RESPONSES OF *PISUM SATIVUM* TO SOIL ARSENATE, LEAD AND ZINC: A GREENHOUSE STUDY OF MINERAL ELEMENTS, PHYTASE ACTIVITY, ATP AND CHLOROPHYLLS

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Academic dissertation

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Front cover: Flowering shoot of *Pisum sativum* cv. ‘Phenomen’.

“There´s no such place as far away” (Richard Bach)

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LIST OF ORIGINAL PUBLICATIONS

The following original publications form the basis of this thesis. In the text, these papers are referred to by their Roman numerals.

I	Päivöke AEA, Simola LK (2001) Arsenate toxicity to *Pisum sativum:* Mineral nutrients, chlorophyll content, and phytase activity. Ecotoxicol Environ Safety (Environ Res section B) 49: 111-121.
II	Päivöke AEA (2002) Soil lead alters phytase activity and mineral nutrient balance of *Pisum sativum.* Environ Exp Bot 48: 61-73.
III	Päivöke AEA (2003) Mineral elements and phytase activity in *Pisum sativum* grown at different Zn supply levels in the greenhouse. Environ Exp Bot 49: 285-294.
IV	Päivöke AEA (2003) Soil pollution alters ATP and chlorophyll contents in *Pisum sativum* seedlings. Biol Plant 46: 145-148.

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ABSTRACT

The responses of *Pisum sativum* cv. 'Phenomen' to arsenate, Pb and Zn were examined in potted soil cultures of 3-12 and 21 days´ duration in the greenhouse. A wide range of soil concentrations of these elements were studied, including those considered to be acceptable for agricultural soils (2-20 mg As, 500 mg Pb and 300 mg Zn kg^{-1} dry soil). Growth, remobilization of cotyledon reserves, chlorophylls, ATP and mineral elements were assessed, with particular interest in interparameter relationships.

The effects of arsenate, Pb or Zn on the growth of seedlings contributed indirectly to the early decline (at and after day 9) of *in vivo* total phytase activity of the cotyledons. Element-specific impacts on the export of individual mineral elements from the cotyledons suggested that requirements of the axis, and some mechanisms mediating element remobilization, may have been altered.

Low levels of soil arsenate, Pb and Zn (40 µmol As, 2 mmol Pb and 5.3 mmol Zn kg^{-1}, respectively) increased ATP concentrations in the seedlings. The parameters of growth and ATP concentrations correlated inversely in plants exposed to arsenate or Zn. Only Zn reduced the chlorophyll concentrations in seedlings, and a surplus of ATP per total chlorophyll concentration occurred. In adult plants, arsenate raised chlorophyll a and b concentrations, but their ratio declined; Pb and Zn had no impact on chlorophyll concentrations in adult plants.

During the 21-day exposure, the shoots were usually more sensitive than the roots. Shoot dry weight (dwt) declined even at low (24 µmol As kg^{-1}) arsenate concentrations, and relatively more As accumulated from lower than from higher soil As concentrations. Under Pb exposure, the soil Pb concentration reducing shoot yield by 10% was lower (1.4 mmol Pb kg^{-1}) than the concentration for 90% (3.5 mmol Pb kg^{-1}) root tolerance index (TI), but the dwt-based TI responded in an inversely linear manner to soil Pb concentration. Medium (6.5 mmol) soil Pb concentration yielded higher plant Pb concentration and total content than low (1.1 mmol) or high (9.4 mmol Pb kg^{-1}) soil concentrations. Shoot dwt declined at or higher than 3.2 mmol Zn kg^{-1} dry soil, but the decline stagnated when soil Zn level rose. The fraction (%) of shoot Zn correlated inversely with soil Zn concentration.

Arsenate, Pb and Zn all caused an increase in plant Mg concentration. The tops of Pb- and Zn-exposed plants had higher Mg concentrations than the average levels found in the shoots, while in arsenate-exposed plants the fraction occurring in the roots rose. Pb and Zn reduced P concentrations in the roots and arsenate those of the shoots. Pb lowered the root, and Zn the shoot N concentrations, but the decline stagnated. The shoot K concentration correlated inversely with soil Pb. In Zn-exposed plants, shoot Fe concentration and total Fe content of the entire plants declined.

Parallel change relationships between a number of elements occurred in plants grown with arsenate, Pb and Zn, pointing to some response mechanisms of *P. sativum* that might be shared by these three elements. The parallel change and correlations suggested that, apart from Mg, Ca and K also played significant roles under Pb and Zn exposure. The results also suggested that the roles played by Mn and Na could have been altered under exposure to arsenate, Pb and Zn.

Significant responses in *P. sativum* cv. ‘Phenomen’ occurred even at or below the maximum allowed soil concentrations of these three elements in agricultural soils. Results confirmed the necessity to consider species- and element-specific toxicity safety ranges, and that low soil concentrations may be even more harmful than higher concentrations. It is concluded that the observed trends and directions of change are relevant and should also be focused on in the field.

ABBREVIATIONS

ATP	Adenosine triphosphate
EC_{10}	Effective concentration to lower yield by 10%
FAO	Food and Agricultural Organization of the United Nations
HELCOM	Helsinki Commission – Baltic Marine Environment Protection Commission
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
OECD	Organization for Economic Cooperation and Development
PAL	Phenylalanine ammonia-lyase
TI	Tolerance index
WHO	World Health Organization

Other abbreviations are used only in sections in which they are explained.

1. INTRODUCTION

1.1 Wider problem context

In 1992, the Earth Summit resolved to safeguard the environment and to maintain the productive capacity of our planet. Improving the management of hazardous substances, wastes, toxic chemicals, and maintaining soil fertility and unpolluted water resources, were among the urgent undertakings listed by the global community (United Nations 1992). Environment and health can be regarded as global public goods (Kaul et al. 1999), and the upkeep of these goods requires international cooperation.

The primary food production capacity and the quality of products in many parts of the world are threatened by contamination of soil and water resources (Macnair 1993; Acharyya et al. 1999; Iyengar and Nair 2000). Even now, over 800 million people go hungry every day. Undernourishment, poor health, vulnerability to toxicity hazards, and contaminated food and beverage, all are significantly linked to poverty, both in the industrialized and developing world (Iyengar and Nair 2000; FAO 2002a). Efforts are ongoing to improve the nutritive value of crops (reviews: Grusak and DellaPenna 1999; Brinch-Pedersen et al. 2002; Grusak 2002).

Being primary producers that accumulate contaminants from the soil and atmosphere (Harrison and Chirgawi 1989; Haygarth and Jones 1992), plants can contribute elements in harmful concentrations to the food chain (Welch 1993; Wierzbicka and Antosiewicz 1993; Smith 1994; Tripathi et al. 1997). As and Pb are among those elements, which at elevated concentrations constitute a potential threat to the environment and human health (OECD 1996; United Nations 1998; WHO 2001a; 2002). Development of crop species able to restrict or control the translocation of As would be an important means to diminish food contamination (Meharg and Hartley-Whitaker 2002).

Despite its role as one of the essential micronutrients Zn, at enhanced concentration in the environment, also becomes a hazard to living things (e.g. WHO 2001b). At the same time, Zn deficiency is common in crop plants, particularly, in agricultural lands with high pH. Currently, efforts are being made to improve the tolerance of crops to Zn deficiency and to improve their Zn efficiency (Rengel and Römheld 2000).

1.1.1 Arsenic

As released from parent material to aquifers and freshwater wells is an acute problem that affects millions of people in many countries (Acharyya et al. 1999; Commission on Life Sciences USA 1999; Pi et al. 2002; WHO 2002). In the Ganges Delta alone, a large portion of the human population is affected by groundwater that contains from 30 to 36 mg As litre^{-1}, while borehole water contains from 3.8 to 12 mg As litre^{-1} (Acharyya et al. 1999; Chowdhury et al. 1999). The WHO provisional value for drinking water is 10 μg As litre^{-1} (WHO 2002). In 1998, the Commission of Sustainable Development of the United Nations called for global action on the overall management of toxic elements and in Decision 6/1 reiterated the need to improve international cooperation in the management of toxic substances, including As in freshwater resources (United Nations 1998). The issue of contamination of freshwater supplies naturally does not only relate to potable water, but it bears a cascade effect on soil and farm produce (e.g. Abedin et al. 2002).

1.1.2 Lead

Despite the fact that the sale of leaded gasoline is being phased out in the OECD countries (OECD 1996) – the sale in Finland ceased in 1996 (Soveri et al. 1998) – Pb is used in fuels in the developing countries (Motluk 1996; George 1999), and in many manufacturing and production processes worldwide (OECD 1996;

George 1999). The fact that, globally, children living in poorer quarters often suffer from acute Pb toxicity (OECD 1996; Needleman 1999), amply demonstrates, not only the vastness of the problem, but also that poverty is a significant contributing factor. House dust, chipping paint and tap water are major Pb sources in children (Lanphear 1998; Oliver et al. 1999; Leroyer et al. 2000). In the USA alone, one child in 20 has a blood Pb level over 10 μg decilitre^{-1} (Lanphear 1998). Pb paints, forbidden in Australia in the early 1990s and in Finland for decades, became permitted again after Finland joined the European Union (Hallanaro et al. 2000). Prenatal and childhood exposure to Pb causes neuropsychological and social problems and anaemia (Lanphear 1998; Needleman 1999). Reduced fertility in women exposed to Pb has, at least partly, been attributed to interaction between Pb and oestrogen metabolism and to the mutagenicity of Pb (Tchernitchin et al. 1998).

