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# The glutathione system as a stress marker in plant ecophysiology: is a stress-response concept valid?

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## Abstract

Environmental stress impacts cause an increased formation of reactive oxygen species (ROS) in the chloroplasts (photo-oxidative stress). The role of glutathione in the antioxidative defence system provides a rationale for its use as a stress marker. However, responses of glutathione concentrations and redox states are not consistent among the large number of available publications. In the present review the hypothesis that stress responses of the glutathione system follow a general ecophysiological stress-response concept is investigated. In this view, an initial response phase would be followed by an acclimation phase where a new steady-state is established. Alternatively, if successful acclimation is not achieved, degradation of the system will follow. Recent publications dealing with responses to photochilling, salinity, and drought are analysed as to whether the results fit the concept. In general, an initial stress response was related to changes in the glutathione redox state, whereas acclimation was marked by increased glutathione concentrations, increased related enzyme activities, and/or a more reduced redox state of glutathione. The latter was interpreted as overcompensation leading to enhanced regeneration of glutathione. Deterioration effects upon strong stress impacts were related to progressive degradation and oxidation of the glutathione pool. A time-course analysis, which has rarely been done in the published literature, showed this sequence of events. When apple trees were subjected to progressing drought, the initial response was a slight oxidation of the glutathione pool, followed by

increased glutathione concentrations. When the stress increased, glutathione concentrations dropped and redox state became more oxidized, which marked the degradation of the system. In spite of the general congruency of these results with the suggested stress-response concept, several limitations have to be highlighted: The importance of the glutathione system relative to other components of the photoprotective and antioxidative defence system, as well as relative to stress avoidance strategies, has to be established. It is suggested that a variety of parameters taking into account alternative protection pathways (e.g. photorespiration, light dissipation) and other components of the antioxidative systems should be measured. Within such response patterns the glutathione system is a valuable stress marker in ecophysiological studies.

Key words: Antioxidative defence system, ecophysiological studies, glutathione, reactive oxygen species, stress marker, stress-response concept.

## Introduction

Glutathione is widely used as a marker of oxidative stress to plants, although its part in plant metabolism is a multifaceted one (Grill *et al.*, 2001; Fig. 1). As it is a non-protein sulphur-containing tripeptide, glutathione acts as a storage and transport form of reduced sulphur. Sulphur compounds such as SO<sub>2</sub> and H<sub>2</sub>S, although naturally occurring as atmospheric trace gases, are major anthropogenic air pollutants. Their toxic effects to plants have been

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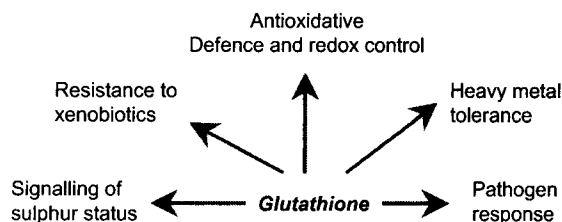


Fig. 1. Multiple roles of glutathione in plant metabolism (Grill *et al.*, 2001).

the subject of many studies without being fully clarified, but they have well-documented effects on plant glutathione metabolism (De Kok and Tausz, 2001). Glutathione is related to the sequestration of xenobiotics and heavy metals and is also an essential component of the cellular antioxidative defence system, which keeps reactive oxygen species (ROS) under control (Noctor and Foyer, 1998). Antioxidative defence and redox reactions play a central role in the acclimation of plants to their environment, which made glutathione a suitable candidate as a stress marker.

Given the multiple functions in metabolism (Fig. 1), the view of glutathione as a stress metabolite related to photo-oxidative stress is obviously a very simplified one. In recent years, huge progress has been made on cellular and molecular aspects of glutathione synthesis, subcellular roles, and regulation. Glutathione has been shown to play antioxidant roles in cell compartments other than chloroplasts, for example, in the mitochondria, cytosol, peroxisomes (Noctor *et al.*, 2002a), and is present in high concentrations in nuclei (Müller *et al.*, 2002). It is presently being discussed as a universal redox sensing and signalling system at the cellular level (Noctor *et al.*, 2002a). Recent results suggest the existence of glutathione transport systems in membranes with fast exchange rates, for example, between chloroplasts and cytosol (Noctor *et al.*, 2002a).

