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The role of biomarkers in environmental assessment (4). Terrestrial plants

W.H.O. ERNST[1] and P.J. PETERSON[2]

[1]*Department of Ecology and Ecotoxicology, Faculty of Biology, Vrije Universiteit Amsterdam, The Netherlands*
[2]*Monitoring and Assessment Research Centre, King's College London, London, UK*

The potential of metabolites, enzymatic processes and changes in plant performance as biomarkers in environmental assessment is reviewed. Biomarkers may be used as an early warning system of specific or general stress at each biological level, from molecules to ecosystems. The sensitivity of a species and, thus, the efficiency of a biomarker will depend on the degree of already present adaptation to environmental stress and on the homogeneity of the investigated population. Biomarkers for specific environmental stresses are scarce; better known are biomarkers for environmental stress complexes such as heavy metals, physiological drought and extreme temperature or biomarkers as a reaction on a full scale of environmental stresses. It is argued that a battery of biomarkers is necessary to evaluate chemical hazards to species.

Keywords: adaptation; damage; drought; fluorinated compounds; heavy metals; phytochelatins; proline; putrescine; seleno amino acids; stress.

Introduction

In recent years there has been increasing concern around the world over chemicals in the environment. Their widespread distribution stemming from human activities has given rise to potentially harmful effects on ecological systems and the environment which sustain human welfare. Contaminated soil, water and the atmosphere from the discharges of chemicals into the environment over many years was highlighted in Agenda 21 adopted by the Plenary of the United Nations Conference on Environment and Development in Rio de Janeiro in 1992, emphasising the need to reduce hazard and better quantify environmental and human health risks.

Plants as all other taxonomic groups have the ability to adapt to such environmental conditions which may cause metabolic and functional injury to non-adapted organisms. In this contribution we restrict the topic to terrestrial plants. This adaptation can be the result of a relatively recent exposure of plants to an unusually high concentration of a substance present in low concentrations in each environment, e.g. heavy metals in the vicinity of metal-processing industries being built in a low-metal environment (Wu *et al.* 1975; Ernst 1976). Another adaptation has evolved for man-made substances released relatively recently to the environment, e.g. herbicides such as atrazine (McCloskey and Holt 1990; Warwick 1991). However, adaptation to environmental stress may also be the

result of a long-term exposure in environments mismanaged by man some 1000 years ago, e.g. Roman metal mines in Europe or some 100 years ago.

These adaptations may camouflage the impact of environmental stress on biota. Such adaptations may not only affect population dynamics and species diversity in ecosystems (Ernst 1993), but also the efficacy of pesticides because the natural fauna has already adapted to toxic concentrations in plants, e.g. fluoroacetate in Australia (Twigg and King 1991).

Therefore, the use of changes in metabolites and/or inorganic substances as biomarkers in environmental assessment may only be possible if we know the adaptation level already present in the populations of plants, animals and microorganisms. Differences in adaptations of populations in response to environmental pollutants will cause positive or negative responses and determine the position of a biomarker.

In this contribution we will elaborate on the consequences of already present adaptation in terrestrial plants on the power of biomarkers in environmental assessment, a subject completely overlooked in recent books on this topic (McCarthy and Shugart 1990; Huggett *et al.* 1992; Peakall 1992). The paper then addresses the potential for biomarkers to reveal not only the status and trends in environmental assessment but also their possible use in a predictive sense to indicate potential effects (damage) arising from defined pollutant releases. In this context we use the term 'biomarker' as biochemical, physiological and morphological changes in plants to measure their epxosure to chemicals. We do not agree with the definition given by the National Research Council (1987) that 'a biomarker is a xenobiotically-induced variation in cellular or biochemical components or processes, structures or functions that is measurable in a biological system or sample', because heavy metals being in low concentrations essential for the functioning of cellular processes are not xenobiotics.