In a resolution of the Council of the OECD, The Declaration on Risk Reduction for Lead, the member governments decided, *inter alia*, to reduce the risk of children´s exposure to Pb, phase down the use of Pb, and implement public awareness and occupational health campaigns and strategies (OECD 1996). In 1997, the Environment Leaders´ Summit of the Group of Eight (G-8) countries declared similar intentions (Canada Department of the Environment 1997).

1.1.3 Zinc

In addition to its use in the metallurgical industry, urbanization and the modern lifestyle are significant contributors to environmental Zn (Soveri et al. 1998; Guidotti et al. 2000; Hallanaro et al. 2000). In Finland, Zn originating from wear of studded tyres and use of road sealants could be reduced if friction-based winter tyres became mandatory (Soveri et al. 1998). Soil fertility and health of soil microflora show sensitivity to soil Zn (Doelman and Haanstra 1989; Chaudri et al. 1993; Bruins et al. 2000). Acidification enhances the solubility of many metallic elements, including that of Zn (Kauppi et al. 1990). The potential for high toxicity of Zn lies in its function as a micronutrient, ready uptake by plants, and high soil and *in-planta* mobility (Longnecker and Robson 1993; Marschner 1993). It is a structural component e.g. of ribosomes, also contributing to their integrity, is involved in protein synthesis and DNA replication, and serves as a catalyst or cocatalyst of many enzymes, thus having access to key metabolic processes in plants, animals and human beings. Zn also plays a significant role in the control of gene expression (reviews: Vallee and Auld 1990; Brown et al. 1993; Berg and Shi 1996; Auld 2001).

1.2 Pea crop

Pisum sativum L., the pea, is one of the old crop plants in the world, which today is also widely cultivated in the temperate regions and at higher elevations in the tropics, being one of the most important of the legumes (Pate 1977a; FAO 2002b). Globally (Sevey 1911; Makasheva 1986; Chapko et al. 1991; Carr et al. 1998; Rauber et al. 2001) and in Finland (Valle 1960; Järvi 1984; Hannukkala 1993; Peltola and Sormunen-Cristian 1997; Salo 1998), extensive research has been undertaken to assess the benefits of intercropping different cultivars of *P. sativum* with other species, usually oats or barley, to be used as animal feed, green manure, fresh forage, silage, and semidry and dry hay. Easy availability of protein-rich imported animal feed supplements, such as soya, has undoubtedly contributed to the stagnation of pea cultivation in Finland. Pea seeds are also expensive, although compensated for by the nitrogen-fixing capacity of this species and diminished requirement for fertilizers (e.g. Salo 1998). Recently, organic farming has rekindled interest in pea cultivation as a supplement in animal feed, green manure (Peltola and Sormunen-Cristian 1997; Khalili et al. 1999) and for human consumption (Leinonen et al. 2000). In 2000, pea production in Finland was 7000 tonnes. Globally, a total of 3.5 million tonnes of pea seeds were produced in 1999; in Western Europe the production in 2000 was 540 000 tonnes, and in Eastern Europe and the USA about 50% and 20%, respectively, of the amount produced in Western Europe (FAO 2002b).

Phytic acid occurs in most seeds and it may constitute from 65% to 80% of the total reserve P of the seeds (review: Raboy 1997). In *P. sativum*, close to 0.9% of the total dry matter content of cotyledons is phytate, and in this species, about 45% of the total seed P occurs as phytate (Adsule et al. 1989). K is the prevalent associated mineral element in this species, and to a lesser extent Mg, Zn and other ions (Crean and Haisman 1963; Raboy 1997). Phytin metabolism during germination serves in the overall homeostasis of P and other phytin-associated mineral elements, which are released during phytate hydrolysis. Concomitantly, intermediary *myo*-inositol phosphates are formed. These compounds have important functions in cellular metabolism, such as secondary messengers and in cell wall synthesis. Hydrolysis of phytate is a major reaction in the overall process of utilization of seed reserve materials, both as organic and mineral constituents (Reddy et al. 1989; Raboy 1997). Thus, phytase activity is of the utmost importance for early development of seedlings, and factors affecting this activity can also be expected to affect the growth of plants.

On the other hand, phytate in pea seeds and in a number of other food crop species also constitutes a major nutritional problem, due to the stability of complexes between phytate and many essential elements (Zn, Ni, Co, Mn, Ca, Fe), which lowers their availability from food and animal feed (reviews: Brown et al. 1961; Reddy et al. 1989; Brinch-Pedersen et al. 2002). To improve the digestibility of phytate-rich food and to enhance the availability of micronutrients in these foodstuffs, efforts are ongoing to genetically modify plants and animals to be able to express phytase and to improve the availability of micronutrients, including that of Zn and Fe, from food (Brinch-Pedersen et al. 2002). Yet, due to some potential anticarcinogenic properties of phytic acid, caution may be needed in the manipulation of phytate content (Welch 1993). Thus, it is important to better understand the impacts of elevated concentrations of metallic elements in soils on phytase activity, not only from the standpoint of their impact on the growth of crop plants, but also of how these elements impact the availability of essential micronutrients from phytate-rich seeds, including those of *P. sativum*.

Efforts to improve the Fe content of pea seeds are also ongoing; this is important, since anaemia is the most prevalent result of nutrient deficiency in the developing countries (Grusak 2000). Zinc deficiency in crop plants and domestic animals is also widespread (WHO 2001b). Uptake of Fe and Zn are interrelated, e.g., in some wheat genotypes, which display similar reactions to Zn and Fe deficiency in their increased exudation of phytosiderophores in the rhizosphere, while genotypes tolerant to Zn deficiency also show increased Fe uptake by roots (Rengel and Römheld 2000).

1.3 Peat

Peat is a natural constituent of agricultural soils in Finland, which have high organic matter contents (Sillanpää 1982). One-third of the area of Finland is wetlands, and about 17% of the total peat resource is suitable for energy and garden peat raw material (Lappalainen 1992). One phenomenon of the 1990s in Finland, was the sale of potted, peat-grown fresh garden produce, such as herbs, lettuce and more recently, also pea sprouts. The overall cultivation of potted vegetables is increasing rapidly in Finland, and amounted to a total of 41 million pots in 1999 (Aakkula et al. 2001).

Peat is rich in organic matter and has high affinity for metallic elements, which it binds. In the usually acid pH of peat-rich soils, the humic substances, including humic and fulvic acids, form stable organic complexes with many divalent cations, such as Zn and Pb. It is likely that complexes of fulvic acid are more easily available to plants than those of humic acids. Thus, organic matter may serve in the regulation of mobility and bioavailability of metallic elements to plant roots. In peat-rich soils, deficiencies of Cu, Zn, Mo and Mn may occur due to the stability of the organic complexes of these elements (review: Kabata-Pendias and Pendias 2001).

1.4 The present study

Fairly little recent information is available on the effect of arsenate, Pb, or Zn on the mineral element status of crop plants, an aspect that may be inadequately expressed when toxicity is measured in terms of yield reduction. It has been estimated that the invisible impacts of toxic elements generate considerable economic losses (Macnair 1993). Since elevated concentrations of arsenate, Pb and Zn contribute to the global problem of soil contamination, and since *P. sativum* is an important crop, it was considered necessary to investigate the responses of this species to these three elements, focusing on mineral element status, accumulation and interelement relationships. The present work draws together the results of four original papers (I-IV) and is structured so that the literature review (section 3) and the discussion of the results presented in these papers (section 5) complement each other. The aim of the study (section 2) describes the hypotheses made, parameters and approaches chosen to test the hypotheses, and the main features of the original papers (I-IV). The materials and methods (section 4) also include preliminary work and other supplementary information. The results are discussed, particularly, in view of the most recent available information. The results show significant mineral element imbalance in *P. sativum* cv. ‘Phenomen’ after exposure to soil arsenate, Pb and Zn in the greenhouse, and indicate trends that bear relevance and also require focusing under field conditions.

2. AIM OF THE STUDY

The aim of the present study was to assess the responses of *P. sativum* cv. ‘Phenomen’ to soil-applied arsenate, Pb and Zn, focusing on mineral element status. Since the responses to elements at elevated concentrations vary (e.g. Wierzbicka 1988; Chaney 1993), species-specific information is required to judge the contribution of any element to the food chain and to decide on the soil and *in-planta* safety ranges for a crop species.

The major hypotheses were: 1. Arsenate, Pb and Zn imbalance the mineral element status of *P. sativum* cv. ‘Phenomen’ (I-III), including below the maximum soil Pb concentration considered acceptable in agricultural soils (II) or at or below the soil and intraplant Zn concentration considered phytotoxic (III). 2. A significant mechanism of action on this species is indirectly, one mechanism being alteration in the plants´ adenosine triphosphate (ATP) status (I-IV).

The scope comprised seedling (3-12 days) and flowering (21 days) plants, since the former time range coincides with active remobilization from the cotyledons and the latter with the usual timing of harvesting intercropped peas (section 4.1). The approach was via assessment of responses in terms of growth, phytase activity of the cotyledons, and chlorophyll, ATP and mineral element concentrations and total contents as well as interparameter relationships. The difficulty of extrapolating results from nutrient solution cultures under field conditions was recognized, and therefore potted soil cultures in a greenhouse were used, with an effort at mimicking the cultivation of *P. sativum* in the field, recognizing however, that potted cultures also possess limitations (e.g. Chaney 1993).