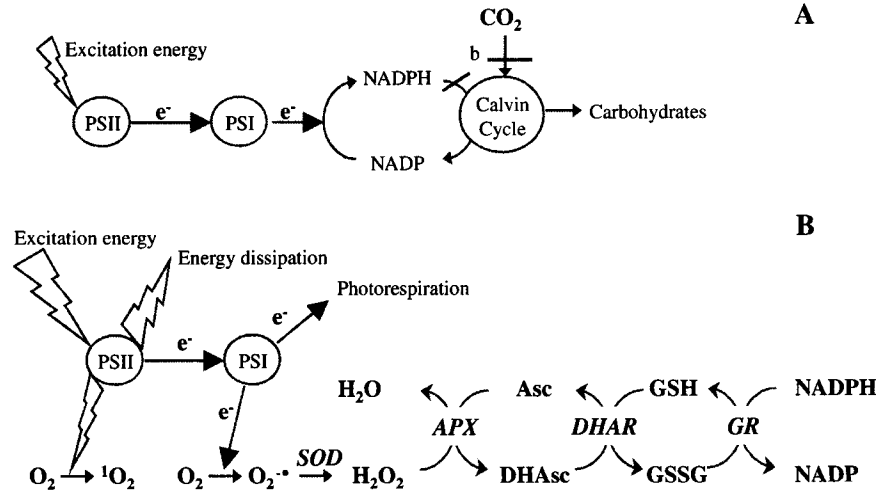
By contrast with the focus on cellular, subcellular, and molecular aspects of glutathione, the importance of measurements of the glutathione system in plant ecophysiological studies has not been re-evaluated in the light of the recent findings. The question arises as to whether it can be justified to investigate the glutathione system as a stress marker in ecophysiological studies, which focus on whole-plant responses. The present paper is not intended as an encyclopaedic review of glutathione responses to different environmental impacts as this has been done recently (Grill *et al.*, 2001; Tausz *et al.*, 2003), but instead it concentrates on the role of the glutathione system in defence against photo-oxidative stress. The objective is to examine whether recent literature results support the suitability of the glutathione system measured in whole-leaf tissue extracts as a stress marker.

## The role of antioxidative defence in plant stress reactions

Biochemically, the metabolism of plant cells under stress is generally characterized by an increased formation of reactive oxygen species (ROS) (Eltner and Osswald, 1994; Foyer and Noctor, 2000). In the chloroplasts, an imbalance between the consumption of reductant (NADPH) in assimilation (mainly carbon fixation), and the need of the electron transport chain for the regenerated electron acceptor at the PSI site (NADP) can lead to the transfer of electrons to alternative acceptors, predominantly molecular oxygen (Fig. 2). Environmental stress factors, which inhibit the function of the Calvin cycle (i.e. CO<sub>2</sub> fixation and NADPH and ATP consumption), exacerbate the situation. For example, drought-induced stomatal closure limits CO<sub>2</sub> availability, or low temperature or chemical agents inhibit CO<sub>2</sub> fixation, while at the same time, light-driven electron transport proceeds at high rates. Light absorption exceeds the demand for photosynthesis and excess excitation energy leads to an over-reduction of the electron transport chains. The formation of ROS is initiated by the univalent reduction of O<sub>2</sub>, or by the transfer of excess excitation energy to O<sub>2</sub> (Fig. 2). This situation, called photo-oxidative stress, is common to most plant stress responses.

