The significance of glutathione in protection of *Brassica pekinensis* against hydrogen peroxide and paraquat toxicity

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Abstract

In oxidative stress there are many antioxidants which protect the plant, glutathione is one of them. Hydrogen peroxide and paraquat (for its generation of superoxide) are two compounds that cause stress in plants and are used in many researches about stress and the resistance of plants against it. In this research the question was searched whether glutathione does protect when the plant is under stress caused by hydrogen peroxide or paraquat and decreased enzyme activity. The conductivity for paraquat and also the fluorescence showed a increase in damage in both temperature treatments but especially the cold (low enzyme activity) treatment. The pigments showed a similar result only the warm leaf discs were bleached and the cold leaf discs had a decrease but still they were green. The thiol-content decreased also more at the warm temperature, and not as much when chilled. For hydrogen peroxide the results for pigment and thiols were the same as the ones for paraquat. There is a decrease, but it is only very slightly in the cold, and more in the warmth. The decrease only happened at the very high concentration of 512 mM H_2O_2. The conductivity showed the same results as with paraquat, but the fluorescence of hydrogen peroxide was in accordance wit the pigments and thiol-content. Again it only happened at the highest concentration. There is no indication that glutathione protects, and that the tests using hydrogen peroxide and paraquat are very trustworthy.

Introduction

In the cells various important processes, like photo-synthesis, have reactive oxygen species (ROS) as a by-product (van Breusegem and Dat 2006). When the consumption of NADPH by the Calvin cycle is not rapidly enough oxygen will serve as an electron receptor and the ROS superoxide may come into existence (Perl Treves 2000). Superoxide will, in its turn cause other ROS. Many environmental factors (Fig. 1), like ozone or heavy metals, can cause increased ROS production and therefore cause oxidative stress in plants (Gechev et al. 2002). In large quantities ROS can react with almost all cellular compounds and cause damage (Fig. 2) by inactivating enzymes, destructing DNA, cause lipid peroxidation and cell death (van Breusegem and Dat 2006). Therefore the plant has a defense system of several antioxidants to keep the reactive oxygen species under control (van Breusegem and Dat 2006).

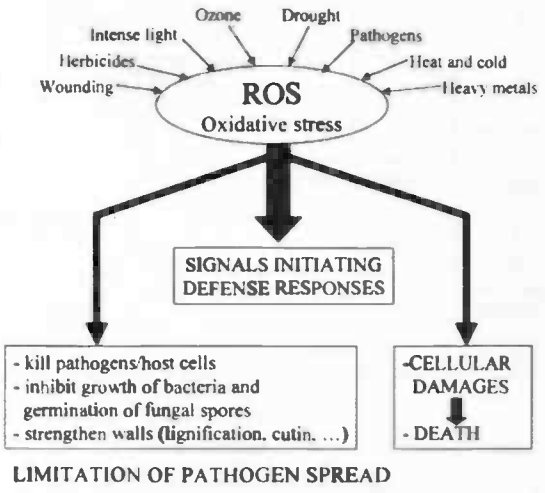


Figure 1. The factors that can cause oxidative stress and the results they initiate.

Reactive oxygen species are; superoxide ($O_2^{\bullet-}$), singlet oxygen ($O^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) (Perl-Treves and Perl 2000). Hydrogen peroxide is the most stable of these compounds and also one that diffuses easily through membranes and aqueous compartments (Perl-Treves and Perl 2000; Willekens et al. 1997). They cause the formation of other radicals by extracting electrons from organic compounds with which they collide causing chain reactions which lead to the destructions mentioned before (Fig. 2) (Perl-Treves and Perl 2000). $OH^{\bullet}$ is the most reactive and destructive radical, therefore the real danger of superoxide and hydrogen peroxide is when they are transformed into hydrogen radical (Gaspar et al. 2002).

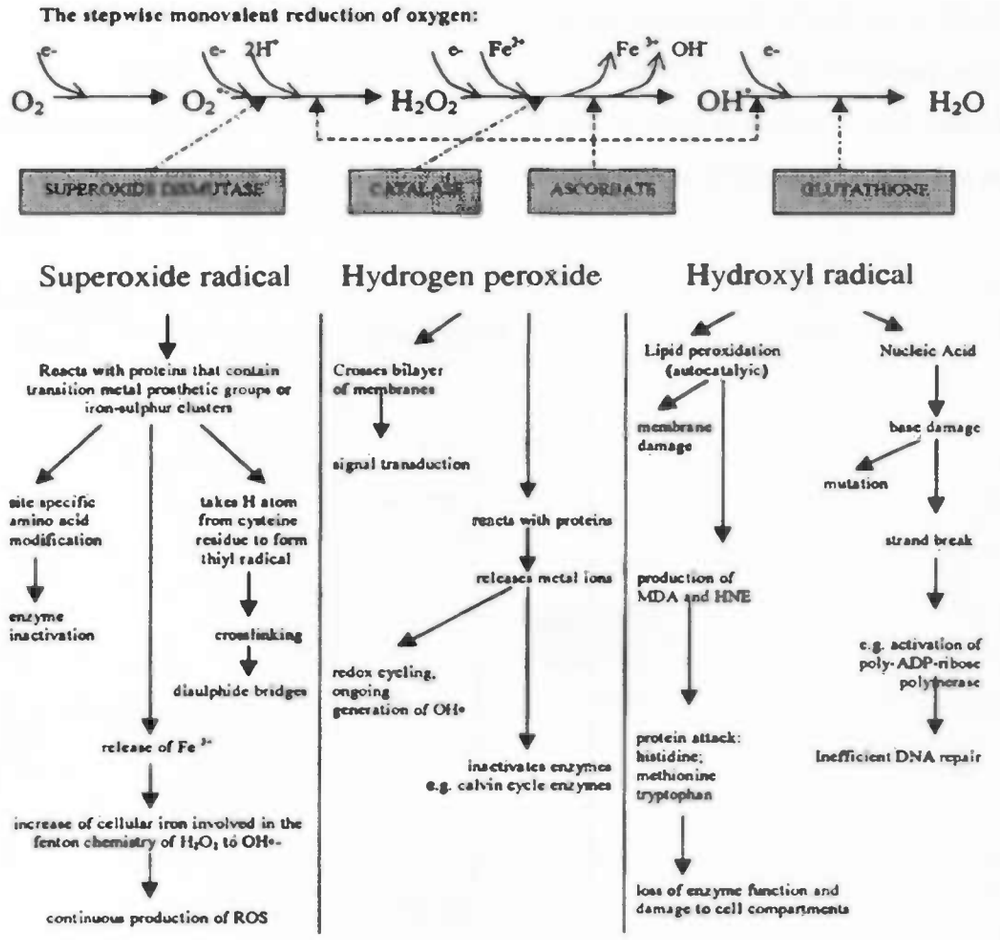


Figure 2. The different ROS species and how they are produced and discarded of in the plant cell and their harmful effects (Cassels Curry 2001).