The responses of *P. sativum* cv. 'Phenomen' to arsenate (I), Pb acetate (II) and Zn acetate (III) are described, after 3-12 and 21 days´ exposure, in terms of growth, *in vivo* and *in vitro* total (I-III) and specific phytase activity (II; III), chlorophyll a and b contentrations (I-III), element accumulation and partitioning patterns, as well as mineral element concentrations, total contents and interparameter relationships (I-III). The ATP and chlorophyll a and b concentrations of 9-day-old seedlings exposed to the three elements are described and the interparameter relationships assessed (IV).

3. REVIEW OF THE LITERATURE

3.1 Sources of soil arsenic, lead and zinc

3.1.1 Arsenic

As toxicity was known as early as in 300 B.C. In metallic form it has been used at least from the 12th century A.D. It bears a sinister connotation linked to suicides, witchcraft and murder (Azcue and Nriagu 1994), and it was a source of despair and inspiration to alchemists wishing to transform metals into gold (Knowles and Benson 1983; Azcue and Nriagu 1994). It has been used to treat syphilis and sleeping sickness, and it is currently still used in developing countries against some tropical diseases (Azcue and Nriagu 1994; Rosen 1999). Even in early 1900, arsenical herbicides were suspected of killing fruit trees (Headden 1910), but arsenates were extensively used in agriculture for many decades thereafter, with a peak from 1930 to the late 1940s (Merry et al. 1983; Murphy and Aucott 1998). Pb arsenate was also used in Finland for some 70 years, until 1959 (Mäkelä-Kurtto 1986). Globally, organic arsenicals have largely replaced inorganic As compounds, and currently pesticides represent about half of the total use of As (Chilvers and Peterson 1987; Merlin 1996).

As is a natural constituent of the lithosphere and occurs in some 200 minerals, frequently as mixed sulphides (review: Matschullat 2000). Granite in Finland contains an average of 2 mg As kg^{-1} and shales 20 mg As kg^{-1} (Koljonen 1992). The oxidation states of arsenic are 5+, 3+, 0, 1– and 2– (Brown et al. 1999). Weathering of parent material, volcanic eruptions and sea spray liberate As naturally, and it can be methylated, demethylated and volatilized by soil bacteria (Siegel 1998; Matschullat 2000). Anthropogenic sources to the air, soil and water are significant (Steinnes et al. 2000; Geiszinger et al. 2002). In mining and industrial areas high (130-4000 mg As kg^{-1}) soil concentrations of As occur (Matchullat 2000; Ogola et al. 2002; Wenzel et al. 2002); e.g. 40 mg As kg^{-1} have been reported in topsoils of an old tannery site (Dankwarth and Gerth 2002). Cu smelting and energy generation (Chilvers and Peterson 1987; Melanen et al. 1999), especially burning of coal (Qafoku et al. 1999), are significant contributors. Discharging of geothermal effluents for several decades to wetlands in New Zealand resulted in peat concentrations of 5800 mg As kg^{-1} (Chagué-Goff et al. 1999). High-temperature urban waste combustion generates particulate emissions of As, Pb and other elements that contribute to dry (particles) and wet deposits (rain) (Haygarth and Jones 1992; Melanen et al. 1999). In Finland, government regulations set limits on emissions of metals and metalloids, including As, Pb and Zn, from municipal waste incineration and burning of hazardous wastes (Valtioneuvosto 1994a, b; 1997). As emissions in the 1990s, decreased by about 90% but there appears to be an upward turn towards the end of the decade. The major contributors are industry and energy generation (Melanen et al. 1999).

Local As contamination by wood preservation plants has also been reported (Speir et al. 1992; Rasmussen and Andersen 1999). As and vehicle traffic are interconnected, but the origin of As is other than fuel combustion (Guidotti et al. 2000). Smokers subject themselves to residues of As in cigarette smoke (WHO 2001a). About 30% of the current human exposure to As comes from drinking water (Matschullat 2000; WHO 2002), and leaching of As from parent material affects the freshwater supplies of millions of people in Bangladesh and Inner Mongolia, and smaller numbers in Austria, Chile, Germany, Sweden, the USA and other countries (Acharyya et al. 1999; Pi et al. 2002; WHO 2002). In Finland, around 10-fold increases in As concentrations have also been identified in borehole wells located in geographic areas of high rock As content (Hallanaro et al. 2000).

3.1.2 Lead

The average Pb concentration of granitic rocks and shales in Finland is about 20 mg kg^{-1} (Koljonen 1992). The global average Pb concentration of soils ranges from 2 to 100 mg kg^{-1} (Siegel 1998), being an average of 17 mg kg^{-1} in Finnish soils (Koljonen 1992). The extractable Pb concentration of agricultural topsoil in Finland is 2.1 mg and in clay soils 3.1 mg litre^{-1} (Urvas 1995). Elevated garden and cultivated soil concentrations have been reported, e.g. in Poland 17-165 mg Pb kg^{-1} (Chlopecka 1996), England and Wales up to 1100 mg Pb kg^{-1} (Davies 1992) and Slovakia over 1820 mg Pb kg^{-1} soil (Krauss et al. 2002). Industrial and mining areas may have concentrations around 30 g Pb kg^{-1} soil (Seidel et al. 2002); e.g., river water next to a mining site in Kenya contains about 14 mg Pb litre^{-1} (Ogola et al. 2002).

Pb has long been used by humans for various purposes. As a soft metal, Pb was easy to work into various utensils (Nriagu 1983; Needleman 1999). The fall of the Roman Empire has been connected with the daily intake of up to 1520 µg Pb by free Roman citizens due to leaded wine and cooking vessels (Nriagu 1983). The connection between Pb exposure and sicknesses, child mortality and infertility was already recognized in the 1700s (Needleman 1999).

Sources of Pb in the immediate environment of human beings include cosmetics, ceramics, toys and house dust (Nair 1999; Oliver et al. 1999). Industry and dumping sites (Prasad 1996; Maurice and Lagerkvist 2000), sludge, waste incineration and energy generation (Prasad 1996), mining and metal smelters (Chlopecka 1996; Steinnes et al. 2000; Ogola et al. 2002), Pb batteries, ammunition, Pb paints and water pipes (Pacyna 1987; Leroyer et al. 2000) all contribute to Pb input in the environment. Recent studies indicate that Pb, Zn and As are all associated with high motor vehicle traffic volumes and probably relate to metal alloys in combustion engines (Guidotti et al. 2000; Maiz et al. 2000). All three elements volatilise in high-temperature combustion of coal or oil and remain in the atmosphere as aerosols that can travel long distances (Pacyna 1987; Kauppi et al. 1990). Short- and long-range atmospheric deposition of Pb is a significant contributor to soil contamination (Haygarth and Jones 1992; Steinnes 2001).

Pb emissions peaked in the 1970s and 1980s (Nriagu 1998; Weiss et al. 1999; Shotyk et al. 2000). In Sweden, the use of Pb increased about 40 times from 1880 to 1960 (Bergbäck et al. 1992), while in England and Wales only about 3 mg Pb kg^{-1} of the present soil Pb concentration of 40 mg kg^{-1} was attributed to fuels after 1946, and about 17 mg Pb kg^{-1} to earlier emissions (Haygarth and Jones 1992). Leaded gasoline was initially introduced in the USA in

1923 (Needleman 1999), and after World War II also in Europe (Bergbäck et al. 1992; Haygarth and Jones 1992). The sale of leaded gasoline is being phased out here (OECD 1996), and atmospheric deposition has declined (Bergbäck et al. 1992; Steinnes 2001). In Finland, Pb emissions have decreased by about 95% from 1990 to 1997, which can be attributed to the cessation of sale of leaded gasoline and to the development of cleaner technologies (Melanen et al. 1999; Hallanaro et al. 2000). A number of parties to the Baltic Sea Commission have managed to curtail the overall emissions, but wet deposition is still a major source of lead in this region (HELCOM 1997). The Baltic Sea bears particular relevance, because fish are one of the four major food-borne sources of Pb to Finns. Yet, our daily Pb dose from food is about 5% of the maximum recommended by the FAO/WHO Joint Committee (Tahvonen 1997).

3.1.3 Zinc

In the lithosphere, Zn occurs most commonly in sulphides, but also in carbonates, oxides, sulphites and phosphates. Globally, granites contain 40-100 mg Zn kg^{-1} (Kiekens 1995), while granite in Finland contains an average of 50 mg Zn kg^{-1} and shales about 100 mg Zn kg^{-1} (Koljonen 1992). The extractable Zn concentration of cultivated surface soils in Finland is 3.4 mg litre^{-1} (Urvas 1995).

As with As and Pb, Zn has long been used by humans for various purposes, and pollution of soil by Zn is widespread (Chaney 1993; WHO 2001b), although history was not always able to distinguish between As, Pb, Sb, Sn and Zn (Nriagu 1983). The growth-improving ability of Zn on *Aspergillus* was recognized in 1869-1870 (short reviews: Brown et al. 1993; Kiekens 1995), and in 1914 Brenchley reported on a certain range of Zn concentrations that promoted plant growth, but Sommer and Lipman (1926) proved the essentiality of Zn to plants about a decade later.

