Physiological Responses to Heavy Metals in Higher Plants; Defence against Oxidative Stress

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Depending on the physiological process investigated heavy metal phytotoxicity can be either inhibitory or stimulatory. Photosynthesis and its partial light and dark reactions are inhibited; the activity of various enzymes, located in several cell compartiments, is increased. These enzymes are mostly induced since metals affect the transcription activity. They appear to be related to the plant defence against oxidative stress caused by metal phytotoxicity. Careful examination of the time course of this induction reveals differences in response between the metals applied.

Introduction

Heavy metals constitute a heterogeneous group of elements; a relatively high density (approximatively 5 g/cm^3) is their common characteristic. From general biological as well as from plant physiological point of view essential and non essential heavy metals can be distinguished.

Essential elements are micronutrients; when available at suboptimal concentration, plants develop deficiency symptoms, generally characterized by leaf bleaching or browning and by growth inhibition. These elements play an essential role as components of metalloproteins, as cofactor in the enzymatic catalysis and in manifold other cellular processes. Therefore deficiency induces plant stress. At supraoptimal concentration however micronutrients become phytotoxic and also induce leaf chlorosis and dwarf growth. Although some growth stimulation might be observed at low concentration of some non-essential heavy metals, they distinctly interfere at high concentration, demonstrating similar effects as phytotoxic amounts of micronutrients.

In this contribution attention will be focussed on several physiological effects of supra-optimal concentrations of essential as well as non-essential heavy metals in higher plants. Depending on the physiological activity considered the response to the same phytotoxic concentration can be inhibitory or stimulatory. The latter effect appears to be the result of a defence mechanism against oxidative stress, imposed by metal toxicity. In general, these responses can be detected before any symptom of phytotoxicity becomes visible.

Inhibition and promotion of physiological activity

Since metal phytotoxicity results in leaf chlorosis and growth inhibition, its interference with photosynthesis was extensively studied. Several review papers are devoted to this aspect (*e.g.* Clijsters and Van Assche, 1985; Vangronsveld and Clijsters, 1994; Krupa and Baszynski, 1995; Krupa, in this volume).

In general light dependent CO_2 fixation is inhibited. This effect can be indirect due to interference with the plant-water relations and with stomatal closure (for a review see Barcelo and Poschenrieder, 1990) or to modification of the source-sink relations within the plant (Ciscato *et al.,* 1997). However direct effects on the chloroplast were also observed: membrane destabilisation, inhibition of chlorophyll synthesis, interference with the photosystems and inhibition of the Calvin cycle enzymes are described. From these observations it appears that the chloroplast is highly sensitive to heavy metals indeed and that inhibition of photosynthesis by these elements is complex.

However, after application of heavy metals an increase in activity is frequently observed for a

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number of enzymes, located in several cell compartments (for a review see Van Assche and Clijsters, 1990; Vangronsveld and Clijsters, 1994). A selection of these enzymes is presented in Table I. This response is observed in a variety of plant species and according to the literature every metal tested (Cd, Cr, Cu, Hg, Ni, Pb, Sn, Sb, Tl, Zn, and even the metalloid As) increases the activity of some if not all of these enzymes. This effect is function of the amount of elements assimilated by the plant (Van Assche *et al.,* 1988).

For several of these metals it was demonstrated that the increase in activity can be the result of modification of gene expression (Kurepa *et al.,* 1997; Thomas *et al.,* 1998; Xiang and Oliver, 1998). Glutathione, reactive oxygen species (Foyer *et al.,* 1997) and jasmonic acid (Xiang and Oliver, 1998) are considered to participate in the signal transduction pathway. Therefore metals can induce enzyme activity as a result of *de novo* protein synthesis (Xiang and Oliver, 1998).