Anti-oxidants like superoxide dismutase, catalase, ascorbic acid and glutathione are important to regulate the amount of ROS in the cells (Fig. 2). Superoxide dismutase (SOD) will change superoxide into hydrogen peroxide by the reaction: $2\ O_2^- + 2\ H^+ \rightarrow O_2 + H_2O_2$, with SOD as catalyst (Perl-Treves and Perl 2000). The reaction between superoxide and hydrogen peroxide, the Haber-Weiss reaction: $H_2O_2 + O_2^- \rightarrow O_2 + OH^{\bullet} + OH^-$, causes hydroxyl radical to form in the presence of metal ions which serve as catalyst (Perl-Treves and Perl 2000). To prevent this $OH^{\bullet}$ formation hydrogen peroxide must be removed. The enzyme catalase is especially important for the regulation of H_2O_2, it acts as a sink for it (Willekens et al.1997; Tsanko et al. 2005). Catalase, catalyst in the reaction: $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$, is present in the cytoplasm and the peroxisome. Ascorbic acid, in the Halliwell-Asada pathway: 2 ascorbate + $H_2O_2 \rightarrow$ 2 MDA + 2 H_2O, with APX as catalyst, is the H_2O_2 scavenger in the

chloroplast (Perl-Treves and Perl 2000). Apart from these scavengers there is also glutathione (Fig. 3) which has several roles. It is the compound which turns DHA back into ascorbate, but it has also a more direct protective role in reactions with oxygen species (Perl-Treves and Perl 2000). When catalase is deficient and cannot remove the H_2O_2 it is found that glutathione takes the role of protection (Willekens et al. 1997).

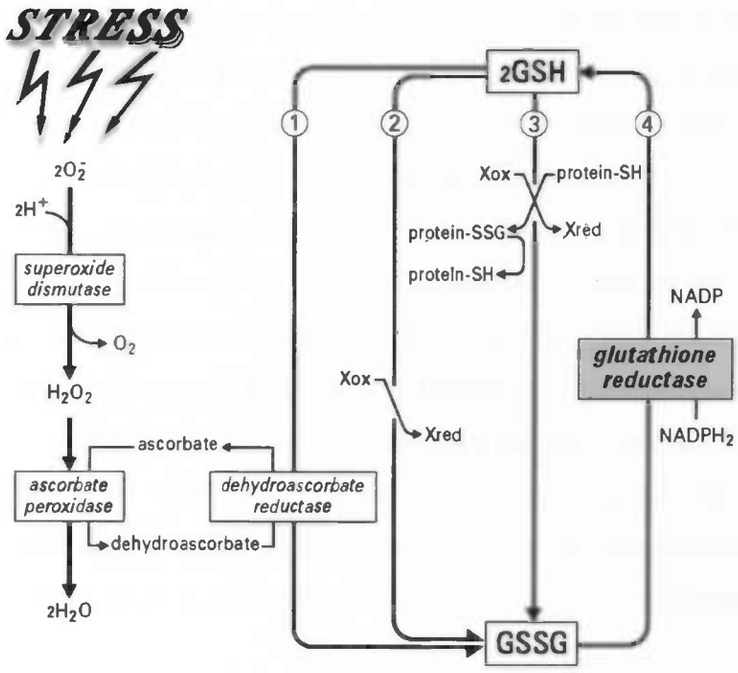


Figure 3. Significance of glutathione and glutathione reductase in the enzymatic detoxification of reactive oxygen species in the glutathione-ascorbate cycle (1) and as thiol buffer in the protection of proteins via direct reaction with reactive oxygen species e.g. superoxide, H_2O_2 and lipid hydroperoxides (2), or by the formation of mixed disulfides (3) (adapted from De Kok and Stulen, 1993).

When there is a limited and controlled amount of the H_2O_2 it can initiate defense responses, and trigger PCD in cells infested with pathogens (Levine et al. 1994; Desikan et al. 1998). However, though much is known about the reactions that take place in the cell with hydrogen peroxide, knowledge about what is does to the entire plant is still lacking despite much research (van Breusegem and Dat 2006). Despite the fact that H_2O_2 is thought to be a signal for cell death, it is not always clear whether the hydrogen peroxide is cause or consequence. Sometimes when the anti-oxidants are not able to remove excess H_2O_2 that was formed under oxidative stress, cell death may be triggered instead of the stress acclimation that was actually intended (Tsanko et al. 2005). In research hydrogen peroxide is sometimes used to cause stress in plants

and to see whether transgenient plants are more resistant to stress. Another compound that is used to cause stress in plants is paraquat. Under the influence of light Paraquat causes a huge production of superoxide: $PQ^{+} + O_2 \rightarrow PQ^{2+} + O_2^{-}$. It has the same effects as hydrogen peroxide but it is a bit more toxic because of this formation of superoxide, which is absent with H_2O_2 (Perl-Treves and Perl 2000).

The question in this research is:

- What is the role of glutathione in the protection of *Brassica pekinensis* against oxidative stress caused by hydrogen peroxide or paraquat? Will the effect of both toxicants be the same or will it differ? And what will be the effect of decreased enzyme activity using temperature?

Since chilling has an effect on the activity of an enzyme, it will be used to diminish the enzyme activity. It is expected that when chilled the plant will be less able to defend itself and therefore will bleach and die more quickly. Also it is expected that the glutathione will be gone first, before the carotenoids and the chlorophyll, because of its protective role. After glutathione the carotenoids will diminish and eventually chlorophyll a followed by chlorophyll b, leaving the leaf discs bleached and dead. Paraquat will have a more toxic effect so the leaf discs treated with paraquat will show an effect before the leaf discs treated with hydrogen peroxide.

Material and method

Brassica pekinensis was first sown on vermiculite and after 11 days put on a 25% hoagland solution. The plants were grown in a climate room of 21°C during the day and 17°C during the night. The humidity was 60-70%, and the light was 14 hours/ day and the light intensity is 350-400 µmol m^{-2} s^{-1}. Leaf discs of 17 mm in diameter were taken after 11 days on nutrient solution from the second and third leaves. The leaf discs were put into glass petridishes. Catalase is of paramount importance for regulating H_2O_2 homeostasis as it can function as a cellular sink for H_2O_2. Catalase deficiency leads to elevation of H_2O_2 levels and triggering cell death (Willekens et al. 1997; Tsanko et al. 2005). It also is very likely to be the cause for the high exogenous concentration necessary to let bleaching and cell death occur 500 mM was necessary (Levine et al. 1994). Therefore the concentrations 512; 128; 32; 8; 2 and 0 mM hydrogen peroxide were used. It is expected that antioxidants are decreased before the

cell is really damaged by bleaching or cell death. In each concentration twelf leaf discs were put, of which two were used for chlorophyll and carotenoid content, three for conductivity measurements and the other seven for thiol content. The petridishes were placed for 24 hours in two cabinets, one with 6°C and the other with 23°C. The enzyme activity goes down with temperature therefore two different temperatures were used to see that at a higher temperature the leaf disc is more protected. The light intensity was about 300 µmol m^{-2} s^{-1}. The leaf discs were illuminated for the full 24 hours. For paraquat the same method was used, but with the concentrations; 10; 5; 1 and 0 µM paraquat. Pigments and carotenoids were measured using the Lichtenthaler method (Lichtenthaler 1987). The leaf discs are weighed, then 10ml 100% acetone is added per gram fresh weight. This is centrifuged at 2500 rpm for 20 minutes. 0.5ml of the supernatant is added to 3.5ml acetone and 0.5ml water, and this is done in duplicate. Then the $E_{663.2}$, $E_{646.8}$ and E_{470} are measured with 80% acetone as a blanc. The chlorophyll content was calculated in mg g^{-1} FW using the following formulas;

- $Chl_a = 12.25\ E_{663.2} - 2.97E_{646.8}$, $Chl_b = 21.50E_{646.8} - 5.10E_{663.2}$,
- $Car_{x+c} = (1000E_{470} - 1.82Chl_a - 85.02Chl_b)/\ 198$ (Lichtenthaler 1987).

