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REVIEW ARTICLE: FIELD TECHNIQUES

Measuring antioxidants in tree species in the natural environment: from sampling to data evaluation

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Abstract

Biochemical measurements of antioxidants and protective pigments have been successfully introduced as markers of environmental stress in field studies (mainly forest studies). A guideline for field sampling and analysis methods is required to allow better comparison of data from different studies. The present review paper recommends HPLC methods for the analysis of ascorbate and glutathione (in oxidized and reduced form), tocopherols, and chloroplast pigments. Methodological variations are substantially lower (coefficients of variance of repeated extractions typically 4–9%) than biological variations of field samples (typical variation coefficients 8–36%), hence special emphasis is put on considerations of sampling standardization in the field with respect to sample time (seasonal and diurnal) and representative sampling of individuals and tissues. Following the suggestions in this paper would enable researchers to produce results that could be compared with those of several forest studies on conifers published in recent years. A larger data-set available for multivariate statistical evaluations (e.g. principal component analysis and cluster analysis) will enhance the diagnostic value of such investigations.

Key words: Antioxidants, ascorbate, carotenoids, glutathione, pigments, principal component analysis, stress markers, tocopherol.

Introduction

In the last decade, measurements of antioxidative and photoprotective defence systems have been successfully introduced into plant ecophysiological field studies (Polle and Rennenberg, 1992; Tausz *et al*., 1996*a*, 1998*a*, *b*). Since plant responses to environmental stress situations are generally linked to the action of active oxygen species (AOS; Elstner and Oßwald, 1994), AOS scavenging compounds can be used as stress markers. Among others, low-molecular weight antioxidants such as ascorbic acid, glutathione or α-tocopherol, and protective pigments such as carotenoids, have been tested as stress indicators in field studies (Tausz *et al*., 1996*a*, 1998*a*, 2001*a*). In green plant tissues, a large part of the stress-dependent AOS generation is produced in the photosynthetic apparatus in a reaction driven by absorbed light energy (photo-oxidative stress; Elstner and Oßwald, 1994). Chloroplast pigment analysis is, therefore, often included in such studies to provide a relationship between light capture capacity versus light protection systems. As found along altitude gradients in the field, concentrations of protective compounds tend to increase with increasing stress levels, whereas light capture potential (chlorophyll concentrations) generally decreases (Polle and Rennenberg, 1992). However, due to the highly variable conditions in field studies, the results are only rarely that clear, and many seemingly contradictory reports are found in the literature, making the use of antioxidant measurements for stress indication difficult. Since the antioxidative defence system is a complex network of compounds and reactions that depend on each other (Fig. 1), the responses in the field are rather complex. The application of multivariate statistical methods greatly enhanced the possibilities of field data evaluation (Tausz *et al*., 2002*a*). Several studies using such evaluation methods re-established the use of antioxidant patterns in stress monitoring in trees in field investigations (Tausz *et al*., 1998*b*, 2001*a*, 2002*a*; García-Plazaola *et al*., 2000). Multivariate statistics usually require quite large sample numbers. The more data that can be included the