Heavy metals induce oxidative stress

The question rises what should be the physiological meaning of this enzyme induction. Actually metal phytotoxicity is considered to result in oxidative stress (De Vos and Schat, 1991; Gallego *et al.,* 1996; Weckx and Clijsters, 1996 and 1997). Elements such as Cu produce various reactive oxygen species (Chaudière, 1994; Koppenol, 1994). Lipid peroxidation products and hydrogen peroxide can accumulate in the plant tissue after metal treatment (Girotti, 1985; Aust *et al.,* 1985; Weckx and Clijsters, 1996 and 1997). Electron paramagnetic resonance measurements demonstrated an accumulation of stable organic peroxides in bean leaves after Cu or Zn treatment (Navari-Izzo and Pinzino, pers. comm.) and of superoxide radicals in the roots after Cu application (Dedonder and Callens, pers. comm.). Plants dispose of an appropriate defence strategy against oxidative stress (Elstner, 1996). The induction of antioxidative enzymes is considered to be one of the defence responses towards this type of stress, imposed by heavy metals.

Enzyme induction as a defence strategy against oxidative stress

The majority of the enzymes, induced by heavy metals, is involved in the plant defence against oxidative stress. They were subdivided into three groups (Table I).

Table I. Three groups of enzymes, which are induced by phytotoxic amounts of heavy metals.

1. *Enzymes metabolizing reactive oxygen species*
   Peroxidases (POD), EC 1.11.1.7 and 11
   Superoxide dismutases (SOD), EC 1.15.1.1
   Catalases (CAT), EC 1.11.1.6
2. *Enzymes involved in the ascorbate-glutathione pathway* (Halliwell-Asada pathway)
   Ascorbate peroxidase (APOD), EC 1.11.1.11
   Monodehydroascorbate reductase (MDHAR), EC 1.6.5.4
   Dehydroascorbate reductase (DHAR), EC 1.8.5.1
   Glutathione reductase (GR), EC 1.6.4.2
3. *$NAD(P)^+$ reducing enzymes*
   Malic enzyme (ME), EC 1.1.1.40
   Glucose-6-phosphate dehydrogenase (G6PDH), EC 1.1.1.49
   Isocitrate dehydrogenase (ICDH) EC 1.1.1.42
   Glutamate dehydrogenase (GDH) EC 1.4.1.2 etc.

The common characteristic of the first group of enzymes is their catalytic reaction with the reactive oxygen species peroxide and superoxide. Plant peroxidases are subdivided in guaiacol and ascorbate peroxidases (Asada, 1992). The former deliver physiologically active reaction products and are mainly located in the cell wall, vacuole and cytosol. The latter detoxify peroxides and are principally found in the chloroplast and cytosol. Both types are induced by heavy metals. In bean leaves the electrophoretic pattern of the guaiacol isoperoxidases varies as a function of the metal applied (Van Assche and Clijsters, 1990). Ascorbate peroxidases will be discussed in the second group.

In plants three types of superoxide dismutase (SOD) isoenzymes are distinguished: the chloroplastic Fe-SOD, the mitochondrial Mn-SOD and the Cu-Zn-SOD, located in chloroplast and cytosol (Bowler *et al.*, 1994). Since they are metalloenzymes, metal deficiency affects their capacity. Metal excess however also modifies these enzymes. In a metal sensitive cultivar of *Phaseolus vulgaris* excess Cd stimulated the Mn and/or Fe SOD but this metal inhibited the Cu-Zn isozyme; the total SOD capacity was not changed. On the contrary phytotoxic Zn concentrations had no effect (Cardinaels *et al.*, 1984; Weckx and Clijsters, 1997). The influence of excess Cu was studied more intensively; inhibition (Weckx and Clijsters, 1996) as well as stimulation of total SOD capacity and/or of some of its isozymes was observed. The effect depended on the plant organ examined, on

its age (for leaves) and on the method of metal application. There was no clear correlation between SOD mRNA levels and enzyme capacity; moreover, excess Cu induced deficiency of other essential elements, which regulate the transcription of SOD genes (Kurepa *et al.*, 1997).