Thiol-content was determined using photospectronomy (De Kok et al.1988). The leaf discs were weighed and then 10ml extraction medium (80mM sulfosalic acid + 1mM Na_2EDTA + 0.15% ascorbic acid) was added to every g FW and homogenized with an Ultra turax. This homogenate is filtrated over 1 layer of miracloth into small plastic centrifuge tubes placed in ice. Then the tubes are centrifuged in a Beckman J2-MC cooling centrifuge at 16.000 rpm for 15 minutes. The reaction is carried out in duplicate, with two blancs per extract and 3 DTNB blancs per series. First 1.0ml is put together with 0.1ml of DTNB, in the sample blancs the DTNB is replaced by 0.1ml demi-water. For the series blancs 1.0ml of extraction medium is put together with 0.1ml of DTNB. Then 2.0ml of Tris-HCL is quickly added to all and vortexed, then the E412 is measured. SH-content is calculated by; ((1-(2 + 3))*10*3.1)/ 13.6 in which 1 = samples + DTNB, 2 = DTNB blancs, 3 = samples + water (De Kok 1988).

Conductivity measurements were done using a Höfelt CDM 2e conductivity meter. Three washed leaf discs were first put in 3 ml of demiwater and put in a warm water bath for 30 minutes at 30°C then the conductivity was measured. Then they were put in 90°C for 10 minutes and then cooled down till 30°C again before the conductivity was measured for the second time. The fluorescence was also measured in a separate

experiment, the same parameters were used, though this time there were only five leaf discs per petridish and all five were used for the fluorescence measurement. The Fv/Fm value which is the measured and showed parameter, it shows the maximum efficiency at which the light absorbed by the chlorophyll in the PSII process is converted into chemical energy (Baker and Rosenqvist 2004). For the analysis of the results a students T-test was used with $\alpha = 0.01$.

Results and discussion

Paraquat

When a plant (or leaf disc) is put into paraquat and it is illuminated, there will be a production of superoxide, which will cause other reactive oxygen species in its turn. The conductivity (Fig. 4) shows the leakage of electrolytes in the leaf discs. Both temperature treatments have an increase in conductivity. The cool treatment has a higher leakage at 0 and 1 μM paraquat then the 23°C, which is not the case in the higher concentrations. This is probably the result of changes in the cell membrane permeability when the temperature is low. However, when the concentration increases the difference disappears, so eventually the concentration damages the cell membrane so much that the difference in permeability has been overcome. The fluorescence (Fig. 5) shows that at every concentration and at both 6 and 23°C the efficiency of the leaf discs to absorb light for photosynthesis is diminished (Baker and Rosenqvist 2004). At a lower temperature the result is also due to photo-inhibition, because cold stress causes photo-inhibition, which is not the case at a higher temperature. This explains some of the lower Fv/Fm value at the 6°C treatment compared to the 23°C treatment. The combination of cold and paraquat, however damages the ability of chlorophyll to absorb light for chemical energy more. So paraquat does damage the leaf discs severely, in all the different concentrations, but especially in combination with cold.

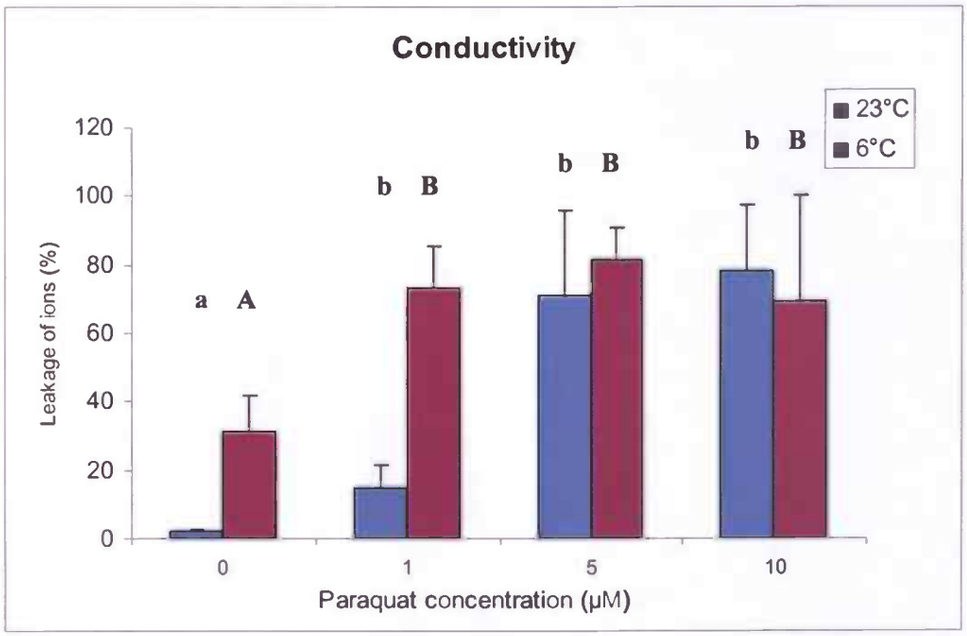


Figure 4. The impact of paraquat on the electrolyte leakage by measuring the conductivity of leaf discs of *Brassica pekinensis* upon incubation at 6 and 23°C in light. Leaf discs were incubated for 24 h. Data on ion leakage represents the mean of 3 measurements with 3 leaf discs in each (± SD).