Burning of fossil fuels releases Zn (Kiekens 1995). Atmosperic Zn is mainly bound to aerosol particles that may travel long distances and be deposited dry or with rain (WHO 2001b). Other sources include mining and refining of nonferrous metals (Steinnes et al. 2000; Seidel et al. 2002), chemical industry (Maurice and Lagerkvist 2000), and fertilizers and other agricultural chemicals (Kiekens 1995). During a period from 1974 to 1987 in Finland, atmospheric deposition was a smaller contributor of microelements to agricultural soils than were fertilizers (Erviö et al. 1990). Vehicle traffic, wearing of tyres and road-sealing materials are significant sources of Zn to the environment (Soveri et al. 1998; Guidotti et al. 2000). In Finland, traffic volumes by private cars are increasing; only about 20% of current transport is by public means (Hallanaro et al. 2000), yet in the 1990s Zn emissions decreased about 85% (Melanen et al. 1999).

Due to tight regulations and control of waste disposal and recycling, the use of waste material, such as sludge, could be expected to rise in the future. In Finland, the use of sludge to improve the Zn balance of soils could also be made attractive, although the presence of other metals, including Pb and Cd, impose limitations (Jokinen 1993; Mäkelä-Kurtto 1993). Current regulations state that the use of sludge should not add more than 1500 g of Zn ha^{-1} y^{-1}, and the total concentration must remain at or below 150 mg Zn kg^{-1} soil (Valtioneuvosto 1994a).

3.2 Bioavailability of arsenic, lead and zinc

A feature common to As, Pb and Zn is that all tend to remain in the upper layers of the soil, which usually also have high organic matter contents, although there are exceptions to this feature (Hodgson 1963; Merry et al. 1983; Andreu and Gimeno-García 1999; Matschullat 2000). Elements are partitioned between soil fractions and between solid, sorbed and dissolved phases, the partitioning depending on chemical and physical factors, such as the characteristics of the adsorbing platforms (Claassen and Steingrobe 1999; Greger 1999). Partitioning of elements between different phases in the soil influences their bioavailability (Chlopecka 1996; McBride et al. 1997). Soluble, exchangeable and chelated fractions, also called labile forms, are bioavailable (Greger 1999; Maiz et al. 2000). High concentrations of free metal ions in soil solution are more toxic to plants than corresponding concentrations of other soluble forms (McBride 1995). A number of biotic and abiotic factors influence the bioavailability of an element, such as the activity of plant roots and soil microflora on the one hand, and on the other the chemical composition of the rhizosphere, soil pH and reduction/oxidation potential (Darrah 1993; McCully 1999).

3.2.1 Soil pH and oxidation/ reduction potential

The pH of soil solutions affects the availability and mobility of mineral elements (Kauppi et al. 1990; Darrah 1993; Greger 1999). Metal toxicity, most commonly caused by Al, is more frequent in acid than in alkaline soils (Aniol and Gustafson 1989), and the low pH of the soil usually increases the solubility of cationic elements (Kauppi et al. 1990; Greger 1999). In acid moraine soils, for example, the solubility of As, Pb and Zn follows the order of Zn > As > Pb, but the type of geological substrate influences the effect of other physical and chemical factors of the soil on the solubility (Blaser et al. 2000). For example, under a controlled microcosm study, As was more soluble from sewage sludge at a pH range of 5.0-6.5 than at higher pH (Carbonell-Barrachina et al. 2000). In another microcosm study on sediments from a cooling water pond of a power generation scheme, As was more available at pH 7.5 than under acidic conditions (Masscheleyn et al. 1991), whereas in a former tannery site, the impact of soil pH on the solubility of topsoil As was small, but in the Fe-rich deeper soil horizons, the solubility increased at and below pH 3 and above pH 6, but was low from pH 3 to 6 (Dankwarth and Gerth 2002). These examples illustrate that the relationship between the solubility of As and soil pH is strongly influenced by other soil factors. Reducing conditions enhance the solubility, probably because the adsorption substrates, Fe-oxyhydrates, are more soluble. Under these conditions As occurs as a trivalent species, whereas under oxidizing conditions, the pentavalent (arsenate) prevails (Masscheleyn et al. 1991; Matschullat 2000; Meharg and Hartley-Whitaker 2002).

The impact of soil pH on the solubility and availability of Pb varies (Davies 1992; McBride et al. 1997; Maskall and Thornton 1998), which rather implies that the soil pH impact is mediated and influenced by other soil components (Davies 1992; McBride et al. 1997). For example, in an extensive study comprising 46 garden plots in former mining regions of England and Wales, soil pH (range 4.6-6.2) was not a significant factor influencing the uptake of Pb into *Raphanus sativus* (Davies 1992). However, soil pH influences the partitioning of Pb between soil fractions (Maskall and Thornton 1998).

Zn is amphoteric in its chemical character (Vallee and Auld 1990). In alkaline soils, it may occur as a hydrated ion or as carbonate, while in acid soils, divalent Zn ion is prevalent (Kiekens 1995; Table 1.2 in Greger 1999). Soil pH and the solubility of Zn are significantly interrelated, and lower pH (Hodgson 1963; Sillanpää 1982; Chaney 1993) and oxidizing conditions (Masscheleyn et al. 1991) enhance the solubility. In moraine acid soils, Zn is highly soluble (Blaser et al. 2000), whereas in garden soils the exchangeable Zn, soil pH and cation exchange capacity are negatively related (Davies 1992). At higher soil pH values, Zn may be associated with clay, Fe- and Mn-oxides (Maskall and Thornton 1998; Maiz et al. 2000).

The oxidation/reduction potential of soils also affects the solubility of other elements, particularly that of multivalent cations, such as Mn. Changing the oxidation status and adsorbing capacities of Fe and Mn particles in the rhizosphere may have cascade effects on a number of elements (Brown et al. 1999).

3.2.2 Soil constituents

In a comprehensive study of forest soils in Switzerland, water-soluble As, Pb and Zn were found in the surface layers, from 0 to 20-40-cm depths, and accumulation of Pb in the topsoil was particularly clear in moraine soils (Blaser et al. 2000). Organic soil constituents are heterogenous, depending on the past history of the soil. Organic matter consists of solid, colloidal and dissolved components and possesses various charged groups including hydroxyl, carbonyl and phenolic groups that function as cation exchange sites (Hodgson 1963; Greger 1999). Binding of metals with organic matter is dependent on many factors, including the amount of suitable metal-complexing compounds, such as humic acids, and pH. In natural soils, the affinity of metallic elements for organic matter is quite strong (Hodgson 1963; McBride et al. 1997), but soluble organic matter may also enhance the solubility and availability of some elements (Hodgson 1963; Sillanpää 1982).

The anionic arsenate forms organic complexes in which it has an affinity for nitrogen groups, although adsorption of arsenate on the surface of colloidal particles is more common than chemical binding (Masscheleyn et al. 1991; Kumaresan and Riyazuddin 2001). As and Zn may be coprecipitated with Fe-oxides or bound onto Fe-oxyhydroxides in the rhizosphere (Doyle and Otte 1997), and the precipitates and bound oxides have been shown to gather in the Fe-rich soil horizon (Dankwarth and Gerth 2002). Arsenate has a stronger adsorbing affinity for the surface of mineral particles than does arsenite (Brown et al. 1999).

The solubility of soil Pb is influenced by both the organic and mineral fractions of the soil to a greater extent than that of Zn (Alloway et al. 1988). Zn mostly adheres to the soil matrix, onto clay particles, organic matter and various hydrous oxides, and it occurs to a lesser extent in soil solutions, even though plants principally take up the Zn found in soil solutions (Marschner 1993). In addition to divalent Zn, plants may use some complexed and chelated forms of it as well (Bingham et al. 1986). Similarly Pb also adheres readily onto soil substrates, such as clay and Fe-/Mn-oxides, depending on the pH conditions (McBride et al. 1997; Maskall and Thornton 1998; Singh et al. 1998), and associates with organic matter and carbonates in the soil (Hardiman et al. 1984; Maskal and Thornton 1998; Blaser et al. 2000). Increasing the soil clay content, cation exchange capacity and adsorptive surface area decreases the soluble fraction of Pb (Hardiman et al. 1984; Urvas 1995), and thus in sludge-amended soils the high adsorption capacity of organic and mineral fractions may decrease the accumulation of Pb in plants (Hooda and Alloway 1993). Pb is usually more soluble from lower soil Pb concentrations, than from higher concentrations (Hardiman et al. 1984), while Pb adsorbed on the surface of mineral particles is more bioavailable than crystallized Pb (Brown et al. 1999).

3.2.3 Manipulation of bioavailability

Other inorganic elements and their salts in the soil affect the availability of arsenate, Pb and Zn, whose characteristics are utilized in phytoremediation and corrective fertilization during efforts to stabilize elements into less available forms (Admassu and Breese 1999; Bothe and Brown 1999; Derome and Saarsalmi 1999). For example, in polluted forest areas application of lime and macronutrient fertilizers reduced the amounts of harmful metals, increased soil solution macronutrient concentrations and promoted the mineralization of P and N in the organic layers of the soil (Derome and Saarsalmi 1999).