The presence of adapted populations

Plant populations are composed of a great diversity of genotypes with different potentials to react to changes in environmental conditions. As a result of this reaction the fittest genotypes will survive and bring the population to another adaptation level than before the environmental change. Resultant changes in genotypic composition of populations can be reflected in isoenzyme composition as shown in some metal-tolerant plants (Porter *et al.* 1981), but not in others (Verkleij *et al.* 1985). In a permanent exposure a population may consist largely of fully tolerant genotypes, where many are unable to colonize metal rich microsites due to physiological constraints (Ernst 1974, p. 64). As soon as soils display a mosaic of concentrations/bioavailability at the micro-level (Kakes 1981) they permit a range of tolerances even on highly toxic soils.

BIOMARKER PRODUCTION IN DIFFERENTLY ADAPTED PLANTS

Population (genotype) specific adaptations are well documented in the case of a surplus of heavy metals (for a review, see Peterson 1982, 1993; Ernst *et al.* 1992). Changes in root elongation processes have been used widely in the procedure of the rooting technique (Wilkins 1978) to restablish genetic differences of the adaptation to metals. Recently phytochelatins P(s), poly (*g*-glutamyl) glycines have been proposed as another overall response of plants to metal exposure (Grill *et al.* 1986). As with the rooting technique, it worked out that the phytochelatin (PC) concentration does not only depend

on the metal exposure, but also on the tolerance level of the investigated plants. In a comparison between Cd-sensitive and Cd-tolerant plants of *Silene vulgaris*, Cd-sensitive plants produced significantly more phytochelatins than Cd-tolerant ones at the same external Cd concentration (De Knecht *et al.* 1992). As soon as Cd-tolerant and Cd-sensitive plants were tested at such external Cd concentrations that inhibit root growth in each population by 50% (EC_{50}), the PC concentration was nearly the same (De Knecht et al. 1993). This result indicates that the cellular, but not the environmental Cd concentration is responsible for the synthesis of the biomarker. In addition, the PC synthesis is also metal specific. With the highest stimulation by small (10 μM) concentrations of Ag, Cd, Hg and Te, the same PC concentration was only achieved by a 100 times higher concentration (1000 μM) of Pb (Grill *et al.* 1987).

Biomembranes are the first barrier which an environmental pollutant has to pass, but only a few seem to affect their integrity. A metal-specific and genotype specific reaction has been found for differences in the K efflux of Cu-tolerant and Cu-sensitive plant species (*Agrostis*, Woolhouse and Walker 1981; *Silene*, De Vos *et al.* 1991) and of As-tolerant and As-sensitive plants (Porter *et al.* 1981).

These examples already indicate the caution which has to be taken in using biomarkers in the field.

ROOT SYSTEMS GOVERN SELECTION PRESSURE

It may be tempting to follow the recent discussions of some animal ecotoxicologists (Baird 1993; Forbes and Depledge 1993) and generalize from the adaptation level of one species to that of a whole community or ecosystem. In the case of soil contaminants the distribution of the contaminant within the soil profile and the extension of the root system inclusive of its mycorrhizae may determine the adaptation level of plants and plant communities. When the contaminant is retricted to the upper zones of the soil profile, shallow-rooting species may have developed a tolerance to the stress factor, while deep-rooting species may still retain a high degree of sensitivity, as shown for *Agrostis capillaris* and *Molinia coerulea* in a grassland ecosystem affected by a Zn/Cd smelter (Dueck *et al.* 1984).

Great differences in the adaptation to flooding have been demonstrated for plant species in wet dune slacks (Schat 1984). Shallow-rooting species were sensitive to oxygen deficiency, whereas deep-rooting species could cope with oxygen deficient soils by well-established aërenchyma.

LONGEVITY OF SEED BANKS MAKES TROUBLE

In ecosystems dominated by annual plant species with a highly persisent seed bank, e.g. weeds in agricultural ecosystems, each year the new generation may be composed of genotypes reflecting the variability of environmental stresses over many decades and even up to a century for highly persistent seeds. In the case of sequential changes of applied herbicides (Le Baron and Gressel 1982) and the recently detected increase of resistance to 15 herbicide families (Warwick 1991) it will be difficult to estimate the adaptation level of a weed population in a field and to differentiate environmental stress compounds. This potential of conservation of adaptation pattern in different genotypes of a plant population is in contrast with the adaptive pattern of insects against pesticides,

because animals have no highly persistent 'egg' banks; each annual population has the same genetic load if immigration is small.