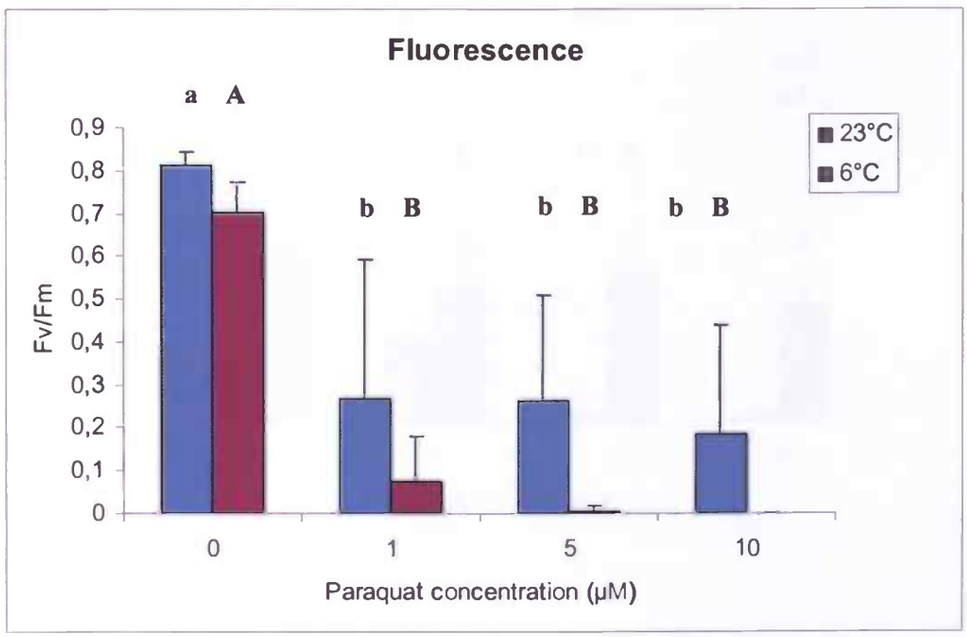
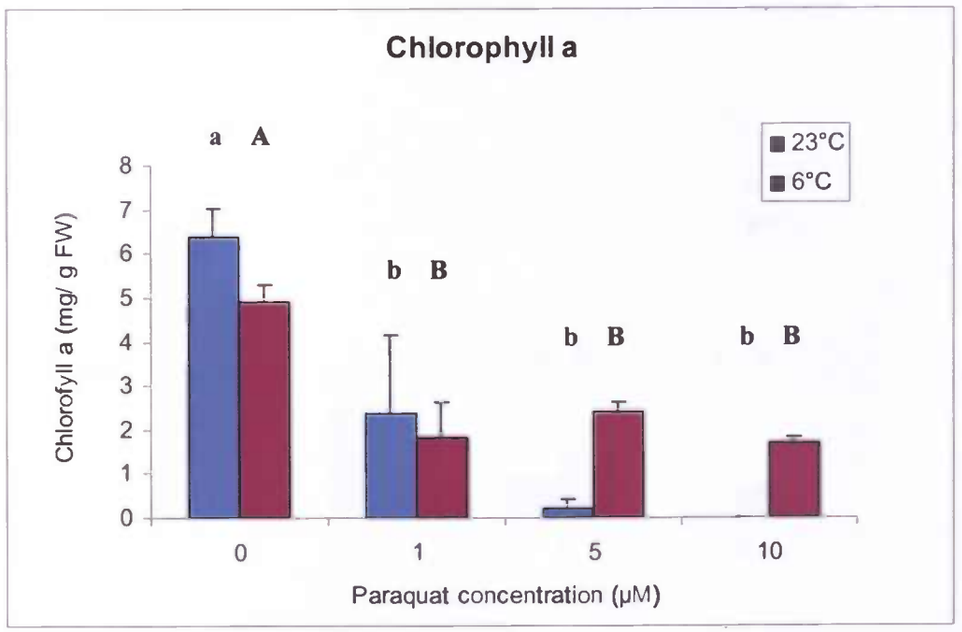
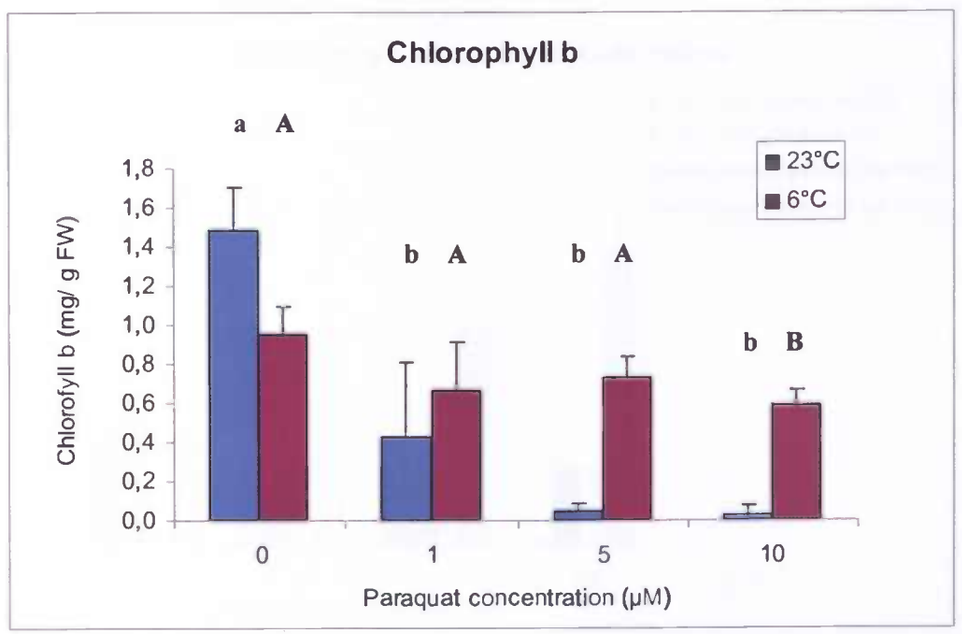


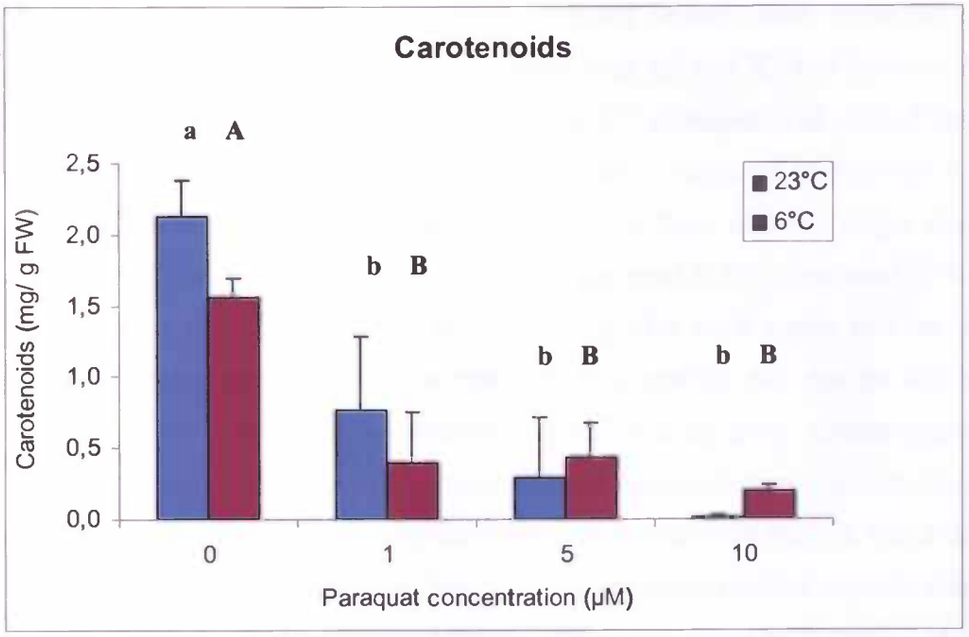
Figure 5. The impact of paraquat on fluorescence of leaf discs of *Brassica pekinensis* upon incubation at 6 and 23°C in light. Leaf discs were incubated for 24 h. Data on fluorescence represents the mean of 5 leaf discs measurements (± SD).