To reduce the mobilization of heavy metals, the potential for low solubility of Pb and Zn phosphates has been investigated, e.g. by addition to the soil of chemically manufactured apatite (Boisson et al. 1999; Traina and Laperche 1999) or ground natural fish bone apatite (Admassu and Breese 1999). Both types of apatite are effective in decreasing the solubility of metallic elements such as Pb and Zn (Admassu and Breese 1999; Boisson et al. 1999; Traina and Laperche 1999), but the uptake of As increased from soils enriched with the synthetic apatite (Boisson et al. 1999). However, Ca in the natural fish bone apatite decreased the mobility and solubility of arsenate, through the formation of calcium arsenate apatite (Bothe and Brown 1999). Similarly, liming reduced the solubility of As from soils contaminated by former tannery activities (Dankwarth and Gerth 2002). In pot trials using soils contaminated with Pb, Zn and Cd (690, 410 and 4.5 mg kg^{-1} soil, respectively), addition of $CaHPO_4$ also markedly lowered the accumulation of all these elements in rape, kohlrabi, kale and celeriac (Leh 1986). The difference between arsenate, Pb and Zn is that arsenate is adsorbed and desorbed rather than precipitated and dissolved (Masscheleyn et al. 1991; Qafoku et al. 1999). Since phosphate can compete with arsenate for the adsorption sites, the availability of arsenate may increase, particularly, under phosphate-rich conditions (Qafouku et al. 1999). Moreover, in a phytoremediation trial in which two waste products were recycled and mixed, Ca carbonate from power generation and anthracite powder from coal mining, Cu and Zn were stabilized in the soil at a pH range of 5.8-6.2, so that their uptake into alfalfa was reduced (Arsova and Raychev 2001).

Species- and toxicant-specific *in-planta* phytoextraction offers the potential for phytoremediation. For example, *Brassica napus* shows better ability to extract Zn and Cd than Cu (Rossi et al. 2002). Hyperaccumulators of As, e.g. some ferns, offer the potential for *in-planta* phytoremediation (Zhao et al. 2002). The *in-planta* partitioning and manipulation of As also offers potential (Vassil et al. 1998; Pickering et al. 2000), and in this respect, crop species able to restrict As translocation, offer the potential to diminish food contamination (Meharg and Hartley-Whitaker 2002). Knowledge of the entire genome of *Arabidopsis* hyperaccumulators is likely to open further avenues, also at the genetic level, to improve the phytoextraction and rhizofiltration capabilities of plants with regard to toxic concentrations of metals in the environment (Schäffner et al. 2002).

3.2.4 Rhizosphere

The microcosm around plant roots, i.e. the rhizosphere, is highly complex and heterogenous. The oxidation status and species composition of mineral elements are influenced by the physical and chemical conditions in the soil, the presence of free-living and plant-associated microflora, and plant roots (reviews: Crowley and Rengel 1999; McCully 1999). In contrast, the uptake of nutrients and water from the soil by plant roots may alter the condition of the rhizosphere, which can differ from that of surrounding soil (Darrah 1993; McCully 1999). For instance, As and Fe concentrations are higher in the rhizosphere of some wetland plants than in the surrounding soil solution (Doyle and Otte 1997). Plant-associated mycorrhizae play an important role in As (Sharples et al. 2000; Meharg and Hartley-Whitaker

2002) and Zn tolerance and toxicity (Faber et al. 1990; Kaldorf et al. 1999). Mycorrhizal associations have also been shown to alter the uptake of essential and harmful elements. For example, in *Viola calaminaria* (DC.) Lej., association with arbuscular mycorrhizal (AM) fungus altered the distribution of some macronutrients, micronutrients and heavy metals (Fe, Zn and Ni) in the root tissues, so that the stelar tissues had less heavy metals than the cortex compared with plants grown without the AM fungus (Kaldorf et al. 1999).

The efficiency of nutrient acquisition is influenced by root morphology and diameter, number of root hairs and length, pattern of growth, and degree of root penetration in the soil (McCully 1999; Williamson et al. 2001). Similarly, the nutrient status of plants and external growing conditions influence the morphology and some other characteristics of the roots (Adalsteinsson and Jensén 1989; Dinkelaker et al. 1995; Williamson et al. 2001). In *Arabidopsis*, the shoot P status and the external and intrashoot phosphate availability together influence the activity of root tips and phosphate acquisition (Williamson et al. 2001). Reduced availability of some other nutrients can also influence root architechture and induce proteoid patterns of growth (Dinkelaker et al. 1995).

Mucilage, which is formed by decaying root cap cells and bacterial activity (Uren 1993; McCully 1999), mediates contact between soil particles and plant roots (Darrah 1993; Uren 1993; McCully 1999). Mucilage may also contribute to the mobilization of mineral elements, such as Mn (Uren 1993). Mucilage protects roots from harmful elements, such as Al, which is bound to it (Marschner 1995). Fine soil particles and mineral elements may be adsorbed onto roots, which affects access of other elements.

Roots can also actively exude protons and various substances, including organic acids and nitrogenous compounds. This phenomenon relates to nutrient acquisition of the plant, in particular, to that of P, Mn, Fe and Zn, but is probably also influenced by other factors, such as pH (reviews: Jones 1998a; Ryan et al. 2001). Organic acids, such as malate, oxalate and citrate form metal ion complexes, and their role with regard to metallic elements may relate to tolerance mechanisms, although this function awaits further clarification (Jones 1998a). Yet, root-exuded oxalate has been reported to enhance Pb tolerance in rice (Yang et al. 2000).

Soil microbial activity may oxidize, reduce and methylate metalloids and metals; e.g. As may be converted by soil bacteria into volatile monomethyl- or dimethylarsinic acids and As-hydride, and also oxidized (Siegel 1998; Seidel et al. 2002). For example, under S-rich conditions of mine tailings, *Thiobacillus* species enhance the solubility of As by oxidation, with a simultaneous production of sulphuric acid and decline in soil pH, which as a cascade effect increases the solubility of Cu, Mn and Zn (Seidel et al. 2002). Pb (Siegel 1998) and Zn (Chaney 1993) may also be volatilized.

A number of nonpathogenic soil bacteria and mycorrhizal fungi are able to improve nutrient availability to plants and may protect plants from toxic elements (Schüepp et al. 1987; Marschner 1995; Díaz et al. 1996; Kaldorf et al. 1999; Carlot et al. 2002), but the mode and extent of plant-mycorrhizal fungus cooperation is dependent on the species (Díaz et al. 1999). Elevated element concentrations are harmful to the activity of soil- and plant-associated microflora (Doelman and Haanstra 1989; Chaudri et al. 1993; Bruins et al. 2000). Yet, the response of soil microorganisms to Zn and Pb is significantly related to soil properties, such as cation exchange capacity, organic C, and total N contents, and is also dependent on the activity status of the soil microflora population; an actively metabolizing soil population is more vulnerable than a dormant one (Ohya et al. 1988).

Rhizobium species appear to differ in their sensitivity to soil metal contamination. *R. meliloti* is unaffected at 1540 mg Zn kg^{-1} (Angle and Chaney 1991), whereas strong reduction in the number and activity of *R. leguminosarum* biovar. *trifolii* occurs in soils at 200-250 mg Zn kg^{-1} (Chaudri et al. 1993). In *Rhizobium* bacteria, resistance to excess Zn involves a proton-cation antiporter protein, which also operates in the formation of root nodules in legume species (Nies and Silver 1995).

In mycorrhizae (Sharples et al. 2000) and some soil and water microorganisms, resistance mechanisms to arsenate, Pb and Zn have been characterized to the level of gene regulation (Nies and Silver 1995; Rensing et al. 1998; Bruins et al. 2000). In addition to *R. leguminosarum*, *P. sativum* lives in symbiosis with an arbuscular endomycorrhizal fungus (Balestrini et al. 1999).

3.2.5 Temperature

Soil and air temperature affects the availability of elements and their toxicity, and influences the transpiration of plants and the rate of mobility of elements in the transpiration stream. In general, active uptake of elements is responsive to temperature change; e.g. low temperature reduced the uptake of Zn in wheat (Hart et al. 1998). In corn, macronutrient concentrations of the ear leaves were higher at 35 °C than at 16 °C, whereas the concentrations of Cu, Ni, Cd or Pb were not affected, yet the concentration of Zn rose with temperature (Sheaffer et al. 1979a, b). Another example is the accumulation of Pb in ryegrass, which increased with rising temperature from 15 to 25 °C both in sludge-amended soils and in soils with added Pb nitrate (Hooda and Alloway 1993). Optimum growth temperature differs between species, but for most crops it is between 20 and 30 °C (Marschner 1995), which is also true for *P. sativum*, although cultivar-specific differences occur (Pate 1977a).

3.3 Early research

In the following a few examples of early research are reviewed with regard to responses of different plant species to As, Pb and Zn and to the problematics of toxicity of these elements. In 1885, Knop listed As, Pb and Zn as nonessential elements for plants.

3.3.1 Arsenic

Arsenite was found to be more toxic than arsenate, and monocotyledons less susceptible than dicotyledons, of which *P. sativum* was the most sensitive species. An amount as low as only 1 mg of As litre^{-1} reduced the growth of *P. sativum*, caused brown coloration of roots, plasmolysis and altered water balance (Nobbe et al. 1884). In 1898, Stoklasa noted a dark-green coloration of plants exposed to As. Pea was more sensitive to arsenite than barley and shoot growth more sensitive than that of the roots in both species (Brenchley 1914).