ONTOGENETIC MODIFICATIONS BY ENVIRONMENTAL STRESS

The development of adapted and non-adapted plants may be modified in different ways by an exposure to a stress factor. The best example is known from triazine-resistant and triazine-sensitive lines of *Brassica napus* (Dekker and Burmester 1992). During the vegetative phase of development photosynthetic carbon assimilation was less in resistant than in sensitive plants and the activity pattern changed in a different way during the day. With the onset of the reproductive phase, resistant plants assimilated more carbon dioxide than sensitive plants. Although triazine resistance is only due to a single base pair mutation of the *psbA* chloroplast gene (Hirschberg and McIntosh 1983) and daily photosynthesis may be a reliable biomarker, ontogenetic aspects can strongly modify the impact of the environmental stress. Pleiotropic effects may even change the competitive ability under various climatic schemes.

CRITERIA FOR FIELD USE OF BIOMARKERS

In advance of all testing procedures, a careful establishment of the adaptation level of the plants under consideration to the environmental stress is necessary together with the development stage of the plants and the exposure of the tissue. In the case of annual plants with persistent seed banks an enormous mixture of adaptation levels may be present and hamper the environmental assessment.

Damage

Environmental factors are only ecologically relevant if they result in a stress and, thus, in effects on the individual, the population and the ecosystem. But environmental stress can damage plants at all biological levels, from molecules to ecosystems. Therefore, damage will be defined as a situation when the stress has passed beyond the repair system operating at each biological level as soon as the final result will affect the fitness of an individual and/or population and the structure and/or function of an ecosystem. Due to the various kinetics and reaction velocities at each level, from disturbance of a biomembrane (De Vos *et al.* 1991) up to changes of the genetical composition of a population (Wu *et al.* 1975; Macnair 1987; Schat and ten Bookum 1992), the time scale of damage may vary from seconds to decennia. Damage at the higher organization level can often be related to visible parameters of plant performance, i.e. plant colour, plant structure, plant growth and reproduction (Ernst 1974). Visible damage is the first registration by eye of a chain of changed biochemical and physiological processes at the subcellular, cellular, tissue and whole plant level. In addition, the classical examples of non-visible damage at the autotrophic level, i.e. 'healthy green' metal-resistant grassland near metal smelters (Dueck *et al.* 1984) and its deleterious effects at the various heterotrophic levels, i.e. herbivores (Vetter and Mählhop 1971), carnivores (Ma 1987) and detritivores (Ireland 1979) demonstrate that biomarkers as instruments of early detection of a stress situation may be elaborated at all biological levels. Nevertheless, it may be difficult to relate changes of biomarker quantities with the concept of visible

damage due to the high diversity of repair systems and the low diversity of visible damage in plants.

Biomarkers in plants

In the past 30 years plant species or specific genotypes of a species have widely been used as biomonitors to localize emission sources or to analyse the impact of pollutants especially gaseous air pollutants, on plant performance (Posthumus 1982). It is regrettable that the well-accepted terms 'biomonitor' and 'bioindicator' are proposed to be substituted by the term 'sentinel' (Lower and Kendall 1990) because such a change contributes only to a semantic discussion, but not to scientific progress. Another difficulty with sentinel organisms relies on the belief that data on one species, i.e. a sentinel for one particular pollutant would provide protection for all other species and even for all other pollutants. Additionally, all life-history stages should be protected with data from the same sentinel, yet species sensitivity varies with stage of development. A meaningful environmental assessment of a stress factor or multiple stress, has to establish the chance of an individual to persist, to maintain its population and to ensure the functioning of its ecosystem.