Because the presence of ROS in cells is an inescapable feature of life in an oxygen atmosphere (Eltner and Osswald, 1994), plants have evolved antioxidative defence systems to keep ROS under control. Excess excitation energy has to be dissipated harmlessly as heat, or the electron transport to alternative acceptors has to be kept under control. Flexible reduction in light use efficiency of photosynthesis mediated by photoprotective pigments, or alternative pathways such as photorespiration or the Mehler-peroxidase-reaction serve as safety valves of the photosynthesis under excess excitation energy conditions (Asada, 1999). ROS formed in spite of, or in the course of, these control measures have to be scavenged by antioxidative defence systems. Glutathione is a water-soluble, low-molecular-weight antioxidant, which plays a central role in these systems (Fig. 2).

The ascorbate-glutathione-cycle (Fig. 2), which uses reduced glutathione (GSH) as an electron donor to regenerate ascorbate from its oxidized form, dehydroascorbate, is considered the main pathway of superoxide and H<sub>2</sub>O<sub>2</sub> removal in the chloroplast (Noctor and Foyer, 1998). The capacity of this cycle is dependent on the concentrations of the antioxidants and the related enzyme activities, which suggests that these parameters would be suitable markers for the antioxidative systems. Changes in turnover rates of this cycle may become manifest in altered redox ratios of dehydroascorbate/ascorbate or reduced to oxidized glutathione (GSH/GSSG). In addition, glutathione is the substrate of glutathione peroxidase (GPX) reactions and



**Fig. 2.** Simplified scheme of photo-oxidative stress and antioxidative defence in chloroplasts (more details in Elstner and Osswald, 1994; Foyer and Noctor, 2000). (A) Unstressed chloroplast: absorbed light energy is used to drive electron transport. Reduction equivalents are used in the Calvin cycle or other reductive reactions at rates balanced with electron transport rates. If the pathways are blocked at the indicated position (b), this will lead to photo-oxidative stress depicted in (B). (B) Excess excitation energy situation under stress: photoprotective systems dissipate part of the excess energy. Excess energy can be transferred to oxygen creating singlet oxygen ( $^1\text{O}_2$ ). Electron transport leads to reduction of molecular oxygen and generation of superoxide ( $\text{O}_2^-$ ) and hydrogen peroxide (Mehler-peroxidase reaction).  $\text{H}_2\text{O}_2$  is detoxified in the ascorbate–glutathione cycle. Asc, ascorbate; GSH, reduced glutathione; GSSG, oxidized glutathione; SOD, superoxide dismutase; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GR, glutathione reductase;  $e^-$ , electrons; PS, photosystem.

glutathione-S-transferases (GST), which also serve in the removal of ROS and their reaction products.

### Responses of plant thiols to environmental stresses

Since virtually all environmental impacts cause photo-oxidative stress in plants (Elstner and Osswald, 1994), responses of the plant antioxidative systems in general, and glutathione-related in particular, were observed upon exposure to, for example, natural abiotic stresses, biotic stresses (pathogens), or pollutant impacts. First observations of higher glutathione concentrations in foliar tissues of plants exposed to environmental stresses were interpreted as an acclimation, which strengthens the antioxidative defence systems (Esterbauer and Grill, 1978; Polle and Rennenberg, 1992). However, although numerous recent publications deal with the role of glutathione in plant resistance, acclimation, and adaptation to photo-oxidative stress, the emerging picture is an increasingly complex one. According to different studies, glutathione levels may or may not increase or may even decrease upon stress exposure. The GSH/GSSG redox state may change towards being more oxidized, more reduced, or not at all. Glutathione-related enzyme activities may be related to higher resistance or higher susceptibility. Among others, there are extensive reviews on the response of glutathione to environmental stress impacts (Grill *et al.*, 2001; Noctor and Foyer, 1998; Polle, 1997; Tausz *et al.*, 2003).

### A general stress-response concept applied to the glutathione system

The ecophysiological reaction of plants to environmental stress factors can be described in a general kinetic concept according to principles given in Larcher (2003). Stress evokes a time-series of responses in metabolic function of the plant. If acclimation to the stress factor is successful, the metabolic parameter will change dynamically until a new steady-state is achieved. If metabolic responses are insufficient or the stress intensity is too high, the system will not reach a new steady-state, but will deteriorate (Fig. 3).