Arsenic compounds were widely used in agriculture by early 1900. But reports started to appear claiming that As-, Pb- and Cu-containing herbicides were causing deaths of orchard trees, grass and grazing animals (Headden 1910). Morris and Swingle (1927) demonstrated that in potted garden soil, the toxicity was dependent on the cation component of the arsenical herbicides, plant species and soil type. The claim that soils accumulated As herbicides (Headden 1910) was supported by sugar growers in Australia in the 1930s (c. f. Clements and Munson 1947). Yet, since As compounds were considered effective soil sterilants, their use was encouraged, but the benefits of matching carefully the dose with soil type were emphasized (Crafts 1935). Early phytoremediation experiments in soils with tomato, Sudan grass and bean suggested that about 100 harvests would be required to remove arsenic from the topsoil (Clements and Munson 1947). Later, As toxicity was doubted again, at least in citrus species, since the deep-rooted growth pattern allowed roots to bypass As-rich layers (Liebig et al. 1959).

As-P interaction interested many researchers. Stoklasa (1897) observed that a low dose of arsenate promoted growth when supplied with phosphate. Later, it was reported that phosphate addition reduced the toxicity of arsenate in a soil type-dependent manner (Hurd-Karrer 1939; Benson 1953). The interaction was suggested to occur at the uptake level (Clements and Munson 1947), or arsenate could replace phosphate in oxidation reactions (Bonner 1950).

3.3.2 Lead

Higher concentrations of Zn were found to be more toxic to *P. sativum* (Nobbe et al. 1884) and *Zea mays* (Knop 1885) than Pb. The latter ‘only’ reduced the dry matter production of otherwise apparently healthy plants (Knop 1885). The low toxicity of Pb was confirmed in *Vicia faba*, in which Pb accumulated in the roots, but one-third of it could be removed with dilute HNO_3, suggesting that Pb was attached to the cell wall apoplastic space. Strong antagonism between Pb and Cu, and a weaker one with Mg, Ca and K were identified (Hevesy 1923). Somewhat later, however, Prát (1927) established strong Pb-Ca antagonism in *V. faba*. Yet, soil-applied Pb was suggested to be beneficial rather than harmful, and Pb nitrate was claimed to be a better fertilizer than Na nitrate (Berry 1924).

3.3.3 Zinc

Zn was found to affect internode development in *P. sativum* (Nobbe et al. 1884). A low Zn concentration range stimulated the growth of *P. sativum* and *Hordeum vulgare* (Brenchley 1914), although no conclusion was drawn on the essentiality of Zn. Based on their research of nutrient solution cultures in several mono- and dicotyledonous plant species, Sommer and Lipman (1926) were able to confirm that Zn was an essential micronutrient for plants. In the following decade, the role of Zn as a micronutrient was investigated intensively, particularly in connection with a little-leaf or rosette disease condition of fruit trees, including citrus species and stone fruit trees, (Chandler et al. 1932; Hoagland et al. 1936). It was realized that amendment of the soil with ferrous sulphate, which had Zn as a significant impurity, removed the problem. After subsequent experimentation with Zn sulphate, Chandler et al. (1932) suggested that it might react with some harmful unidentified chemical in the soil and prevent the occurrence of the disease. Later observations made on a number of herbaceous and woody species showed that species reacted differently to a certain external Zn dose and to different Zn salts. Moreover, the amount of *in-planta*, metabolically available Zn was suggested to be altered during plant growth, because the response of seedlings to a certain external Zn dose showed toxicity, but plants later recovered and grew well at the same external Zn concentration (Hoagland et al. 1936).

3.4 Uptake of essential and nonessential elements

To become metabolised by the plant, elements need to be transported, at some point, through the plasma membrane of the roots. Membrane transport is powered by the electrochemical potential created by H^+-ATPases (section 3.5.1; Kennedy and Gonsalves 1987; Palmgren 2001). An excess of positively charged ions passing through the plasma membrane depolarizes the membrane potential and acidifies the cytoplasm (Cumming and Taylor 1990; Axelsen and Palmgren 2001). The membrane potential is susceptible to disturbance by arsenate, Pb and Zn (Kennedy and Consalves 1989; Ullrich-Eberius et al. 1989; Barlian Aidid and Okamoto 1992). For example, in the plasma membrane fraction of the roots of *Zea mays*, low Zn concentration enhances the H^+-ATPase activity by competing with Mg (Kennedy and Consalves 1989; Axelsen and Palmgren 2001), but 3 mM Zn is inhibitory. Pb does not activate the ATPase, although it slowly depolarizes the membrane potential (Kennedy and Gonsalves 1989). In *Lemna gibba,* depolarization by arsenate is dependent on the phosphate level (Ullrich-Eberius et al. 1989), and in *Impatiens balsamina* stem sections, Pb (0.5 mM) depolarizes the parenchyma-xylem membrane potential, i.e. xylem loading (Barlian Aidid and Okamoto 1992).

3.4.1 Uptake of arsenate

Competition between phosphate and arsenate at the uptake level (Clements and Munson 1947) has been confirmed in more recent studies, e.g. in *Oryza sativa* (Tsutsumi 1983; Abedin et al. 2002), *Holcus lanatus* (Macnair and Cumbes 1987; Meharg and Macnair 1992a; Hartley-Whitaker et al. 2001b), *L. gibba* (Ullrich-Eberius et al. 1989), *Brassica juncea* (Pickering et al. 2000) and *Pteris* ferns (Zhao et al. 2002). Meharg and Hartley-Whitaker (2002) have recently reviewed the uptake and metabolism of As in plants.

Due to the negative charge present, root cells absorb anions instead of their moving in the apoplastic space of the root cortex (Clarkson 1996). In *L. gibba,* arsenate uptake is characterized as proton-anion cotransport (Ullrich-Eberius et al. 1989). In higher plants (Meharg and Macnair 1990; 1992b; Meharg et al. 1993; Abedin et al. 2002), mycorrhizae (Sharples et al. 2000) and bacteria (Nies and Silver 1995; Bruins et al. 2000), arsenate and phosphate have been suggested to share the uptake system, although further details of the relationship are being investigated (Meharg and Hartley-Whitaker 2002). Arsenate accumulation follows Michaelis-Menten kinetics at a concentration range that coincides with the level of activity of the high-affinity phosphate uptake system (Sharples et al. 2000; Abedin et al. 2002). Two types of arsenite transporters have been identified in microorganisms, but they operate in pumping arsenite either into the vacuole or in efflux from the cells (review: Rosen 1999).

3.4.2 Uptake of lead

Roots usually accumulate Pb (e.g. Ernst 1974; Cseh 2002; Krupa et al. 2002), but it also translocates in the transpiration stream (Tanton and Crowdy 1971; Malone et al. 1974; Donnelly et al. 1990). Pb moves in the apoplastic space of the root cortex, and it can bypass the endodermis and gain symplastic access in the young root zone and in sites of lateral root initiation (Wierzbicka 1987; Eun et al. 2000). Pb has been shown to enter and move within the cytoplasm (Malone et al. 1974), and its uptake has been thought to be by passive absorption (Tung and Temple 1996), although more recently, proteins have been identified that mediate cross-membrane movement of Pb (Kerper and Hinkle 1997; Rensing et al. 1998; Arazi et al. 1999). Apart from a channel type of protein identified in the root plasma membrane of tobacco (Arazi et al. 1999), mechanisms implicated in Pb transport have mainly been demonstrated in cell cultures and in microorganisms (Kerper and Hinkle 1997; Rensing et al. 1998; Binet and Poole 2000).

3.4.3 Uptake of zinc

Zn is an essential micronutrient and is mobile in plants (e.g. Longnecker and Robson 1993). The usually negative membrane potential in the roots could enable passive intake of Zn, although it is very unlikely to occur. Recently, low- and high-affinity active uptake systems of Zn have been described, and several membrane transporters are known to traffick Zn (Guerinot 2000; Lasat et al. 2000; Pence et al. 2000; Assunção et al. 2001). Some of these proteins also transport other metallic elements (e.g. Guerinot 2000; Lasat et al. 2000; Pence et al. 2000; Williams et al. 2000; Assunção et al. 2001; Mäser et al. 2001).

3.5 Transporters

A number of recent reviews have been published on different types of metal transporters operating in different cellular compartments and functions (e.g. Guerinot 2000; Lasat et al. 2000; Williams et al. 2000; Axelsen and Palmgren 2001; Clemens 2001; Dietz et al. 2001; Maeshima 2001; Mäser et al. 2001; Palmgren 2001; Gaxiola et al. 2002).

Many molecular mechanisms have been discovered that mediate cross-membrane metal traffic in plants (Eng et al. 1998; Grotz et al. 1998; Curie et al. 2000; Lasat et al. 2000; Pence et al. 2000; Assunção et al. 2001). It is, however, presumed that high- and low-affinity uptake systems exist for nutrient elements and that external and *in-planta* element concentrations influence the activation of these systems (Guerinot 2000). Nutrient elements taken up by plants are translocated to the sites where they are needed, which implies the presence of highly sophisticated intraplant coordination networks and signal transduction and cooperative mechanisms, making the whole issue of element balance extremely complicated (Guerinot 2000; Williams et al. 2000; Clemens 2001; Dietz et al. 2001), a fact already indicated by the Zn compartmentation model (Ernst et al. 1992).