Biomarkers should go beyond the visible parameters of sentinel species. They should establish such processes and products of plants, which enable an early recognition of environmental stress in a dose- or time-dependent manner earlier than visible damage. Biomarkers must therefore be able to predict the environmental outcome and consequential environmental damage. At the (sub)cellular level metabolic damage may be caused by a loss of the integrity of biomembranes of roots (De Vos *et al.* 1991), followed by the depletion of the glutathione pool (De Vos *et al.* 1992), a diminished uptake of nutrients (Weber *et al.* 1991) and diminished photosynthesis (Clijsters and van Assche 1985). After sufficiently long exposure the disturabance of these biochemical processes will impair cell division and, thus, growth.

The more specifically an environmental stress affects a metabolic process the more exactly the signal can be perceived. Ideally, biomarkers should be selected from the events of biochemical or physiological pathways. But the reality is still a random selection process that starts somewhere in the metabolic machinery. Therefore, at the moment, the specifity of a biomarker is more a chance process of the investigative approach, than the results of an understanding of signal perception and signal transduction to reaction.

SPECIFIC BIOMARKERS IN SENSITIVE PLANTS

In only a few cases is it known that an environmental stress will give rise to the production of a metabolite which is different between tolerant and sensitive plants.

Seleno proteins

In the presence of a surplus of selenium, Se-sensitive plants cannot differentiate between S and Se. They incorporate Se in sulphur amino acids such as selenomethionine and selenocysteine in contrast to Se-tolerant plants which biosynthesize and accumulate non-protein seleno amino acids such as selenocystathioneine and Se-methylselenocysteine

(Peterson and Butler 1967; 1971; Burnell 1981). After incorporation of seleno-methionine and selenocysteine into proteins, enzyme activity generally and those of sulphur metabolism decrease which may result in plant death (Brown and Shrift 1981). Thus, the occurrence of seleno proteins in plants provides excellent biomarkers for Se stress although their use in the field has not been widely reported.

Fluorocitrate

After an exposure to a surplus of fluor, biota synthesize fluoroacetyl-CoA and then convert it, via the tricarboxylic acid cycle (TCA) to fluorocitrate. The latter compound blocks the metabolic pathway by inhibiting the enzyme aconitase. As a result of this process, fluoricitrate accumulates and is a very reliable biomarker for fluor poisoning (Twigg and King 1991). Fluor acetate-accumulating plants can prevent the incoportion of fluoroacetyl-CoA into the TCA cycle by a specific enzyme, fluoroacetyl-CoA hydrolase (Meyer *et al.* 1992). Therefore, once more genetic differentiation makes it necessary to determine first the adaption level not only of plants, but also of microorganisms and animals. Due to an associated co-evolution of herbivores to fluoroacetate-bearing vegetation, populations of the brush-tailed possum in Western Australia are nearly 150 times more resistant to fluoroacetate than populations in Southern Australia (Mead *et al.* 1979). The indicate power of a unique biomarker is diminished by adaptation.

Perspectives Instead of the exposure of F-sensitive plants as bioindicators near F-emission sources, the analysis of F-citrate may offer a quick and very specific application of biomarkers in an economic context.

BIOMARKERS FOR A GROUP OF ENVIRONMENTAL STRESSES

Various types of environmental stress may result in the same metabolic reaction at the cellular level. Both drought due to shortage of water in the environment and salinity diminish the free water which is necessary for the optimal performance of plants, i.e. water shortage at the cellular level (see the following subsection). A surplus of the various heavy metals in the environment causes several very specific reaction patterns, however, a surplus of free metals in the cytoplasm allows synthesis of a metal-binding product (see the second subsection). The analysis of metabolites as reaction products on a group of environmental stresses may be a further step in the recognition of specific biomarker sets.