In the present paper, the hypothesis is tested that the response of the glutathione system to photo-oxidative stress undergoes different phases according to the following concept. (i) When dissipation of excess excitation energy is not sufficient, increased rates of ROS production lead to an increased load on the ascorbate–glutathione cycle. The GSH/GSSG redox state, which is usually tightly controlled (at GSH/GSSG ratios near 9 or 10), may transiently shift towards a slightly more oxidized value. This phase corresponds to the initial reaction phase in Fig. 3. (ii) In an acclimation reaction, antioxidant concentrations and related enzyme activities increase. There is growing evidence that this can be triggered directly by the GSH/GSSG redox state itself (Baena-Gonzalez *et al.*, 2001; Noctor *et al.*, 2002a) and/or ROS such as  $\text{H}_2\text{O}_2$  (Neill *et al.*, 2003; Vranová *et al.*, 2002). The ROS scavenging capacity of the ascorbate–glutathione cycle increases, and a new steady-state is reached. This stage

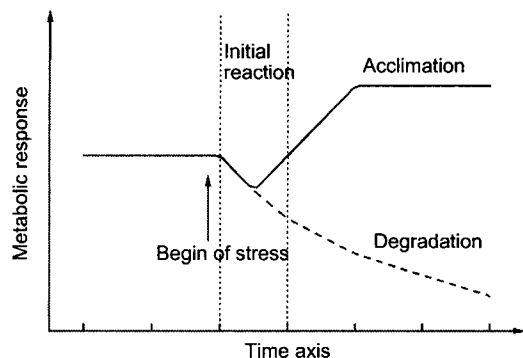


Fig. 3. General scheme of stress plant response (modified after Larcher, 2003).

would be detectable as higher concentrations of ascorbate and glutathione, a restored GSH/GSSG redox balance, and/or increased enzyme activities compared to non-stress/pre-stress values. However, in a study on UV responses of maize it has been shown that only turnover rates of glutathione increased, whereas total tissue concentrations remained unaffected (Masi *et al.*, 2002). 'Over-compensation' may occur in longer term responses, which could lead to higher (more reduced) GSH/GSSG ratios in stress-adapted plants due to strong activation of the defence cycle. (iii) If acclimatory responses are too slow or too weak, photo-oxidative stress will eventually deplete the antioxidative system, and the balance between oxidative load and scavenging will tip towards degradation. Progressive oxidation and degradation of the glutathione and ascorbate pools and, eventually, senescence and death follow.

In the following section literature results on the responses of the glutathione system of plants to three different environmental factors causing photo-oxidative stress are investigated: photo-chilling, salinity, and drought. These factors were chosen because they have been subjected to detailed investigations in recent papers, partly using novel chemical or transgenic approaches. The concept of the stress responses of the glutathione system as outlined above (Fig. 3), is examined for consistency, and the suitability as a stress marker in plant ecophysiological studies is tested.

### Photo-chilling

At high light intensities, temperatures low enough to decrease carbon fixation rate in the Calvin cycle lead to photo-oxidative stress. This phenomenon is called photo-chilling and often occurs at 5 °C and below, but at higher temperatures for sensitive species. Maize, as a chilling-sensitive species, responds at temperatures as high as 11 °C with an increased production of GSH and other antioxidants, whereas the GSH/GSSG ratio dropped upon short-term chilling in young plants. Chilling-sensitive cultivars further increased their GSH concentrations upon long-term chilling and their GSH/GSSG ratios were lower than those

of more tolerant lines (Hodges *et al.*, 1996). Field experiments showed that the chilling tolerance of maize cultivars may be correlated with the capacity of increasing the GSH concentrations and GR activities under stress conditions (Leipner *et al.*, 1999).

Causal approaches confirmed the correlation between glutathione concentrations and chilling sensitivity in maize. Increasing the glutathione concentrations by herbicide safeners, substances that increase glutathione biosynthesis, lead to increased chilling tolerance (Kocsy *et al.*, 2001), whereas the inhibition of glutathione biosynthesis reduced chilling tolerance (Kocsy *et al.*, 2000). Higher glutathione concentrations in chilled maize plants are the result of an induction of key enzymes of glutathione synthesis, as well as sulphate reduction, which also increases cysteine levels (Kopriva *et al.*, 2001).