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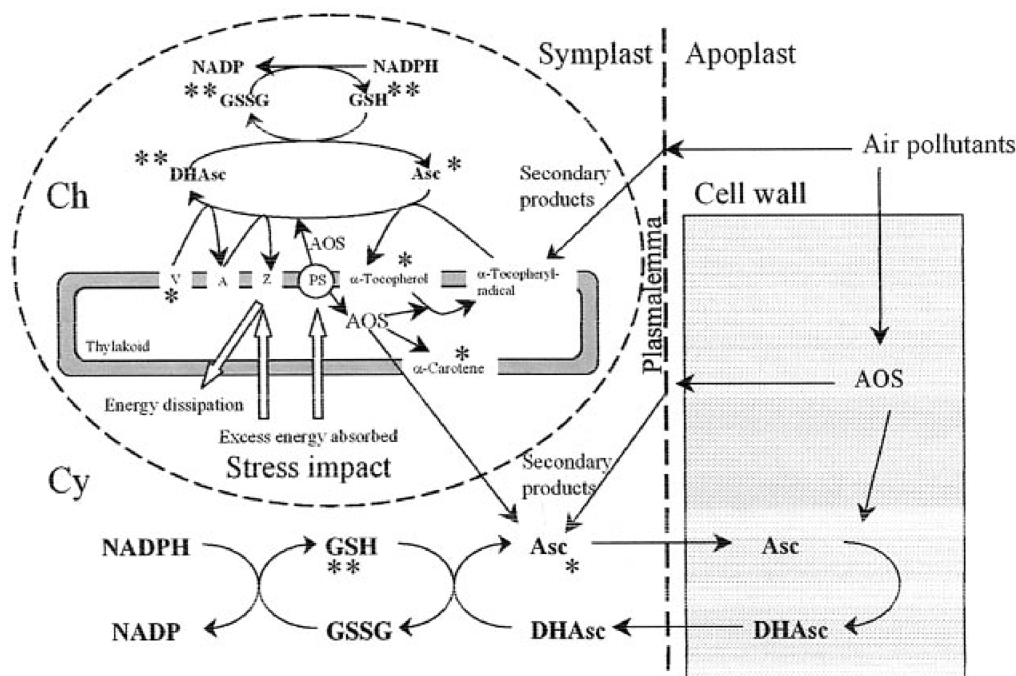

Fig. 1. Scheme of antioxidant and photoprotective defence systems in plant cells. AOS, active oxygen species; Ch, chloroplast; Cy, cytoplasm; Asc, ascorbate; DHAsc, oxidized ascorbate; GSH, glutathione; GSSG, oxidized glutathione. *,** Variables grouped together in a principal component analysis on field data (Tausz *et al*., 1998*b*) representing concerted actions of the respective compounds. Note: The immediate oxidation product of Asc is monodehydroascorbate (MDHAsc, not shown in this figure) which can disproportionate to Asc and DHAsc; MDHAsc can also be regenerated directly Asc (e.g. at thylakoid membranes), a process which is not included in the measurements presented in this work.

better. Although for other tree physiological field measurements the integration of large data-sets in common databases has been successfully implemented (for example, foliar nutrient data of forest trees all over Europe; De Vries *et al*., 2000), for measurements of antioxidant defence systems this is not yet the case.

In the present paper, methods for the determination of ascorbate, glutathione, α-tocopherol, and chloroplast pigments that have been applied in several field studies carried out by our working group (Tausz *et al*., 1998*a*, *b*, 2001*a*, *b*, 2002*a*, *b*) have been reviewed. This paper should enable readers to use these methods to produce comparable data and, in the future, help to establish larger databases on biochemical stress markers in field studies.

Biochemical analyses

In the literature, several methods of antioxidant and pigment determinations have been applied to ecophysiological field studies. In the present paper, the established HPLC determination methods summarized in Table 1 are recommended, because they are accurate and allow a high sample throughput. HPLC methods for α-tocopherol (Wildi and Lütz, 1996), chloroplast pigments (Pfeifhofer, 1989), ascorbate (Tausz *et al*., 1996*b*), and glutathione (Kranner and Grill, 1993) have a relatively low methodological error that is composed of variations in extraction procedure and variation in repeated HPLC injection (Table 2).

The determination of native redox states of antioxidant pools is especially sensitive. Methods for the glutathione (Kranner and Grill, 1993) and ascorbate (Tausz *et al*., 1996*b*) system include derivatization procedures which allow the determination of oxidized and reduced forms.

