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PHOTO-OXIDATIVE DAMAGE TO TRIPHENYL-TETRAZOLIUMCHLORIDE (TTC) REDUCING CAPACITY OF CUCUMIS LEAF DISCS DURING CHILLING

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SUMMARY

The effect of light, during pretreatment at 1 °C, on the capacity of Cucumis leaf discs to reduce TTC[1] was studied. Light decreases the capacity of the cells to reduce TTC by a photo-oxidative process. The kinetics show a lag phase followed by a rapid decrease. 6% oxygen as compared to 100% oxygen lengthens the lag phase but does not alter the rate of damage during the rapid phase. DCMU[2] has an inhibitive effect. A crude action spectrum of the decrease in TTC reduction shows that the blue and the red region of the spectrum are most effective, while the green and the far red region are relatively ineffective. It is suggested that chlorophyll as well as the carotenoids absorb the light energy which causes the harmful effect on the mechanism of TTC reduction.

1. INTRODUCTION

Tetrazolium salts are water soluble colourless substances which are easily reduced in living cells to water insoluble coloured formazans. Reduction takes place in the mitochondria (DRAWERT 1968), probably by cytochrome-oxidase (NACHLAS et al. 1960; PALMER & KALINA 1968).

LAKON (1942) first used TTC to demonstrate the viability of seeds while LARCHER & EGGARTER (1960) used TTC to demonstrate frost damage to cambium of *Pirus* species. STEPONKUS & LANPHEAR (1967) determined quantitatively the frost damage to *Hedera helix* using a refined TTC test. In experiments with the thermophilic plant *Cucumis sativus* damaging photo-oxidation of leaf pigments was found during chilling (VAN HASSELT 1972) and determination of TTC reduction after exposure to light at 1 ° C seemed a useful method for measuring light-induced injury during chilling. This paper describes the effect of light during chilling pretreatment on the capacity of leaf cells to reduce TTC.

2. MATERIAL AND METHODS

Cucumis plants (cv. Kleine Groene Scherpe) were grown in a greenhouse as described before (VAN HASSELT 1972). Discs 7 mm in diameter were punched between the largest veins of the first leaves of ± 14 days old plants and samples

Abbreviations
[1] TTC = 2,3,5-triphenyl-tetrazoliumchloride
[2] DCMU = 3-(3,4 dichlorophenyl)-1,1-dimethyl-urea

of discs were placed at 1 °C, in the dark or at a light intensity of 20,000 lux, as previously described (VAN HASSELT 1972). After pretreatment TTC reduction was measured using a modified version of the test described by STEPONKUS & LANPHEAR (1967): samples of 10 discs were dried between filter paper and placed in 10 ml calibrated test tubes having ground glass mouths which fitted condensor cones during extraction. The tubes were placed in plastic jackets to exclude light and 3 ml TTC-reagent (0.6% w/v TTC in 0.05 M Tris-HCl buffer pH 7.4, containing 0.05% v/v Tween as wetting agent) was added. The discs were kept below the surface of the reagent by a flattened glass rod and were vacuum infiltrated three times. The vacuum was released slowly by allowing entry of air through a glass capillary of 0.2 mm diameter. After infiltration samples were placed in an incubator at 30 °C for 24 hours, the TTC reagent was then drained and samples rinsed twice with distilled water. Next 6 ml 96% ethanol was added and the red formazan was extracted during 5 minutes in a boiling water bath. Extracts were cooled under running tap water and made up to a 10 ml volume with 96% ethanol. After mixing, the formazan concentration was measured spectrophotometrically at 530 nm.

A crude action spectrum was determined as follows: samples of 14 discs were placed at 1 °C in light of different wavelenghts.

Leitz Prado Universal slide projectors equipped with a Hektor objective 1:2.5/200 mm were used as light source. Light of sufficient energy was obtained with broadband interference filters type Filtraflex K, 5 × 5 cm, from Balzers, Liechtenstein.

Wavelenght of maximum transmission (λ max.) of the filters is for K_1 (410 nm), K_2 (450 nm), K_3 (510 nm), K_4 (610 nm), K_6 (663 nm) and K_7 (700 nm). The bandwidth of the filters is about 50 nm at 50% of the maximum transmission.

Light intensity was measured by means of a compensated thermopile type CA 1 combined with a galvanometer type AL 1 (both of Kipp & Zn., Delft, Holland). Light energy during the experiments was adjusted to 51×10^3 ergs cm^{-2} sec^{-1} for filter K_2. About equal quanta for the different spectral regions transmitted by the filters were calculated by multiplying the energy transmitted by K_2 with 450 divided by λ max of the other filters. The calculated energy was obtained by adjusting the light source with a Variac.