In cotton, the increase of the activities of glutathione-ascorbate cycle enzymes in chloroplasts by genetic manipulation increased resistance to chilling-related photo-oxidative stress under laboratory conditions (Payton *et al.*, 2001). This effect was due to maintained rates of photochemistry, not due to increased light dissipation. Presumably, the transported electrons reduced molecular oxygen in the Mehler-peroxidase reaction, and higher rates of the ascorbate-glutathione cycle enabled the detoxification of these ROS (Korniyev *et al.*, 2003). However, field trials did not confirm a higher chilling tolerance of these transgenic plants under ambient conditions (Logan *et al.*, 2003).

By contrast with longer term acclimatory effects of chilling on the antioxidative systems, increased glutathione concentrations due to external feeding did not improve the chilling tolerance of rice leaves in the short-term (2 h of photo-chilling) treatment. Photoinhibition was even stronger, because glutathione inhibited the photoprotective xanthophyll cycle (Xu *et al.*, 2000). In that experiment, glutathione redox state GSH/GSSG decreased progressively during the treatment.

In general, these results fit into the stress-response concept of the glutathione system. Higher glutathione concentrations seem to provide better protection. Increase of cysteine and glutathione biosynthesis may be triggered by changes in the redox state, which reflects an initial stress response. As an important lesson it becomes clear that manipulations of single, glutathione-related components of the antioxidative systems do not necessarily improve the resistance of plants, but may even lead to increased susceptibility (compare also Creissen *et al.*, 1999) due to interactions with other components of the photoprotective systems.

### Salinity

Salinity can induce photo-oxidative stress in plant cells, because Calvin-cycle enzymes can be inhibited. Since

salinity is a growing problem in large parts of the world's agricultural area, research into salinity tolerance of cultivated plants is a priority in many programmes. Furthermore, molecular studies showed the up-regulation of cysteine synthesis in *Arabidopsis* in response to salinity (Romero *et al.*, 2001) and drew further interest to the role of thiols in salinity resistance.

Comparisons of cultivated tomato (*Lycopersicon esculentum*) to its salt-tolerant wild relative *L. pennellii* revealed differences in glutathione metabolism: While the salt-tolerant species was able to increase glutathione synthesis and increase GSH/GSSG redox state within 2 weeks in response to salt stress, the salt-sensitive agricultural species was not. Furthermore, GST and GPX (but not GR activities) increased in *L. pennellii*, suggesting a concerted response of several glutathione-related metabolic pathways (Mittova *et al.*, 2003). Correspondingly, glutathione concentrations increased in a salt-resistant rice variety, but not in a susceptible one (Vaidyanathan *et al.*, 2003).

In view of the stress-response concept of the glutathione system (Fig. 3), higher concentrations of glutathione would confer better antioxidative protection and would be considered an acclimation. An increase in the GSH/GSSG ratio (more reduced), as observed in the tolerant tomato species, would indicate an 'overcompensation' by intensified recycling of glutathione to keep it in its active, reduced form. However, in particular, salt tolerance studies remind researchers of the necessity to look at whole plant responses to the environmental factor in question: salt exclusion or sequestration mechanisms may avoid photo-oxidative stress to these plants and render antioxidative responses obsolete. As an example, wild-type *Brassica napus* accumulated glutathione and cysteine upon salt stress, whereas transgenic plants with a high capacity to sequester sodium into the vacuoles did not show these antioxidative responses (Ruiz and Blumwald, 2002).