Proline and cellular water deficit

A well-known example of a metabolite in environmental stress analysis is the amino acid proline. As soon as a plant suffers water stress the proline concentration increases, independently of whether the cellular water deficit is caused by drought (Singh *et al.* 1973), salinity (Bar-Num and Poljakoff-Mayber 1977), low temperature (Naidu *et al.* 1991; Jouve *et al.* 1993) or heavy metals (Bassi and Sharma 1993). The physiological principle of this proline accumulation is obviously based on the reduced cell elongation and cell division during water stress. One of the options of biomarker research is a further analysis of the pathway of the chosen biomarker so that a precursor being more specific than the metabolite under consideration can help to specify the reaction to the environmental stress. In plants with various degrees of adaption to soil salinity, there is a

positive relation between the concentration of insoluble proteins in cell walls and the degree of NaCl tolerance (Bressan *et al.* 1990). In this case a low cell wall protein concentration may be a good biomarker, however the great variety of salt tolerance in plants may hamper its effectiveness. In the case of drought the first place of water deficit perception is the roots, resulting in a rapid synthesis of abscissic acid, which is transported as a chemical signal to the leaves (Quarry 1989). Recent developments have suggested a physical information, which is passed to the shoot prior to all involvement of biochemical processes.

Perspectives At the moment there is no reliable indication that proline is a specific biomarker which can be used as a specific early warning system for cellular water deficit. A high proline concentration may even be a constitutive character in some copper-tolerant plants (Farago 1981).

Phytochelatins and phytochelatin synthase at the cellular surplus of free heavy metals

As mentioned earlier, phytochelatins (PCs) are synthesized during exposure to a large group of heavy metals and multiatomic anions such as SeO_4, SeO_3 and AsO_4. Available data show a dose- and time-dependent relationship under laboratory conditions for copper (Schat and Kalff 1992), cadmium (De Knecht *et al.* 1993) and zinc (Harmens *et al.* 1993) during short-term exposure. For monitoring purposes, research is needed into the PC production of plants in the field. The most relevant tissue will remain the root tips. Another biomarker for metal exposure may be the sulphide-containing phytochelatin complex (Verkleij *et al.* 1990; Reese *et al.* 1992; Speiser *et al.* 1992), biogenesis of which requires adenylosuccinate synthetase and succino amino imidarole carboxamide ribonucleotide synthetase (Juang *et al.* 1993). The presence of mycorrhizal fungi in the roots of most plant species – *S. vulgaris* is one of the few plants without mycorrhiza – and the impact of heavy metals on vesicular–arbuscular mycorrhizal fungi (Griffioen *et al.* 1993) may interfere with a straightforward dose–exposure relationship.

PC synthase, i.e. *g*-glutamylcystine dipeptidyl transpeptidase, is the enzyme that synthesizes the metal-binding peptides by removing a glutamylcysteine moiety from one molecule of glutathione (GSH) and coupling it to another GSH. It is activated by free metal ions (Grill *et al.* 1989). It may be a potential biomarker for free metal ions in the cell. In the case of this enzyme the laboratory studies are not yet so far developed that the enzyme can be tested in a routine procedure; field data are absent.

Perspectives The use of stabdardized plant material in field exposure studies may open some perspectives for the use of PCs or PC synthase in biomonitoring metal exposure. However, the very metal-specific amount of PC synthesized will demand the simultaneous analysis of all metals which are responsible for the induction of PC synthesis so that a calculation of their metabolic impact can be made. The advantage of simultaneous analysis of PC synthase and metal content may give a reliable judgement of the degree of free metals and the potential of sublethal damage.

Heat-shock proteins

Heat-shock proteins (HSPs) may be another group of biomarkers, because the synthesis of HSPs is dramatically increased by exposure to heat (Brodl 1990). Unfortunately, the

synthesis of HSPs is often not related to the heat sensitivity of genotypes (Fender and O'Connell 1989). In addition, HSPs can be induced by other environemntal stress compounds such as arsenite (Lin *et al.* 1984), so that the perspective for HSPs as biomarkers is very limited. Their presence in low concentrations under normal conditions may demand a high standardization during sampling of field material. The advantage may be the long-term presence of HSPs even after completion of the heat stress, as shown for desert succulents (Kee and Noble 1986). Simultaneous exposition of plants to soil drying and high temperature have revealed intraspecific differences in the synthesis of HSPs in maize (Ristic *et al.* 1991), so that perhaps a combination of water shortage and high temperature may be indicated by HSPs.