3. RESULTS

3.1. Effect of temperature during pretreatment

Fig. 1 shows that pretreatment at 1 ° C in the light decreases the rate of TTC reduction to 6% of the initial rate. Pretreatment at 5 °C in the light decreases reduction to 37%. However, a dark pretreatment at those temperatures results in a slight increase in TTC reduction rate. TTC reduction is approximately equal to the initial rate after pretreatment at 10 °C in the light as well as in the dark.

Pretreatment at 15 °C, 20 °C and 25 °C, in the light, causes an increase in TTC reduction, while dark pretreatment at those temperatures causes, on the other

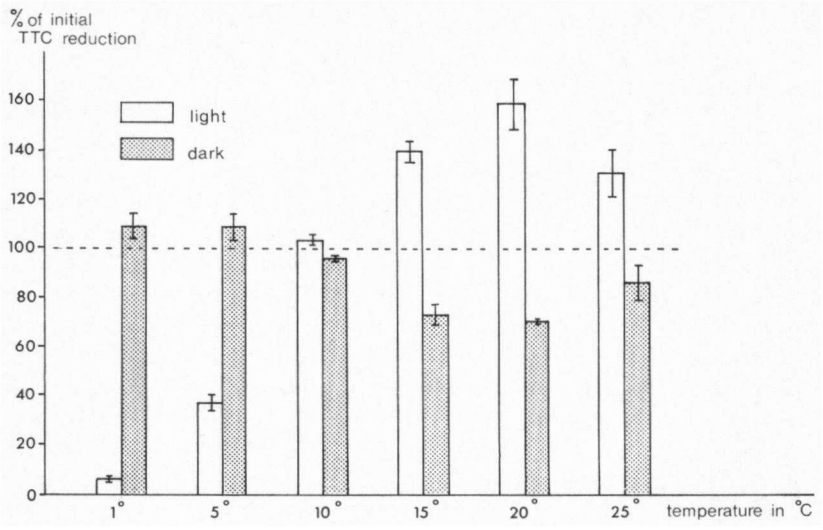

Fig. 1. TTC reduction after 24 hours pretreatment at different temperatures in the light and in the dark.
Large differences in TTC reduction were observed between experiments performed under the same experimental conditions. However, the overall picture was always the same. Therefore in *figs. 1, 2, 3,* and *4,* the data of one representative experiment are shown. Mean values of three samples together with their standard error are given.

hand, a decrease, which is maximal at 20 °C, with a value of 70 % of the initial reduction. It can be concluded that during pretreatment at low temperatures, especially at 1 °C, light causes a decrease in the rate of TTC reduction.

3.2. Kinetics of the decrease in reduction capacity

Fig. 2 demonstrates that there is little decrease in TTC reduction during the first 6 hours of pretreatment at 1 °C in the light. After 6 hours there is a rapid decrease while in the dark there is a constant slow decrease. It can be concluded

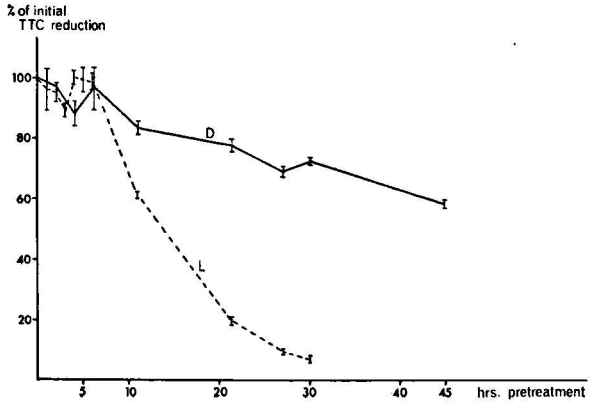

Fig. 2. TTC reduction after different times of pretreatment in the light (L) and in the dark (D) at 1 °C.

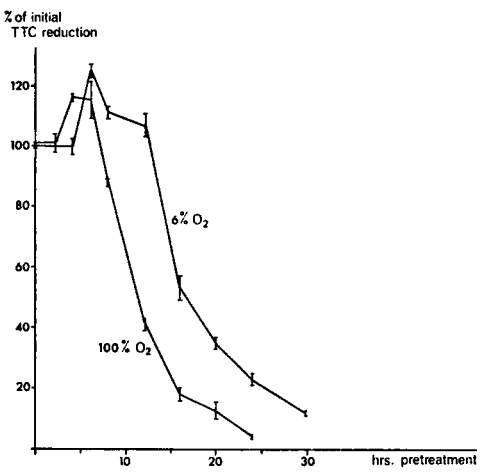

Fig. 3. TTC reduction after pretreatment in an atmosphere of 6% and of 100% oxygen in the light at 1 °C.

that there is a lag phase in the damaging effect of light on TTC reduction at 1 °C.