Chlorophyll a
a A
b B
b B
b B
23°C
6°C
Chlorofyll a (mg/ g FW)
8
7
6
5
4
3
2
1
0
0
1
5
10
Paraquat concentration (µM)

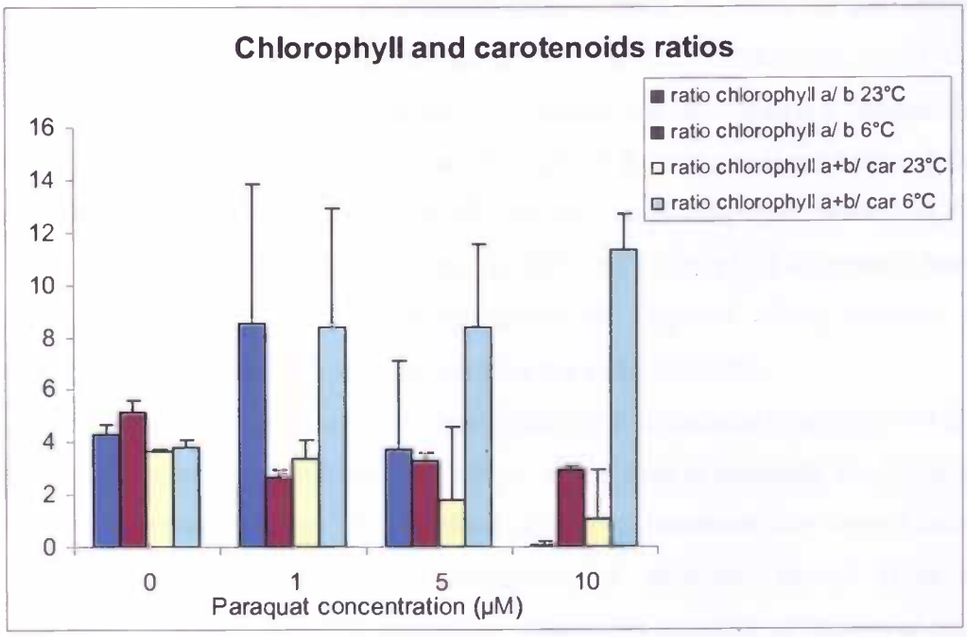
A


Chlorophyll b
a A
b A
b A
b B
23°C
6°C
Chlorofyll b (mg/ g FW)
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
1
5
10
Paraquat concentration (µM)

B



C



D

Figure 6. The impact of paraquat on chlorophyll a, b and carotenoid content of leaf discs of *Brassica pekinensis* upon incubation at 6 and 23°C in light. Leaf discs were incubated for 24 h. Data on chlorophyll a (A), chlorophyll b (B) and carotenoid content (C) represents the mean of 3 measurements with 2 leaf discs in each (± SD). The ratios (D) of chlorophyll a/ b and chlorophyll a and b/ carotenoids (± SD).

The chlorophyll content seems to give a different picture. Both chlorophyll a and chlorophyll b diminish when paraquat is added (Fig. 6A and B), and in both cases the decrease is greatest in the 23°C compared to the 6°C treatment. The chilled treatment, however, shows a more profound decrease in the content of chlorophyll a, while chlorophyll b only shows a very slight decrease. So there is still a larger amount of chlorophyll a and especially b in 5 and 10 µM paraquat at 6°C compared to the same concentrations at 23°C, as seen in the ratios (Fig. 6D). So it seems that the damage shown in fluorescence in at least the 6°C treatment is not due to the loss of chlorophyll a or b. With the carotenoids (Fig. 6C) it is the same, though slightly less visible. In the high temperature treatment the carotenoids decrease till almost nothing in the highest concentration. In the low temperature treatment there is also a decrease along the different concentrations, but still the carotenoid content remains more then at 23°C. These results are reflected by the differences found in the ratios (Fig. 6D), especially those found at the highest paraquat concentrations and the differences between the two temperature treatments. At the control the ratios for both chlorophyll a/b and chlorophyll/ carotenoids are similar for 6 and 23°C. But then the 23°C shows first a rise in ratio and then a decrease. This means that chlorophyll b diminished more at first, but over all the concentrations chlorophyll a decreases more bringing the ratio down again till it is very low at the 10 µM. For the chlorophyll/ carotenoid ratio the temperatures cause different reactions. At 23°C the chlorophyll disappears more then the carotenoids. The cold treatment shows the opposite, more decrease of the carotenoids then of the chlorophyll, because the ratio increases.

The thiol content of the leaf discs diminishes with increasing concentration (Fig. 7). The 23°C treatment has a immediate effect, when there is paraquat; the SH-content is reduced to almost nothing. The leaf discs in the 6°C treatment also show a decreasing thiol-content with increasing concentrations of paraquat, though it is not as immediately as with the 23°C treatment. This is the opposite of what was expected, because the lower enzyme activity should result in a faster decrease of thiol-content in the low temperature treatment. But also the pigments had this anomaly. It seems that the cold has an effect on both the leakage and fluorescence that has not got much to do with the damage caused by paraquat. The decrease in enzyme activity is perhaps also diminishing the reactivity of paraquat, so that the pigments and glutathione are not scavenged as much as in the 23°C treatment. At 23C there might be bleaching, but every compound decreases at the same moment. It is not so that there is an order in

the disappearance of the pigments, the thiol-content does decrease very much already in the 1 µM but this is not enough to say that glutathione protects the pigments.

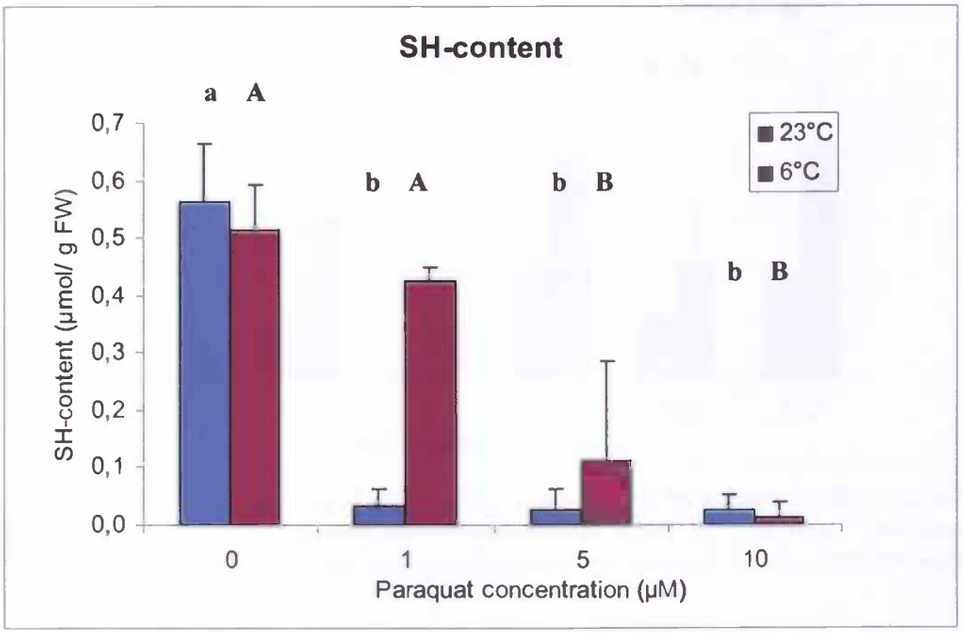


Figure 7. The impact of paraquat on SH-content of leaf discs of *Brassica pekinensis* upon incubation at 6 and 23°C in light. Leaf discs were incubated for 24 h. Data on SH-content represents the mean of 3 measurements with 7 leaf discs in each (± SD).