Sample preparation

In general, two main procedures for the preparation and conservation of plant tissue samples are reported in the literature. (1) Samples taken in the field are frozen in liquid nitrogen immediately (within seconds) and stored in liquid N_2 or at –80 °C until extraction. Extraction is then carried out on frozen material using a mortar and pestle, laboratory mixers or dismembrators in order to homogenize the plant material. (2) Samples conserved in liquid nitrogen are lyophilized prior to analysis. Lyophilization is commonly used for the commercial production of sensitive biochemicals and provides some adavantages for the methods discussed here. If thoroughly protected from moisture, lyophilized material is rather robust compared with frozen samples. It can even be stored at room temperatures for days allowing easy transportation. Lyophilized plant material can be pulverized in a dismembrator and the resulting homogeneous dry powder can be easily extracted,

Table 1. *HPLC methods recommended for the determination of antioxidants and pigments in plant tissues*

Reference	Chloroplast pigments Pfeifhofer, 1989	Ascorbate system Tausz *et al*., 1996	Glutathione system Kranner and Grill, 1993	Tocopherols Wildi and Lütz, 1996
Plant material	Lyophilized dry powder	Lyophilized dry powder	Lyophilized dry powder	Lyophilized dry powder
Extraction	Acetone	1.5 % (w/v) metaphosphoric acid	0.1 mol l^{-1} HCl	Acetone
Pre-column derivatization procedure	None	Labelling of dehydroascorbate with *o*-phenylene-diamine; simultaneous determination reduced/oxidized form	Labelling of thiols with monobromobimane; removing of reduced thiols with *N*-ethylmaleimide and toluol extraction; differential determination reduced/oxidized form	None
HPLC solvents	(A) acetonitrile:water: methanol (100:10:5 by vol.); (B) acetone:ethylacetate (2:1 v/v)	Water:methanol (7:3 v/v) containing 1% hexadecylammoniumbromide (ion pairing reagent)	(A) 0.25% acetic acid (v/v) in water:methanol=95:5 (v/v, pH 3.9); (B) water: methanol (pH 3.9, 1:9 v/v)	Methanol
Column	25×4.6 mm Spherisorb ODS2 5 µm	25×4.6 mm Spherisorb ODS2 5 µm	25×4.6 mm Spherisorb ODS2 5 µm	25×4.6 mm Spherisorb ODS2 5 µm
Gradient	10% (B) to 80% (B) in 17 min, hold 5 min, Return within 5 min	(None) isocratic	95% (A) to 85% (A) in 20 min; 100% (B) for 6 min; 95% (A) for 8 min	(None) isocratic
Detection	Photometric (visible) 440 nm	Photometric (UV) at 248 nm (ascorbic acid) and 348 nm (DHAsc)	Fluorescence (excitation at 380 nm, emission at 480 nm)	Fluorescence (excitation at 295 nm, emission at 325 nm)

Table 2. *Typical variation coefficients (CV=standard deviation in % of mean) of antioxidant and pigment (chlorophylls and lutein as a representative xanthophyll) determinations [based on tissue dry weight] in green plant tissues*

	Replicate extraction from homogeneous lyophilized powder	Replicate individuals (young trees, garden conditions)	Replicate individuals (field plot, old trees within one microsite)
Ascorbate	4[a]	23–26[a], 23–29[c)]	8–22[e]
Dehydroascorbate	7[a]	25–32[a], 23–37[c]	17–31[e]
GSH	9[b]	26–36[c], 10–14[d]	12–18[e]
GSSG	20[b]	36–40[c], 12–21[d]	33–36[e]
α-Tocopherol	4[f]	17–28[c], 13–16[d]	12–13[e]
Total chlorophyll	4[f]	18–31[c], 22–24[d]	14–19[e]
Lutein	6[f]	17–23[c], 11–13[d]	14–16[e]

[a] Tausz *et al*. (1996*b*); spruce needles, two different clones grown in the garden, *n*=20.
[b] Kranner and Grill (1996); lichens, *n*=6.
[c] Data re-evaluated from the study published by Tausz et al. (2001*b*); pine needles, *n*=12, at two consecutive days under garden conditions.
[d] Data re-evaluated from the study published by Wonisch *et al*. (2003); clonal spruce needles, *n*=10, at glasshouse and garden conditions.
[e] Tausz *et al*. (2002*b*); pine needles from uniform microsite conditions, *n*=8, at two sampling dates.
[f] Re-extractions of uniform spruce needle material, *n*=10.