3.5.1 ATPases

H^+-ATPases constitute a large group of cross-membrane transporters that differ among the types of membrane acted upon, i.e. P-type in plasma membrane, V-type in vacuole and F-type in mitochondria (reviews: Axelsen and Palmgren 2001; Maeshima 2001; Palmgren 2001; Gaxiola et al. 2002).

The plasma membrane P-type H^+-ATPase is an approximately 100-kDa polypeptide that operates the transmembrane ion traffic, using the energy of covalently bound P derived from ATP. Mg^{2+} is a necessity for active functioning of these ATPases and is believed to facilitate the transfer of the energy-rich phosphate from ATP to the protein (Palmgren 2001). In both eukaryotes and prokaryotes, these ATPases have been suggested to function, apart from transport, also in the homeostasis of metals, such as Cd^{2+}, Co^{2+}, Pb^{2+}, Ag^{2+} and Zn^{2+} (Rensing et al. 1998; Binet and Poole 2000; Williams et al. 2000). The P-type ATPases also transport K^+, Na^+, Ca^{2+} and Mg^{2+} (Palmgren 2001).

The P-type H^+-ATPases constitute a family of 45 members (‘superfamily’). The entire genome of this transporter family is known in *Arabidopsis*. The large group is divided into subfamilies according to the metals they transport, and subfamily P_{1B}-ATPases are involved in heavy metal transport (Axelsen and Palmgren 2001). These enzymes are also called CP_x-ATPases, due to the shared sequences (‘CP_x motif’) of cysteine-proline-cysteine, cysteine-proline-histidine or cysteine-proline-serine (Williams et al. 2000). This subfamily has two affinity groups, the Cu^{2+} and Ag^{2+}, and the Zn^{2+}, Co^{2+}, Cd^{2+} and Pb^{2+} clusters (Rensing et al. 1998; Axelsen and Palmgren 2001) that display a high degree of selectivity towards the particular metal they traffic. The *Arabidopsis* heavy metal-transporting ATPases resemble those identified in *Escherichia coli* (Axelsen and Palmgren 2001). In *E. coli*, the transcriptional expression of the Z*ntA* gene is more strongly induced by Cd^{2+} and Pb^{2+} than by Zn^{2+} (Binet and Poole 2000).

The V-type ATPases, which also require ATP and Mg^{2+} (review: Maeshima 2001), have been implicated in tonoplast metal transport and in homeostasis-building in plants grown under normal conditions, as well as in responses to toxic levels of metals (Williams et al. 2000; Dietz et al. 2001). Vacuolar H^+-pyrophosphatase (V-PPase), which derives P from inorganic phosphate instead of ATP, may also serve as an electrochemical H^+ pump in vacuolar metal transport (Dietz et al. 2001; Maeshima 2001; Gaxiola et al. 2002). These proton pumps have been identified in a number of crop species (Maeshima 2001). However, the complete role, function and specificity of vacuolar proton pumps and antiporter systems are still being investigated (Dietz et al. 2001; Maeshima 2001). V-ATPase transporters have, however, been shown to serve in vacuolar Zn trafficking in *Silene vulgaris* (Chardonnens et al. 1999). Apart from transport, indications exist of the involvement of V-ATPases in other functions, such as morphogenesis or photomorphogenesis (Maeshima 2001). These transporters may also occur in types of plant membrane other than the tonoplast (Dietz et al. 2001; Maeshima 2001), while the tonoplast probably harbours other, yet unidentified, ion channels and transporters (Maeshima 2001).

ABC-type ATPases (ATP-binding cassette transporters) have been suggested to be involved in the transport of Cd^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+} and Mo^{2+} and in cross-tonoplast transport of complexed elements, such as metal-phytochelatin and metalloid-phytochelatin complexes, and some other organic molecules (Vögeli-Lange and Wagner 1990; Ortiz et al. 1992; Nies and Silver 1995; Axelsen and Palmgren 2001). These ATPases are found in both eukaryotic and prokaryotic organisms (Paulsen and Saier 1997). In addition to tonoplast, various isoforms of ABC-ATPase transporters occur in the plasma membrane and endoplasmic reticulum (Maeshima 2001).

3.5.2 Cation Diffusion Family

The Cation Diffusion Family (CDF) of transporters comprises 13 proteins that occur in eukaryotes and prokaryotes, although members of this family differ with respect to the number of amino acids and sequences, as well as location and direction, in which they operate the catalytic function. The CDFs are regarded as highly specilized and have so far been implicated in the transport of Cd^{2+}, Co^{2+} and Zn^{2+} (Paulsen and Saier 1997; Williams et al. 2000). For example, the ZAT proteins in *Arabidopsis* have been suggested to function in the cellular sequestration of Zn and probably also in that of other metals (Williams et al. 2000; Maeshima 2001; Hall 2002). The overall role of CDFs may be more directed towards the maintenance of cellular homeostasis than towards element uptake (van der Zaal et al. 1999; Williams et al. 2000).

3.5.3 ZIP and ZNT transporters

ZIP (<u>Z</u>RT, <u>I</u>RT related <u>P</u>rotein) metal transporter proteins (Guerinot 2000; Pence et al. 2000; Assunção et al. 2001; Mäser et al. 2001) have been implicated in the uptake and transport of a number of cations, such as Cd^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+} (Eng et al. 1998; Grotz et al. 1998). The iron transporter (IRT1) protein, which belongs to this family, operates in the uptake of Fe^{2+}, and in the roots of *Arabidopsis*, the respective mRNA is induced under low Fe status (Connolly et al. 2002; Vert et al. 2002). It can be artificially induced by over-expression of genes regulating the synthesis of ferritin, a protein involved in cellular Fe storage (van Wuytswinkel et al. 1998). IRT1 also transports Mn and Zn, and the concentration of these two elements usually rises under low Fe status in plants (Welch et al. 1993; Cohen et al. 1998).

ZNT proteins constitute another group of transporters, which are encoded by a family of genes with similarity to the *ZIP* gene family (Eide et al. 1996) of transporters (Lasat et al. 2000; Pence et al. 2000; Assunção et al. 2001). The ZNT proteins have been shown to be involved in the uptake and transport of Zn (Pence et al. 2000; Assuncão et al. 2001) and Cd (Lasat et al. 2000; Pence et al. 2000). In the hyperaccumulator *Thlaspi caerulescens*, expression of this gene family is higher than in the non-hyperaccumulator *T. arvense*. Both in *T. caerulescens* and *T. arvense*, the ZNT1 protein has been shown to facilitate high-affinity Zn uptake, and in the former species also low-affinity Cd uptake (Lasat et al. 2000; Pence et al. 2000), although in *T. arvense* Zn deficiency was required for the expression of ZNT proteins (Pence et al. 2000; Assunção et al. 2001).

The rapid pace of progress in discovering new members of the ZIP family is illustrated by figures showing that in 1998, 15 members were known (Eng et al. 1998), while in 2000, the number was more than 25, presumably occurring in all eukaryotes, and identified in many crop plants (Guerinot 2000; Pence et al. 2000; Assunção et al. 2001). ZIP and ZNT transporters occur at least in the plasma membrane and plastids (Grotz et al. 1998; Guerinot 2000; Lasat et al. 2000; Pence et al. 2000; Assunção et al. 2001). In plasma membranes, their structure is believed to include from 5 to 8 (Grotz et al. 1998), or 8 (Guerinot 2000; Pence et al. 2000; Assunção et al. 2001) cross-membrane domains, and a “variable region of amino acids” with high numbers of histidine residues is presumed to dwell in the cytoplasmic side (Guerinot 2000; Pence et al. 2000; Assunção et al. 2001). It has been postulated that the histidyl area is involved in heavy metal binding (Pence et al. 2000; Assunção et al. 2001) and contributes to the construction of a channel-type avenue servicing the cross-membrane metal traffic (Eng et al. 1998; Guerinot 2000). The toxic external Zn concentration down-regulates the expression of ZIP proteins (Grotz et al. 1998; Connolly et al. 2002). Four types of ZIP transporters have been identified, and these proteins occur in different plant organs and are likely to differ in their functions (Grotz et al. 1998; Clemens 2001). There is great similarity between the ZIP4 and IRT1 proteins of *Arabidopsis* (Eide et al. 1996; Grotz et al. 1998) and the ZNT1 protein of *Thlaspi* (Lasat et al. 2000; Pence et al. 2000; Assunção et al. 2001).

3.5.4 NRAMP

NRAMP (natural resistance associated macrophage proteins) proteins occur in bacteria, fungi, plants and animals (Clemens 2001) and are likely to be involved in metal-ion homeostasis, particularly that of Fe, rather than uptake (Curie et al. 2000).

3.6 Tolerance and toxicity

Agriculture today and in the future will need to make use of contaminated and naturally enriched soils, with higher concentrations of one or more elements (Macnair 1993; Abedin et al. 2002). Therefore, improvement of metal resistance in crop varieties is important only as long as the metal concentrations in food plants do not exceed health levels. The difference in response of crop plants to soil toxicants also extends itself to their nutrient efficiency (Aniol and Gustafson 1989).