3.3. Effect of different oxygen concentrations during pretreatment

In order to test if a photo-oxidative process causes the decrease in TTC reduction, the effect of different oxygen concentrations during pretreatment was studied. As demonstrated in *fig. 3*, there is a shorter lag phase after pretreatment in 100% oxygen than in 6% oxygen. At both concentrations there is an increase in reduction during the lag phase. Such an increase, observed in several experiments, was not found in the experiment of *fig. 2* and its cause is unknown. The rate of decrease after the lag phase is the same after pretreatment at 6% oxygen and at 100% oxygen.

It is concluded that 6% oxygen, as compared to 100% oxygen, delays the beginning of photo-oxidative damage to the TTC reducing mechanism.

When, however, pretreatment in the light at 1 °C takes place in an atmosphere of pure N_2, the decrease of TTC reduction is inhibited to a large extent (*fig. 4*). It can be concluded therefore that the decrease of TTC reduction caused by light during pretreatment at 1 °C is to a large extent due to a photo-oxidative process.

3.4. Effect of DCMU

The effect of different concentrations of DCMU during pretreatment at 1 °C is shown in *fig. 5*. DCMU inhibits the light-induced decrease of TTC reduction. Maximum inhibition is reached at 5×10^{-6} M. Pretreatment at 10^{-4}M in the dark results in a decrease, while lower concentrations have little effect in the dark. This decrease is not an effect of ethanol (*fig. 5*). It may be concluded that there is some direct inhibiting effect of 10^{-4}M DCMU on the mechanism of TTC reduction.

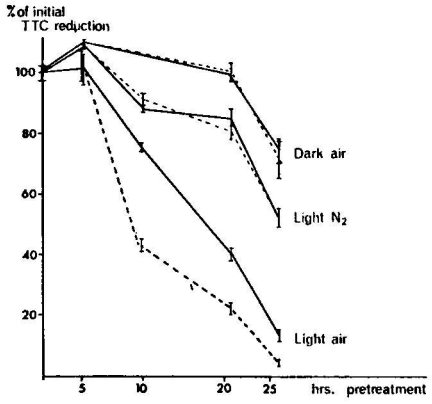

Fig. 4. TTC reduction after pretreatment on demineralized water (– – – –) and on 10^{-5}M DCMU (———) in an atmosphere of air or of pure nitrogen (N_2).

Fig. 4 demonstrates that 10^{-5}M DCMU is ineffective in the dark in air as well as in the light in nitrogen. When, however, both light and oxygen are present, DCMU has an inhibiting effect. It can be concluded that DCMU inhibits the damaging photo-oxidative process.

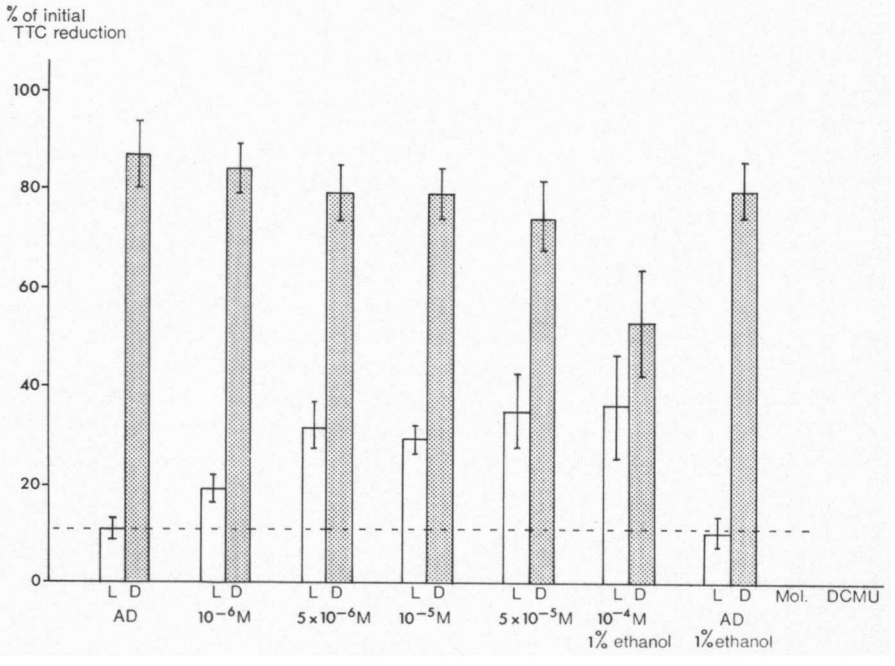

Fig. 5. TTC reduction after 24 hours pretreatment at different DCMU concentration in the light and in the dark at 1 °C. Mean values of five experiments are given together with their standard error.