used for repetitions and for determinations of different compounds on the same material. Some important points must be guaranteed for lyophilization. (a) Samples should be taken in paper bags (it is better not to use aluminum foil) to avoid obstacles to water removal in the lyophilizer; (b) samples are taken directly from liquid N_2 or the –80 °C freezer to the lyophilizer's container; (c) After closure of the container, a vacuum must be established quickly without allowing samples to thaw (dependent on the make of the lyophilizer); (d) the sample container of the lyophilizer is kept at room temperature to allow water to be removed quickly and efficiently. In strict terms, this process is desiccation, not lyophilization, because water removal is done at temperatures above the triple point of water. However, any liquid water is removed immediately from the samples and as long as water evaporates the samples are kept frozen due to evaporation energy; (e) the duration of the process is dependent on the material and the lyophilizer—for conifer needles 72 h is a good estimate. However, samples must be completely dry.

After lyophilization, the samples are sealed in air-tight plastic bags with silica gel to absorb any moisture and then

transported or stored in the freezer. Plant material is ground in a dismembrator and the resulting dry powder is used for all extraction procedures. At any point, samples must be protected from humidity. In particular, when taken from the freezer or using a dismembrator to pulverize the material, adjustment to room temperature before opening is necessary to avoid any condensation of air humidity on the cold samples.

If applied correctly, lyophilization procedures, which were controlled in detail for the more sensitive water-soluble antioxidants glutathione (Kranner and Grill, 1996) and ascorbate (Tausz *et al*., 1996*b*), will not change the resulting concentrations and redox states. In some cases, the resulting concentrations of the measured substances are greater, compared with extractions on frozen material, probably due to better homogenization and easier extraction (Kranner and Grill, 1996).

Lyophilization is optional, because the measured antioxidants are stable at –80 °C and extractions can be made directly from frozen material. However, it is advantageous when the shipping of samples or re-extractions of homogeneous material is required.

Field sampling standardization

In spite of the standardization of the analysis procedures from material conservation to HPLC determination, variations between individuals, particularly in field studies, are typically high (Table 2). The numbers in Table 2 show that methodological errors of biochemical analyses are much less important than individual differences. In particular, biochemical traits of biological samples may vary strongly between or even within individuals, but also because of changing environmental conditions. For use as biomarkers of stress impacts it must be decided which part of this variation should be excluded as far as possible. The selection of sampling procedures will depend on the aim of the study. The guidelines given below are best suited for field plot characterizations and comparisons between plots and years, but modifications will be required for the development of typical syndrome patterns. In the latter case, individuals of different visual appearance or damage classes are selected for correlational analysis.

A number of publications have shown the dependence of pigments and antioxidants on leaf age (Tausz *et al*., 1999), plant age (Tegischer *et al*., 2002), light exposition (Wieser *et al*., 2002), season (Esterbauer and Grill, 1978), and time of day (Schupp and Rennenberg, 1988), in addition to the potential relationship to the stress situations in question. Hence, it is crucial to standardize the sampling of field plots with respect to these sources of variation.

Selection of sampled trees and sampled foliage

In order to characterize a particular field plot, individuals to be sampled should be as similar and as typical as possible. A good basis is the guideline developed for nutrient analysis in tree foliage (De Vries *et al*., 2000). This suggests that dominant individuals or, for open stands, individuals of average height should be chosen. The foliage sampled must have the same canopy position and light exposition. In closed canopy stands, such as temperate spruce forests, samples from the fifth to eighth whorl from the top are commonly used whereas in more open stands (as in Mediterranean type pine forests) sampling from branches exposed to the sun is recommended.