Hydrogen peroxide

Hydrogen peroxide has a less toxic effect then paraquat, because there is not so huge a production of superoxide. Furthermore there are high doses of catalase in the cells to remove much of the H_2O_2 when it gets too much. There is leakage of electrolytes in the concentrations of hydrogen peroxide, also in the lower concentrations. There is more leakage at the 6°C treatment. This is, as seen before with the paraquat results, likely to be result from a difference in the permeability of the cell membrane when the leaf discs are chilled. Here the difference remains till the end. The leakage is less then with paraquat, here it is not more then 40% for 6°C and 20% for 23°C. With paraquat it was eventually 70% for both treatments.

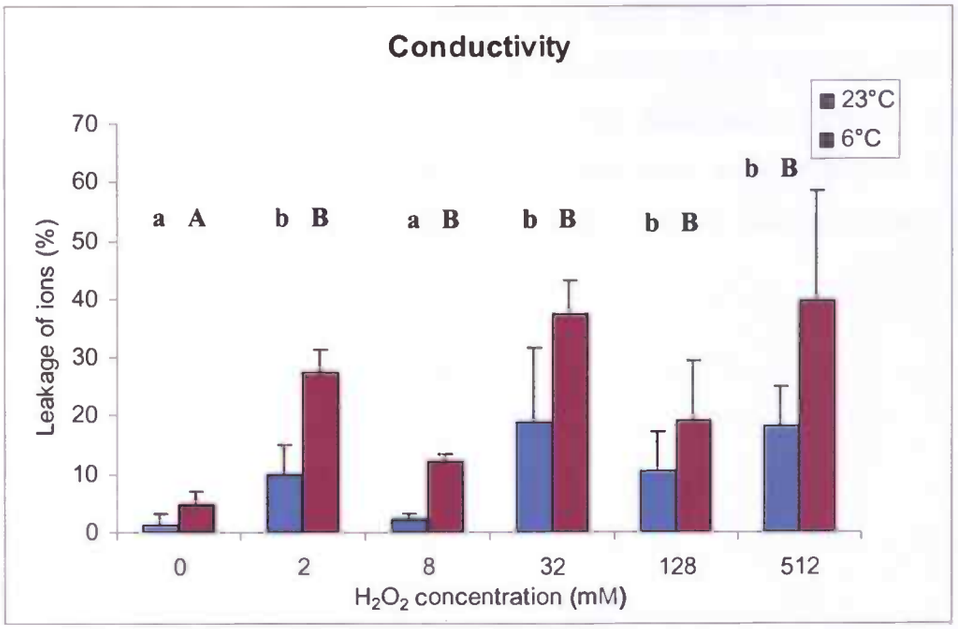


Figure 8. The impact of paraquat on the electrolyte leakage by measuring the conductivity of leaf discs of *Brassica pekinensis* upon incubation at 6 and 23°C in light. Leaf discs were incubated for 24 h. Data on ion leakage represents the mean of 3 measurements with 3 leaf discs in each (± SD).

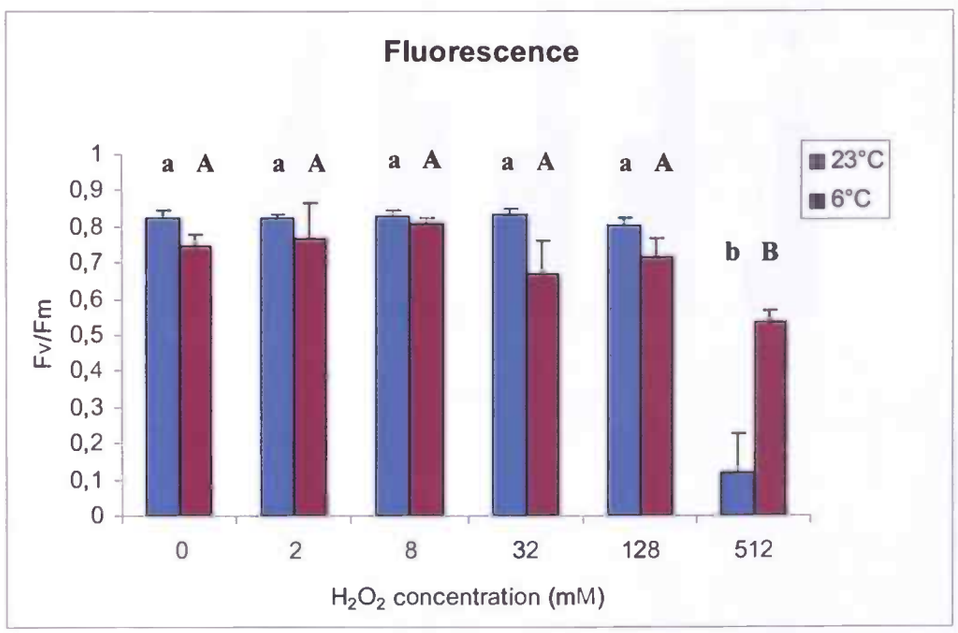
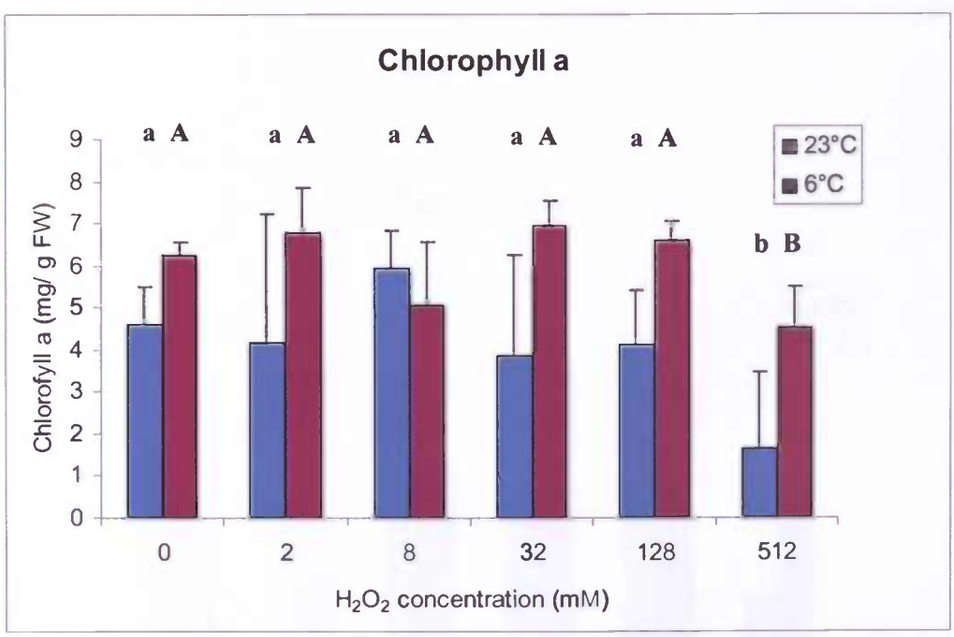
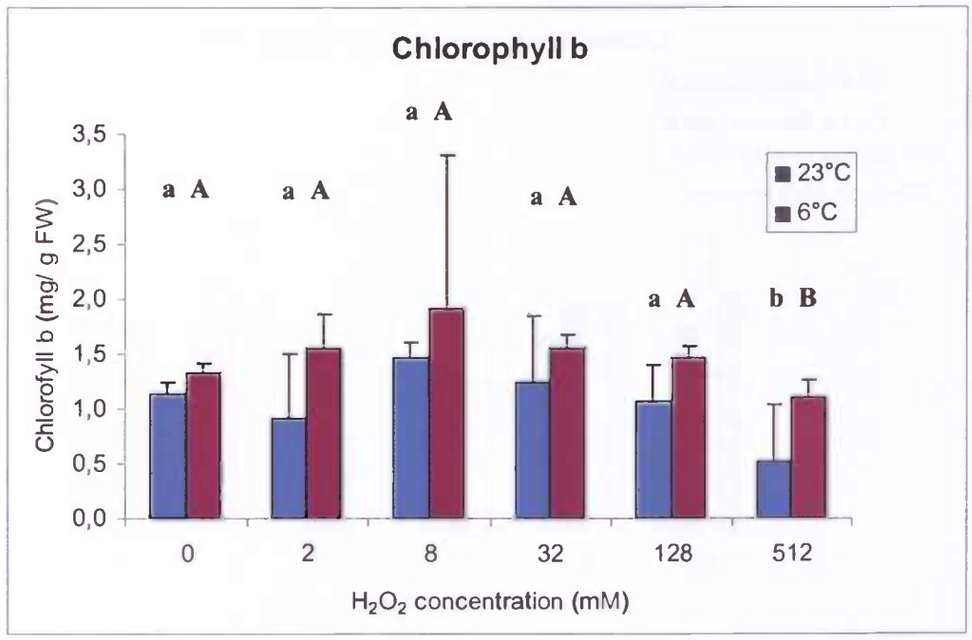