3.5. Action spectrum

Fig. 6 shows a crude action spectrum of the light-induced decrease of TTC reduction. Maximal decrease is caused by blue light (K1). A second maximum is observed in the red region (K6). The decrease is relatively low in the green (K4) and in the far red (K7) spectral region. The relative quantum efficiency of blue light is more than 1.5 times higher than that of red light.

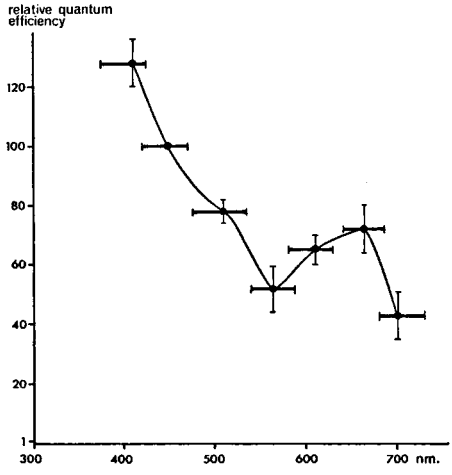

Fig. 6. Action spectrum. The decrease of TTC reduction after 16 hours at different wavelenghts at 1 °C. Horizontal lines show the spectral regions where transmission is 50 % or more of the maximum transmission. Dots indicate wavelenghts of maximum transmission.
The relative quantum efficiency is expressed as the difference between the reduction value of the dark control and the reduction value after pretreatment in light of the indicated wavelenghts. The mean value of three experiments with their standard deviation is given, after adjusting K_2 to 100.

4. DISCUSSION

Kinetics of the decrease of TTC reduction, induced by photo-oxidation during pretreatment at 1 °C, resemble kinetics of the photo-oxidative degradation of leaf pigments at 1 °C in *Cucumis* leaf discs (VAN HASSELT 1972). In both cases a lag phase precedes a rapid, damaging phase. In 6 % oxygen, as compared with 100 % oxygen, there is a considerable increase in the lag phase but no alteration in the rate of the rapid phase. This suggests that during the lag phase a substance which protects the TTC reducing mechanism against photo-oxidation is oxidized.

Such a substance may be carotene. There is circumstantial evidence for a protective function of carotene against aerobic photosensitization (KRINSKY 1968).

In addition, it was observed previously (VAN HASSELT 1972) that photo-oxidative degradation of carotene in *Cucumis* leaf discs at 1 °C shows a shorter lag phase than the lag phase of the photo-oxidative damage to the TTC reducing mechanism observed in the present experiments. It is well established that DCMU inhibits electron flow close to the reducing side of photosystem II in the chloroplast. This, and the fact that the action spectrum of the light-induced damage resembles the absorption spectrum of chlorophyll, suggests that the first stage of photo-oxidation occurs in the chloroplast. However, TTC reduction occurs in the mitochondria.

The different localization of the site of TTC reduction and the primary effect of light suggest an inhibition of the supply of substrate and coenzymes required for the reduction of TTC in the mitochondria. Photo-oxidative damage in the chloroplast could cause such an inhibition, supposed to be the origin of the decrease of TTC reduction caused by frost damage (KOENIGS 1966). LUNDEGÅRDH (1965) described an inhibitive effect of DCMU on respiration, especially on the autoxidation of cytochrome b3. The inhibiting effect of 10^{-4}M DCMU in the dark (*fig. 5*) may be attributed to a similar inhibition of respiration. This would imply that TTC is not only reduced by cytochrome c but also by cytochrome b3.

The high relative quantum efficiency in the blue-green region of the spectrum, where chlorophyll absorbs relatively little energy, suggests that carotenoids, beside chlorophylls, are sensitizers of possible harmful light energy. The relatively high damaging effect of blue light can be related to the results of KANDLER & SCHÖTZ (1956) who showed that red light is much less effective than blue light in bringing about photo-oxidative damage to α- and β-carotene-lacking *Chlorella* mutants.

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