Selection of sampling date and time

To compare different field stands, the sampling time must be standardized in order to guarantee comparable physiological activities within annual and diurnal courses. In temperate ecosystems the preferable date is late summer, when foliage development is accomplished at all elevations, but winter hardening has not yet commenced (in Middle Europe, August/September). In Mediterranean conditions, the summer drought period is a stable period (in Southern Europe, end of August).

Time of day mainly influences the light environment and light-dependent activities. In particular, glutathione, tocopherol, xanthophylls, and ascorbate systems may vary within short time-spans (even minutes) under changing conditions. Since sampling in full sunlight is sometimes impossible in the field (especially in rainy climates), a variation was developed for some studies (Tausz *et al*., 1998*b*): larger branches were sampled and kept in the dark overnight to provide comparable conditions ('artificial predawn conditions') prior to the harvest of tissue material. However, some recent studies have shown that some parameters to be investigated show differences only under sunlight conditions and, therefore, will not be detected in the dark (Tausz *et al*., 1999, 2001*a*).

The following points are a suitable checklist for the development of field sampling protocols. (1) Select the sampling season at a stable physiological stage. In many forest ecosystems late summer (before winter adaptation sets in or before seasonal drought ends) is a good choice. (2) Sampling time of day must be closely standardized because some of the parameters to be investigated are clearly light dependent (e.g. chloroplast pigment composition). Take samples on clear days from 11.00 h to 14.00 h solar time. (3) Select individuals as representative of the plot as possible. In forest ecosystems, dominant trees without (foliar) injury symptoms are suitable. (4) Select sun-exposed foliage of similar developmental stage, canopy position, and exposition. For evergreen trees, the previous season's sun foliage from the southern branches of the top canopy is best suited, requiring climbing of the tree or the use of pole pruners. (5) Foliar samples must be directly, within seconds, frozen in liquid N_2 in the field and transported therein or in dry ice (solid CO_2).

Data evaluation

Data measured according to the protocols above result in representative data-sets consisting of information on light capture and protection pigments (chlorophylls, carotenoids), lipophilic (tocopherol) and water-soluble antioxidants (glutathione system, ascorbate system). Since pigments are exclusively, and tocopherols are mainly located in thylakoid membranes, their concentrations are strongly correlated when based on tissue weight. Expressing data for carotenoids and tocopherols on chlorophyll content eliminates correlations originating from a common location in thylakoids and relates defence capacity to light harvest potency.

How reproducible are biochemical defence parameters?

The stability of the measured biochemical components is evaluated on field-grown Norway spruce trees in the Austrian Alps. The field plots included in Fig. 2 have been part of an extensive forest decline study (Wonisch *et al.*, 1999). Differences between investigated plots were shown to be governed by stable edaphic factors (Wonisch *et al.*, 1999) which did not change much between repeated samplings. If biochemical analysis is a useful marker of environmental influences, the results must correlate between repeated samplings, because the dominant environmental factors remain the same.

The results confirm the suitability of the variables measured (Fig. 2). The correlations between ascorbate and chloroplast pigments of two investigated years are significant. Similar results were found for glutathione, but since a method different from the one recommended in the present paper was applied in that study, the results are not shown.

It is clear that with a change in the dominant environmental conditions (e.g. pollution impact, meteorology etc), the results must be different.

Multivariate data evaluation

Since in most field studies environmental impacts are complex, the results of the biochemical indicators will also be complex. The use of such multivariate plant responses without the application of multivariate techniques is limited (Tausz *et al.*, 2002*a*). The aim of multivariate statistics may be a reduction in the complexity of the data-set, but there is also a gain in direct knowledge due to pattern recognition algorithms.

While Wild and Schmitt (1995) theoretically suggested the application of factor analytical techniques to biochemical data-sets, Grulke and Lee (1997) related a number of morphological canopy attributes of Californian pines to ozone damage with the help of multivariate statistics. Consequently, the application of principal component analysis and cluster analysis has led to new insights into the complex biochemical responses of conifers to environmental conditions in temperate (Tausz *et al.*, 1996*a*, *b*, 1998*b*; Wonisch *et al.*, 1999) and Mediterranean (Tausz *et al.*, 1998*a*, 2001*a*, *b*) climates. Recently, in a number of other studies on biochemical plant defence (García-Plazaola *et al.*, 2000) and tissue chemistry data of tree foliages (Seidling, 2000, multivariate statistics was used. In general, two classes of multivariate methods are used for the analysis: factor analytical techniques (including principal component analysis) and clustering techniques.

