samples of 10 discs together with their standard deviation, the dark values represent the mean of 3 samples of 10 discs. The data are expressed as percentage of the initial chlorophyll concentration. The chemicals were dissolved in 0.15 M phosphate buffer at pH 6.0.

Treatment (10^{-2} M)	Control	Light			Control	Dark		
		Az.	BV	SAL		Az.	BV	SAL
Control	80 ± 2	35 ± 3	28 ± 1	31 ± 2	99 ± 2	95 ± 4	99 ± 2	99 ± 2
DCMU, 10^{-5} M	91 ± 2	33 ± 1	22 ± 2	32 ± 1	101 ± 1	102 ± 4	97 ± 2	92 ± 3
TTC, 10^{-2} M	95 ± 3	62 ± 2	83 ± 3	78 ± 3	102 ± 1	102 ± 1	100 ± 4	94 ± 3
BQ, 10^{-2} M	98 ± 3	95 ± 1	104 ± 3	91 ± 3	99 ± 4	105 ± 3	101 ± 2	102 ± 2

Combined treatments with compounds which accelerated photo-oxidative damage (SAL, BV, and Az.) and compounds which inhibited photo-oxidative damage (DCMU, TTC, and BQ) were studied to obtain more information about the mechanisms of their action. The results are shown in *table 5*. All combinations were without effect in the dark. In the light DCMU alone inhibited photo-oxidative chlorophyll degradation. This inhibitor, however, was ineffective in the presence of Az., BV, and SAL. TTC and BQ alone did inhibit chlorophyll degradation. However, the accelarating compounds Az., BV, and SAL did not alleviate the inhibition by TTC and BQ to a large extent.

4. DISCUSSION

Photo-oxidation of *Cucumis* leaf pigments at 1 °C is affected by application of various substances in three ways. It is prevented by eg. benzoquinone and DCMU; it is unaffected by compounds like ferricyanine and KCN and it is accelerated by SAL and Diquat. The effects of these three groups will be discussed briefly.

4.1 Reagentia which prevent photo-oxidation at low temperature

Benzoquinone very effectively quenches both chlorophyll fluorescence (singlet state) and chlorophyll triplet energy in benzene solution (FUJIMORI & LIVINGSTONE 1957). Also AMESZ & FORK (1967) and AMESZ et al. (1969) show that a number of quinones strongly quench chlorophyll fluorescence in Swiss chard chloroplasts and in the intact algae *Ulva lobata* and *Porphyra perforata*. Quenching occurred in the presence of DCMU as well as in the absence of this compound. Quinones apparently do not stimulate electron transport but interact with the chlorophyll molecules of PS II by formation of traps for the excitation energy.

A similar mechanism may explain why benzoquinone (BQ) and related compounds (*table 4*) prevent photo-oxidative degradation of leaf pigments in *Cucumis* leaf discs. Interaction between benzoquinone and the excited

chlorophyll may prevent formation of triplet chlorophyll and therefore, may inhibit the production of damaging singlet oxygen (because oxygen quenches triplet chlorophyll). A similar mechanism is suggested for the protective action by DAB and benzidine.

Another possibility is that these substances and quinones prevent photo-oxidation by acting as a preferential substrate for light induced singlet oxygen. NOACK (1925) observed that benzidine is oxidized both in vivo and in vitro while at the same time chlorophyll is protected against photo-oxidation. Photo-oxidation of DAB in chloroplast lamellae was shown to be insensitive to DCMU (NIR & SELIGMAN 1970). It seems therefore that this type of photo-oxidation is not coupled to electron transport but occurs directly in the presence of chlorophyll, light, and oxygen.

TTC and related compounds act as electron acceptors in the Hill reaction (DYAR 1953). Inhibition of photo-oxidation of chlorophyll and carotene by TTC *(fig. 2)*, however, may be related with the mechanism proposed for the inhibition by quinone and DAB since chlorophyll fluorescence in benzene is efficiently quenched by TTC (FUJIMORI & LIVINGSTONE 1957). Moreover, this suggestion is supported by the finding that both TTC and benzoquinone inhibit the effect of accelerators of photo-oxidative damage *(table 5)*.