Figure 9. The impact of paraquat on fluorescence of leaf discs of *Brassica pekinensis* upon incubation at 6 and 23°C in light. Leaf discs were incubated for 24 h. Data on fluorescence represents the mean of 5 leaf discs measurements (± SD).

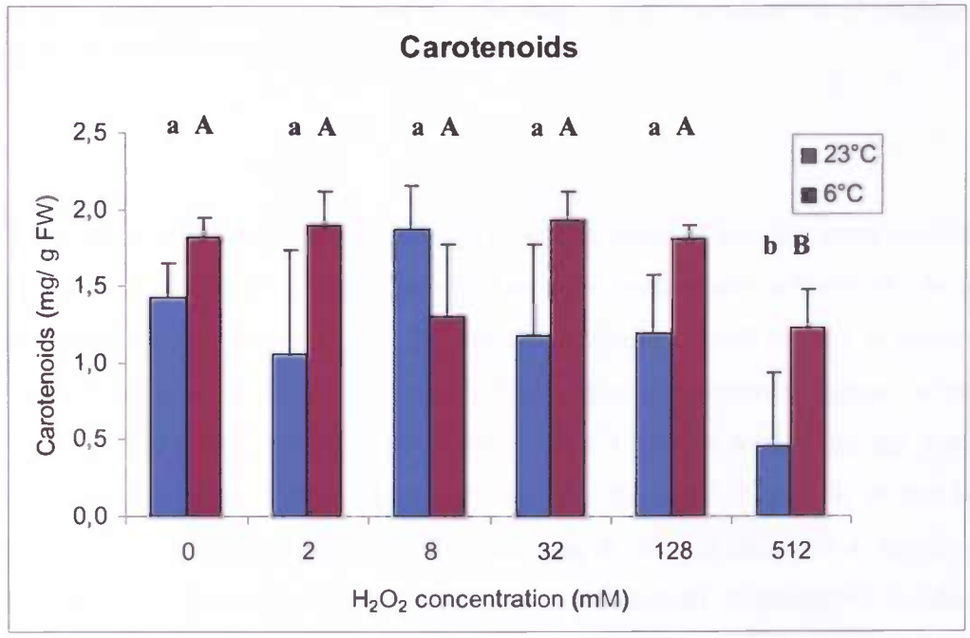
The damage done to the ability to absorb light energy for photosynthesis in the leaf discs is not much, only at the highest concentration of H_2O_2 there is a decrease at 23°C. This was already visible when the leaf discs were removed from the concentrations; only the 512 mM at 23°C leaf discs were bleached. The cold treatment shows less damage, though there is also a slight decrease in the 512 mM concentration.



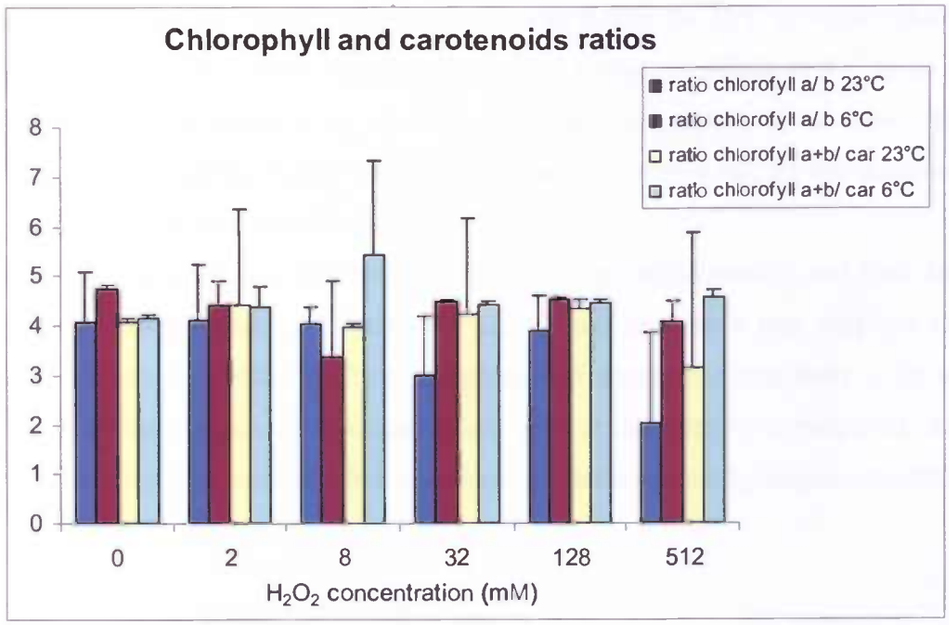
A


Chlorophyll b
a A
a A
a A
a A
a A
b B
23°C
6°C
Chlorofyll b (mg/ g FW)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
2
8
32
128
512
H2O2 concentration (mM)