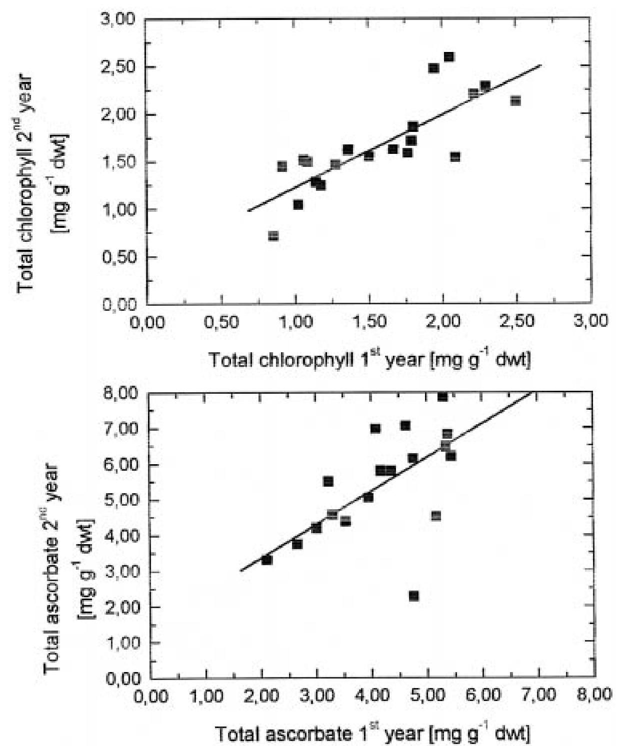

Fig. 2. (Top panel) Total chlorophyll (chlorophyll *a*+*b*) in the previous season's spruce needles sampled in two consecutive years at field plots in the Austrian Alps. (Bottom panel) Total ascorbate (reduced+oxidized form) in the previous season's spruce needles sampled in two consecutive years at field plots in the Austrian Alps. In that field study, stable site-related factors were governing the plant response, hence data from the two years are closely related (Wonisch *et al.*, 1999). Samples are taken from spruce trees without visual symptoms of decline.

Principal component analysis (PCA) groups variables into subsets that are relatively independent from each other. Accumulated variables, called principal components, represent underlying processes responsible for inter-correlations of variables in the original data-set. Examples of PCA application to field data on biochemical defence systems are given in Tausz *et al.* (1996*a b*, 1998*a*, *b*, 2001*a*, *b*, 2002*a*, *b*). The set of physiological variables described above was grouped in PCs, which could be interpreted as complex stress physiological responses. In Fig. 1, symbols with asterisks refer to principal components extracted in a field study on pine needles

(Tausz *et al*., 1998*a*, *b*). Closely related compounds are grouped into the same accumulated variable (principal component).

Cluster analysis comprises methods of grouping individual samples together according to their similarities in multivariate patterns. It serves well as a tool for pattern recognition, an important task in field studies. In several studies (Tausz *et al*., 1998*a*, *b*, 2002*a*, *b*), the assignment of trees to clusters with similar biochemical response patterns to field plots allowed the identification of typical patterns of the measured biochemical variables at certain plots and plot conditions.

Conclusions

As found in a number of studies, biochemical defence systems may provide sensitive tracers of environmental impacts on plants. In order to use them as a diagnostic tool in field studies, standardization of field sampling is required. Most important is the choice of individuals to be sampled, tissues to be sampled, and the standardization of the daily sampling time, whereas a variation in biochemical analysis methods is much less critical. Study design and sampling protocols should be designed with the possibility of multivariate data evaluations in mind.

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