## Drought

If plants employ stomatal closure to minimize water loss, lack of CO<sub>2</sub> will promote photo-oxidative stress in the chloroplasts, hence much attention was paid to the role of antioxidative defence in drought resistance (Smirnov, 1993). Severe drought at low relative water contents caused an oxidation of the glutathione pool in barley leaves (Smirnov, 1993). Short-term responses to mild drought in pine trees also included a slight decrease in the GSH/GSSG ratio, which was suggested as a possible signal for longer-term acclimation processes. Changes in the glutathione system were only followed for two days in that study (Tausz *et al.*, 2001b). While corresponding results have been reported repeatedly, different ecophysiological responses of different species and life forms (e.g. trees versus herbaceous) make generalizations difficult.

Comparisons between susceptible and resistant cultivars of the same species (or species within a genus) are an approach to overcome these difficulties. When Loggini *et al.* (1999) compared two wheat cultivars with different drought tolerance, they found that both cultivars responded with a decline in total glutathione concentrations and showed a higher (more reduced) GSH/GSSG ratio after a month of drought. Leaf water potentials of drought-stressed plants reached between  $-1.95$  and  $-2.32$  MPa. The GSH/GSSG redox state recovered quickly (within 3 d), but glutathione concentrations remained lower after re-hydration. An increase in glutathione-related enzyme activities was only observed in the more susceptible cultivar. The authors concluded that the more resistant cultivar did not show increases in enzyme activities because high constitutive levels were enough to resist the stress, whereas the more susceptible variety reinforced its defence systems. In their view, the decline of total glutathione concentration in both cultivars was moderate and indicative of sufficient defence capacities. By contrast with these results, Herlinger *et al.* (2002) reported an increase in total glutathione concentrations in wheat flag leaves upon drought exposure. This increase was only observed when water potentials reached values below  $-3.0$  MPa and was only significant in the more susceptible cultivar, which reached lower leaf water potentials. Lascano *et al.* (2001) found no clear differences between four different drought-tolerant wheat varieties after one month of drought exposure in the field, but the two more-resistant cultivars responded with an increase in total glutathione during the rewatering period after the treatment. Leaf water potentials were not measured in this study, but relative water contents in leaves were as low as 50%. Surprisingly, only the two more-resistant cultivars showed a tendency towards declining activities of glutathione-related enzymes. The authors subjected leaf tissue of these varieties to a short-term osmotic stress treatment and only found an increase in glutathione at less than  $-0.5$  MPa within 48 h in the resistant varieties. The responsiveness of glutathione synthesis upon osmotic stress was suggested as a screening technique for the selection of cultivars (Lascano *et al.*, 2001).

While the papers cited above used long-term drought protocols (progressing drought over weeks), a shorter-term drought treatment (24 h at a quickly established low soil water potential) already increased reduced glutathione concentrations and GR activity in wheat leaves (Bartoli *et al.*, 1999). In that study, leaf water potentials reached  $-2.0$  MPa and the glutathione redox state is not reported.

These results on wheat do not fit easily in the suggested stress-response concept, because data on short-term or transient changes in GSH/GSSG ratios are not reported, and longer-term responses of glutathione concentrations were not consistent. Furthermore, different leaf water potentials indicate differences in stress intensity among the studies. It has to be taken into account that alternative

protective systems play important roles. For wheat, the importance of drought-related increases in zeaxanthin-related energy dissipation (Herbinger *et al.*, 2002; Tambussi *et al.*, 2002) and photorespiration (Noctor *et al.*, 2002b) has been demonstrated. These processes could decrease the oxidative load on the glutathione–ascorbate cycle in the chloroplast.