GENERAL BIOMARKERS

General biomarkers, which respond to a variety of environmental stresses, may be useful to indicate that something in the environment is a hazard to plant life. Changes in enzyme activities were one of the first biomarkers to establish the exposure of plants to air pollution with peroxidase (PO) as a robust enzyme system (Keller 1974). Although PO gave a good response for SO_2-exposure, it was demonstrated relatively soon that plant species and the chemical-specific reaction (Wellburn *et al.* 1976) as well as the population-specific reaction pattern (Ernst *et al.* 1985) make this biomarker less reliable. Similar results were received with other enzyme systems, such as glutamate dehydrogenase. In addition to or instead of enzymes, metabolites are often used as biomarkers of general stress, for example, an increase in putrescine after a plant's exposition to K^+-deficiency (Smith 1979), to a surplus of chromium (Jacobsen *et al.* 1992) and sulfur dioxide (Priebe *et al.* 1978) and increased levels of UV-B radiation (Kramer *et al.* 1992).

Perspectives Due to changes in nearly all enzyme systems within the development stage of an individual, seasonal and climatic processes and the activity of enzymes in the general metabolism will not enhance their reliability as biomarkers. Metabolites and enzymes of specific pathways, however, may have some promising features.

Conclusion

Biomarker research in plants to date has consisted primarily of short-term exposures under laboratory conditions. Future research needs to analyse plant material grown in the field so that the impact of the various environmental conditions and the role of genotypic adaptation in the quality of the biomarkers can be understood.

In the definition given by McCarthy and Shuggart (1990), biomarkers are defined as 'measurements of body fluids, cells or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response'. Measurements of the presence of basic chemical elements as contaminants in plants have a long standing. The development of internationally available reference materials has even ensured an international comparison of data with comparable chemical matrixes (Griepink 1990).

Measurements of metabolites and assays of enzymatic activities have the disadvantage that a lot of laboratories follow their own procedures. Therefore, comparison of data is

very restricted. In addition, the ultimate goal of monitoring the environment depends on field material. Experience of sampling plant tissues for analysis of biomarkers is still in an initial stage so that a lot of basic research needs to be done for standardization of sampling, sample preparation and exclusion of undesired matrix effects.

Irrespective of whether enzymes or metabolites are used as biomarkers, the concept of a threshold arises. Does the threshold mark changes in environmental stress arising from the stressor or do the values measured reflect normal variation from climatic, edaphic or life-cycle differences? Another way of looking at the threshold is to evaluate what is often referred to as the signal-to-noise ratio. What change in response to the natural variation (noise) constitutes the environmental effect (signal)? Again, field-collected material and studies on field systems are an essential component in establishing reliable biomarkers. The use of biomarkers in a predictive sense must also be validated in natural systems.

The usefulness of plant biomarkers for environmental assessment relies on how well the test system responds to and can be predicted from environmental stress under natural field conditions. To date, single-species tests only are considered and often only lower levels of organization such as organs, tissues or cells are used. Can such tests be used at higher levels of organization, such as the population level, the multispecies level or the community level? As few plant species are universally distributed in ecosystems, whether of natural origin or man-induced, single-species biomarkers may only provide information on restricted geogrpahical coverage. One approach could be to examine biomarkers of plant and/or soil processes rather than of individual species differences.

The greatest challenge lies in how to adapt the biomarker concept to complex ecological situations such as communities and ecosystems where species richness, species succession and other ecological processes dominate. For environmental toxicology to progress, biomarkers of various types, i.e. a battery of biomarkers, will be needed to evaluate chemical hazards to species, communities and ecosystems, within the environmental assessment process. Protection of ecosystems from damage from the wide array of potentially hazardous substances often present as chemical mixtures will be an extremely difficult goal to achieve especially in view of differences between plant species within communities and ecosystems around the world.

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