DCMU inhibits the photo-oxidative degradation of leaf pigments *(tables 2* and *5)*. A similar inhibition of photo-oxidative damage by DCMU in *Cucumis* leaf discs at 1 °C was described earlier (VAN HASSELT 1973, 1974). DCMU inhibits electron transport at the reducing side of PS II between the first reduced substrate Q and the electron transport intermediates between PS II and PS I (DUYSENS & SWEERS 1963). As a consequence reoxidation of Q is prevented. DCMU possibly prevents photo-oxidative damage during chilling because it may prevent oxidation of PS II by enabling a back flow of electrons from the reduced to the oxidized side of the photosystem.

When electron transport beyond Q is blocked by DCMU more electrons may flow back to the oxidized side as a consequence of the relative excess of electrons at the reduced side of the photosystem. It has been suggested that such a cyclic pathway operates around PS II in the presence of DCMU via high potential cytochrome b 559 (CRAMER & BOHME 1971).

4.2 Reagentia which do not affect photo-oxidation

Application of 10^{-2}M KCN did not influence the rate of photo-oxidation of chlorophyll during chilling *(table 3)*, in agreement with the results of MYERS & BURR (1940) with *Chlorella*. 10^{-2}M KCN does however inhibit photosynthesis of *Chlorella* completely. KCN primarily inhibits photosynthesis by affecting the activity of carboxy dismutase (KANDLER & LIESENKOTTER 1963) and under the conditions used in our experiments, 1 °C, this enzyme will not function. This explains the ineffectiveness of KCN to prevent photo-oxidative damage.

The inability of ferricyanide to influence photo-oxidative damage suggests that during chilling no electron flow between the two photosystems occurs, in

agreement with the observation that DCMU was unable to inhibit the action of BV which functions at the reducing end of PS I *(table 5)*.

4.3 Reagentia which accelerate photo-oxidation

BV accelerated photo-oxidative damage in a similar way as Diquat. This herbicide is reduced at the reducing side of PS I resulting in a free radical which is in turn reoxidized by oxygen. The last process gives rise to hydrogen peroxide production (BALDWIN 1969). Electrons needed for the reduction of Diquat and BV will possibly originate from some endogenous electron pool, since DCMU did not inhibit the stimulation of photo-oxidation by BV *(table 5)*. Such a pool may also account for the DCMU insensitive oxygen uptake in irradiated chloroplasts (SETCHENIA et al. 1971, EGNEUS 1972).

Diquat and BV may accelerate photo-oxidation for two reasons, by production of H_2O_2 and by the concomittant inhibition of protecting electron transport from the reduced to the oxidized side of PS I.

The last mechanism may also explain the effect of DSPD which appears to inhibit cyclic electron flow from PS I (RAVEN 1969). However, an inhibitive effect of DSPD on the oxidizing side of PS II, similar to the effect of SAL, cannot be excluded.

The accelerating effect on photo-oxidative damage of SAL, Az., TRIS, EDTA and high pH during chilling may be related to the inhibitive effect of these substances at the oxidizing side of PS II (KATOH et al. 1972, TREBST 1970, CHENIAE 1970). In addition to their inhibitive effect on the electron flow from H_2O to PS II, which will be very low at chilling temperature, these inhibitors may also inhibit the suggested back flow of electrons from the reducing to the oxidizing side of PS II, thus explaining the accelerating effect on photo-oxidative damage. HAM appears to inhibit electron transport between H_2O and PS II at a site different from the inhibition site of other inhibitors (KATOH et al. 1972). The ineffectiveness of HAM in photo-oxidation may be explained if HAM only inhibits electron flow from H_2O to PS II and not the back flow of electrons.

The existence of a cyclic pathway operating around PS II resembling the cyclic pathway around PS I would offer an explanation for the protection against photo-oxidative damage of leaves under low temperature stress. In addition this protective mechanism could function also under other stress conditions when the flow of energy absorbed by the pigments into the enzymatic photosynthesis reactions is inhibited and electrons accumulate at the reducing side of the photosystems.

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