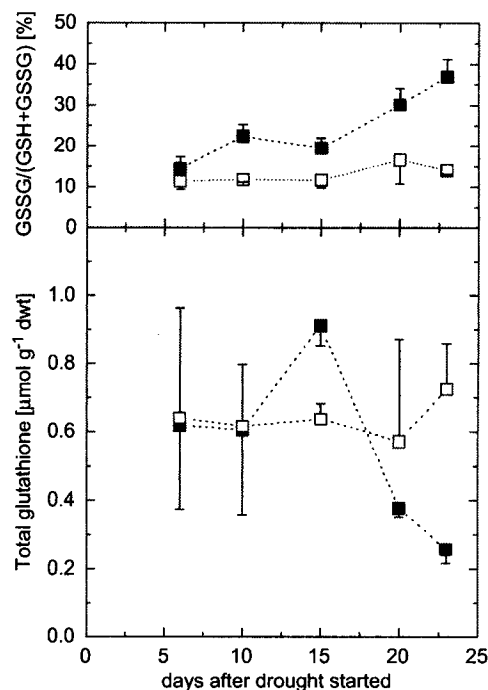
To clarify drought-related responses on the glutathione system according to the concept given in Fig. 3, time-courses of reactions should be recorded. This has been done in a recent study on apple trees which were subjected to progressive drought. First results showed that changes in the glutathione system of the leaves were consistent with the stress-response concept. First, the GSH/GSSG ratio decreased significantly after 10 d, followed by an increase in total glutathione concentrations increased after 15 d. Later on, when the stress became more intense, degradation and further oxidation of glutathione was observed (Fig. 4).

### Conclusions

The recent literature confirms a central role of glutathione metabolism in plant responses to environmental stress. Although knowledge of subcellular changes in the glutathione system could facilitate interpretation, for practical reasons measurements of average tissue values of glutathione concentrations, redox state, and glutathione-related enzyme activities are used to mark photo-oxidative stress responses. In addition to the sulphur nutrition status, which can impact the glutathione system and has to be taken into account, critical limitations directly related to the oxidative stress response also apply.

One major obstacle in the use of the glutathione system as a stress marker is uncertainty as to whether the observed response is dynamic (or at the initial phase of a stress response) or at a steady-state acclimation stage. Comparisons between species, plots, or experimental variants should only be made when a comparable steady-state is reached. In many cases, this is difficult to determine. The alternative would be the study of the kinetic of stress responses. The example in Fig. 4 clearly shows consistency with the stress-response concept.

The second major obstacle is the uncertainty about the importance of the glutathione system relative to other potential stress responses, whether these are part of the photoprotective and antioxidative defence systems (photoprotective energy dissipation, role of other antioxidants such as ascorbate or tocopherol) or not (e.g. stress avoidance strategies independent of photo-oxidative stress). According to the results reported in this paper, it is imperative to include multiple parameters, possibly covering light capture, light use, energy dissipation, photorespiration, and antioxidative defence, in plant-stress physiological studies. For example, in studies on animal



**Fig. 4.** Response of leaf glutathione concentrations and redox state in leaves of apple trees (*Malus domestica* cv. Jonagold Wilmuta) to progressive drought. Youngest, fully expanded, and sun-exposed leaves of 1-year-old trees grown in 50 l pots at an experimental field in Ljubljana, Slovenia, were sampled around midday. Means and SDs of four trees per treatment. Black squares, drought-stressed; empty squares, well-watered controls. Drought was applied by completely withholding irrigation (and protection from natural rainfall), hence increasing time also reflects increasing stress intensity. Predawn leaf water potentials of drought-stressed trees decreased progressively to  $-2.0$  MPa at day 23, whereas control water potentials stayed constant at about  $-0.3$  MPa.

tissues there is, at present, an attempt to measure as many systems related to redox and energy state of cells as possible (Lazzarino *et al.*, 2003). Attempts to use multiple components of antioxidative and photoprotective defence systems in plant tissues use multivariate statistical approaches, which were successfully applied to identify typical patterns of stress responses of forest trees at field plots (Grulke *et al.*, 2003; Tausz *et al.*, 2001a). The first steps towards an integration of such information have been made by metabolic modelling, which potentially gives information about the flux through protective pathways (Polle, 2001). Ecophysiological tools in gas exchange and chlorophyll fluorescence provide further possibilities for the evaluation of photoprotective energy dissipation, photorespiration, and the capacity of the Calvin cycle, which would supply information about the relative importance of ROS scavenging. From this perspective, measurements of antioxidants, and glutathione, as stress markers will remain an important part in assessing the stress response of plants, but only if included in a wider range of stress–physiological parameters.

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