Only a restricted number of data is available on heavy metal mediated induction of the peroxisomal catalase. Cu, Hg and Pb are described to demonstrate at least a temporary stimulation; this enzyme should play a protective role after metal intoxication (Vangronsveld and Clijsters, 1994). This was recently confirmed for Cu (Weckx and Clijsters, 1996) but not for Zn toxicity (Weckx and Clijsters, 1997).

The second group of enzymes considered is involved in the ascorbate-glutathione pathway. It was first described in the chloroplast and later also detected in several other cell compartments (Foyer *et al.*, 1993 and 1994). In this pathway peroxides are detoxified by ascorbate peroxidase; the oxidized substrate is recycled by dehydroascorbate reductase, which receives the necessary electrons from glutathione. The reduction of the latter substrate is catalyzed by glutathione reductase with NADPH as the final electron donor. It was recently shown that the activity of every enzyme involved in this pathway is enhanced by several metals (Gupta *et al.*, 1999; Geebelen *et al.*,1999).

Since NADPH is the electron source for this pathway, it is not surprising that several enzymes of the intermediary metabolism, which reduce $NADP^+$ or NAD^+, are induced by various heavy metals. Several of these enzymes are listed in the third group of Table I.

Differential response as a function of the metal applied

Since a large variety of heavy metals demonstrate enzyme induction (vide supra), the question rises whether this plant response towards metal phytotoxicity is independent of the nature of the metal applied. There might be at least two reasons why this response should be different:

(1) several of these metals (Cu, Hg, ...) easily perform one electron oxidoreduction; others (Cd, Pb, Zn, ...) do not demonstrate this behaviour. Contrary to the latter, the former group easily generates reactive oxygen species by autooxidation, reduction of H_2O_2 (Fenton reaction), lipid peroxidation, etc.

(2) the affinity towards biomolecules in general differs as a function of the metal considered (Nieboer and Richardson, 1980). The production of phytochelatins, polypeptides specifically binding some heavy metals, increases with the metal affinity to SH-groups (Grill *et al.*, 1987).

This differential response was recently illustrated by comparing the effect of Cu and Zn on induction of the enzymes involved in the ascorbate-glutathione pathway (Cuypers *et al.*, 1999). Table II shows that these enzymes were induced by both metals, but the response to Zn was generally slower. Moreover there was a very early induction by Cu of the enzymes MDHAR and GR. The early induction of the former enzyme could be due to the direct monovalent oxidation by Cu of ascorbate to monodehydroascorbate (Van der Zee and Van den Broek, 1998), which is recycled by the early induced MDHAR. The early induction of GR indicates that reduced glutathione is essential when Cu phytotoxicity occurs. This metabolite is a precursor of phytochelatins, which detoxifiy Cu. Glutathione also undergoes direct oxidation by Cu, not by Zn, and its oxidized form is recycled by GR; depletion of reduced glutathione can lead to oxidative stress indeed (De Vos *et al.*, 1992).

Conclusion

Photosynthesis is highly sensitive to heavy metals. Their interference with this process is complex. It can be direct at the chloroplast level by affecting several partial reactions of this organelle depending on the metal applied. Indirect effects on photo-

Table II. Induction kinetics of the enzymes involved in the ascorbate- glutathione pathway in primary leaves of *Phaseolus vulgaris*.
The data represent the time (h) necessary to induce significant enzyme capacities after application of 50 µM $CuSO_4$ or $ZnSO_4$ in the root nutrient medium (adapted from Cuypers *et al.*, 1999).

Enzymes	Cu	Zn
APOD	48	96
MDHAR	5	96
DHAR	24	24
GR	5	no induction

synthesis are also observed. Identification of the primary site of action of heavy metals on this process *in vivo* therefore remains speculative.

Plants subjected to phytotoxic concentrations of heavy metals generally demonstrate symptoms of oxidative stress and develop defence reactions against this stress factor, e.g. induction of antioxidative enzymes. Since this response was observed for a large number of metals tested and even for the metalloid As, at first glance it appears to be a general physiological reaction towards these elements. However, careful analysis of the enzyme induction observed reveals specific differences in response to oxidative stress in function of the element applied.

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