B


Carotenoids
a A
a A
a A
a A
a A
b B
23°C
6°C
Carotenoids (mg/ g FW)
2,5
2,0
1,5
1,0
0,5
0,0
0
2
8
32
128
512
H2O2 concentration (mM)

C



D

Figure 10. The impact of paraquat on chlorophyll a, b and carotenoid content of leaf discs of *Brassica pekinensis* upon incubation at 6 and 23°C in light. Leaf discs were incubated for 24 h. Data on chlorophyll a (A), chlorophyll b (B) and carotenoid content (C) represents the mean of 3 measurements with 2 leaf discs in each (± SD). The ratios (D) of chlorophyll a/ b and of chlorophyll a and b/ carotenoids (± SD).

The content of chlorophyll a (Fig. 10A) remains more or less the same until the 512 mM concentration, there the chlorophyll a drops consistently in the 23°C. In the low temperature treatment, however, the content decreases as well but not as much. When it is cold the amount of chlorophyll a is higher then at the more preferred temperature for enzyme activity. Chlorophyll b behaves in a similar way. First the content of chlorophyll b (Fig. 10B) remains more or less the same, before it, at the last and highest concentration, decreases. This decrease is, like chlorophyll a, more at 23°C then at 6°C. The cold treatment has more chlorophyll b compared to the warm treatment. So the behaviour of the chlorophyll in hydrogen peroxide is similar to the behaviour in paraquat. H_2O_2 is definitely less toxic, but eventually both chlorophyll a and b decrease. They decrease at the same time as well, though chlorophyll a seems to diminish somewhat more. Carotenoids also show the same pattern as the chlorophyll (Fig. 10C). At 512 mM there is a decrease for both treatments, not at the other concentrations. The carotenoid content is higher at 6°C then at 23°C, except at the 8mM concentration were it is the other way around; more carotenoids at 23°C.

The ratio (Fig. 10D) for chlorophyll a/ b shows that in the 23°C treatment chlorophyll a diminishes a little more than the chlorophyll b content. While at 6°C there is not a preference as to which of to two disappears first, it happens at the same time. The ratio for chlorophyll/ carotenoids tells us that the carotenoids do not decrease more quickly then the chlorophyll does.

The SH-content (Fig. 11) increases slightly (but not significantly) and then decreases again for both 6 and 23°C. But only at 512 mM is there a real decrease of thiols compared to the control for both temperature treatments. Again there is no order in which all the compounds decrease. It all goes at the same concentrations, therefore there is nothing to conclude that glutathione protects against hydrogen peroxide.

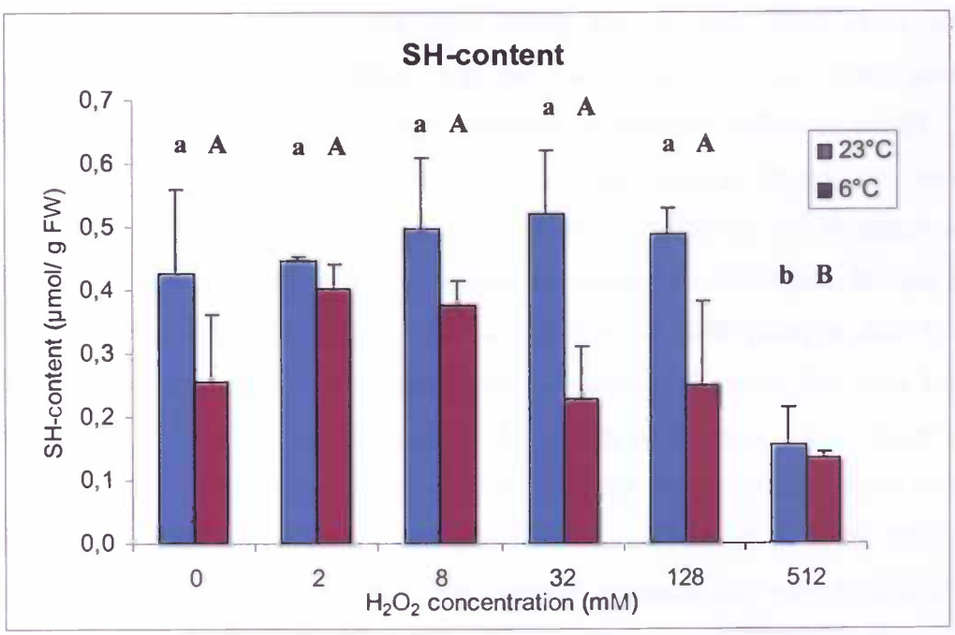


Figure 11. The impact of hydrogen peroxide on SH-content of leaf discs of *Brassica pekinensis* upon incubation at 6 and 23°C in light. Leaf discs were incubated for 24 h. Data on SH-content represents the mean of 3 measurements with 7 leaf discs in each (± SD).

Conclusion

Paraquat and the hydrogen peroxide have eventually the same effect. Both treatments and up in a decrease of glutathione, carotenoids and chlorophyll, but the leaf discs are less sensitive to hydrogen peroxide then to the superoxide caused by paraquat. The effect of paraquat is already visible at 1 μM, while it takes 512 mM for H_2O_2 to show an effect. Other research also pointed out that sufficient concentrations of hydrogen peroxide in a sufficient time space will cause cell damage and cell death and there does not seem to happen much before that triggering concentration (Levine et al. 1994). But there is also no visible order in which glutathione, carotenoids and chlorophyll are removed; it all seems to happen only in this highest concentration of hydrogen peroxide. It might be different if you look at different time periods, because all the leaf discs were treated with different concentrations temperature and illumination for 24 hours. The disappearance of the compounds in time might show different results, but it is not very likely that it will differ from different concentrations. There is a difference in the reaction of leaf discs which are treated with different temperature treatments. With the temperature difference of 20°C, those enzymes which are the coldest will work about four times as slow as the ones at the warmer environment, nevertheless, cell damage, reflected in fluorescence and conductivity is triggered at the same rate as warmer environments, though leaving more of the compounds intact. This is observed in both paraquat and hydrogen peroxide concentrations. These results do not support the idea that with hydrogen peroxide it is possible to regulate more. Hydrogen peroxide is not necessarily better regulated then paraquat, you only need a very high concentration before something happens, but that something is eventually cell death (Levine et al. 1994) while before that nothing really happens to the glutathione or pigments that were measured in this research. The results could be in accordance with the idea that H_2O_2 functions as a cell death triggering signal (Desikan et al. 1998), simply because of the lack of order in which glutathione, carotenoids and chlorophyll are taken away.

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