According to Macnair (1993) tolerance “represents a genotype-environment interaction”. Plants have been grouped into ‘accumulators’ or ‘excluders’ (Baker 1987). Many crop plants are accumulators (Aniol and Gustafson 1989). Excluder plants diminish the uptake of elements (Baker 1987), although exclusion capacity in higher plants is poor or absent (Ernst 1976), whereas bacteria (Nies and Silver 1995) and some mycorrhiza (Sharples et al. 2000) are able to efflux toxic elements.

Tolerance is under genetic control, although the number of genes varies from one to a smaller or larger number of genes, the action of which may further be influenced by ‘modifier’ genes (Macnair 1993; Schat et al. 1996). Tolerance is constitutive i.e. present, and adaptive, i.e. responsive to an external factor, yet both types of tolerance are intertwined (Macnair 1993; Meharg 1994). For example, an elevated level of soil metal concentration (toxicity) causes “selection pressure”, which plays a role in tolerance (Schat et al. 1996). Metal tolerance can be separate, meaning tolerance of each metal regulated by separate gene(s), or cotolerance (pleiotropy), while multiple tolerance is only developed in plants growing on soils with more than one metal in excess (Macnair 1993). For example, in populations of *Silene vulgaris,* originating from metalliferous sites in Ireland and Germany, and from a nonmetalliferous site in the Netherlands, two major coadditive genes control Zn tolerance in this species (Schat et al. 1996). Metal tolerance of crops is believed to be of the separate type (Aniol and Gustafson 1989). With the rapid discovery of metal-trafficking proteins, the understanding of cellular-level processes has progressed, but less is known of the relationships between element homeostasis and tolerance to toxic metals or metalloids at the level of entire organisms (Clemens 2001).

The usual parameter describing toxicity is yield reduction, in terms of shoot and root length, or fresh and dry matter production (Berry and Wallace 1981), but reversibility of plasmolysis (Ernst 1974), enzyme activities, chlorophyll contents and other physiological parameters are also employed (Baker and Walker 1989). To quantify tolerance/toxicity, several indices have been developed. The tolerance index (TI) expresses the relationship between root growth with and without a toxic element (Wilkins 1957). The LD_{50} expresses the dose of a toxicant that causes the death of 50% of test objects. Plant yield reduction may be expressed as EC_{10}-EC_{50}, i.e. ‘effective concentrations (EC)’ to lower yield by 10-50% (Ernst 1997b; Kooijman 1997). The critical tissue toxicant concentration is also used (Davis and Beckett 1978; Macnicol and Beckett 1985). For example, in soil culture of soybean and cabbage, 10% toxicity (EC_{10}) occurs at the upper critical tissue As level of 1 and 1-4 mg of As kg^{-1} plant shoot and leaf dwt. The values for Zn as an essential micronutrient are higher; for example in soybean, bush beans and pea, these values are 450, 250 and 380-500 mg Zn kg^{-1} shoot or leaf dwt (Macnicol and Beckett 1985).

3.7 Physiological response mechanisms

In the following ‘response mechanism’ is used in the meaning of modes of action of plants under exposure, instead of the term ‘tolerance mechanims’. Neither tolerance nor toxicity mechanisms are fully defined as yet (Verkleij and Schat 1989; Macnair 1993; Schat et al. 1996), and mechanisms likely to be involved in tolerance may also include responses such as altered membrane permeability, enhanced metal-binding capacity of the root apoplasm and root exudates. Cellular mechanisms include synthesis of organic acids, phytochelatins, proteins, as well as compartmentalization, and metabolism- and membrane-adjusting functions, including the synthesis of specific transporters (e.g. Verkleij and Schat 1989; Harmens et al. 1993; Meharg 1994; Hall 2002). There is still question as to which mechanisms contribute to the primary and which to the secondary response. The activation of these mechanisms displays element- and plant species-dependency, and presumably more than one mechanism is active simultaneously in a species (Ernst 1976; Harmens et al. 1993; Macnair 1993). Fodor (2002) has recently reviewed responses of higher plants to heavy metals.

3.7.1 Phytochelatins

Synthesis of phytochelatins (PCs) and metallothioneins (MTs) is one of the responses of plants to elevated concentrations of a number of metals or metalloids. Due to their similarity with MTs, PCs have been called class III MTs (Rauser 1995; Cobbett and Goldsbrough 2002). The precise role of PCs, and allocation of tasks between PCs and MTs, is currently evolving and has been repeatedly reviewed (Reddy and Prasad 1990; Steffens 1990; Rauser 1995; Cobbett 2000; Cobbett and Goldsbrough 2002). Currently, PCs are believed primarily to function in detoxification, whereas MTs have been allocated other roles, e.g in chaperoning the translocation of some metallic elements. A gene family encodes MTs, whereas PCs are enzymatically produced (Cobbett and Goldsbrough 2002). Since PC synthase genes have now been identified in higher plants, the role of PCs may be further clarified (Clemens et al. 1999; Ha et al. 1999; Vatamaniuk et al. 1999).

PCs are small-molecular weight, cysteine-rich polypeptides, in which n = 2-11 (e.g. Grill et al. 1985; 1987; Reddy and Prasad 1990) or n = 2-5 (Cobbett and Goldsbrough 2002). PCs were first detected in cell suspension cultures of *Rauwolfia serpentina* exposed to 0.2 mM Cd sulphate (Grill et al. 1985). PCs are induced in response to Ag, Au, Cd, Cu, Hg, Ni, Pb, Sb, Sn, Te and Zn (Grill et al. 1987), and arsenate and selenate (Grill et al. 1986). The intensity of induction is metal-specific, with the proposed sequence: Hg > Cd > As > Te > Ag > Cu > Ni > Sb > Au > Sn > Se > Bi > Pb > W > Zn (Grill et al. 1987; Ernst 1997a). There are exceptions to this sequence; e.g. in some legumes, Pb is a strong inducer of PCs (Tomaszewska et al. 1996; Piechalak et al. 2002). This was also confirmed in root cultures of *Rubia tinctorium*, in which the sequence Ag > Cd > Pb > Hg > As(III) > Cu > As(V) > Zn > Pd > In > Ga > Se > Ni has been suggested (Maitani et al. 1996). Production of PCs implies toxicity, but not necessarily tolerance (Schat and Kalff 1992; Gawel et al. 1996; Ebbs et al. 2002). In fact, the information currently available supports the view that PCs do not significantly contribute to Zn, Cd (Schat and Kalff 1992; Harmens et al. 1993; de Knecht et al. 1994; Ebbs et al. 2002; Schat et al. 2002) or Cu tolerance (De Vos et al. 1992; Schat et al. 2002). In *Silene cucubalus*, Cu tolerance is related to the ability of root cells to maintain glutathione levels, e.g. by restricting the influx of Cu to these cells, and subsequent reduction of the level of PC synthesis (De Vos et al. 1992).

Apart from the inactivation of toxic elements, PCs probably also serve in other functions, including micronutrient homeostasis (Reddy and Prasad 1990; Robinson et al. 1992; Schat et al. 2002), S metabolism (Tomaszewska et al. 1996), maintenance of enzyme activity (Kneer and Zenk 1992), translocation of metals (Vögeli-Lange and Wagner 1990; Cobbett and Goldsbrough 2002) and transport and storage of As (Hartley-Whitaker et al. 2001b). It has been proposed that the inactivation function is secondary to PCs´ primary role in homeostasis (Steffens 1990) a view supported by the fairly rapid turnover of PCs (Tomaszewska et al. 1996; Piechalak et al. 2002), but which is currently the subject of further debate (Cobbett and Goldsbrough 2002).

The cross-tonoplast trafficking of PC-complexed metals is energy-consuming and in microorganisms and higher plants, the transport is mediated by ABC-type cassette-binding ATPases (Vögeli-Lange and Wagner 1990; Ortiz et al. 1992; Nies and Silver 1995). In the presence of Mg^{2+}, these ATPases have been shown to transport Cd-PCs in tonoplast vesicles of the roots of *Avena sativa* (Salt and Rauser 1995). Presumably, higher plants possess a gene homologue to the *hmt1* gene, which regulates the production of these transporter proteins in *Schizosaccharomyces pombe* (Ortiz et al. 1992).

Five types of PC have been identified (PC-PC_4), according to the C-terminal amino acid and the length of the chain (Rauser 1995), although desglycyl PC peptides, which lack the C-terminal glycine, have also been suggested to carry out metal-binding (Maitani et al. 1996). In addition to PCs, i.e. the polymers of γ-glutamyl-cysteinyl-glycine, also homo-phytochelatins (h-PCs), polymers of γ-glutamyl-cysteinyl-β-alanine, occur in legumes (Gekeler et al. 1989; Klapheck et al. 1995; Piechalak et al. 2002). Hydroxymethyl-PCs have been reported in Poaceae (Klapheck et al. 1994).

Strong evidence points towards glutathione and homo-glutathione serving as precursors of PCs and h-PCs (Scheller et al. 1987; Klapheck et al. 1995; Cobbett and Goldsbrough 2002). γ-Glutamyl-cysteine synthetase catalyses the polymerization of glutathione and PC synthase the metal-PC reaction (Grill et al. 1987; Klapheck et al. 1995; Piechalack et al. 2002). The PC synthase is regulated by the end product, the PC-metal complex (Loeffler et al. 1989; Cobbett 2000). Since the PC synthase is constitutive in the cytoplasm of many plants, and also in the roots of *P. sativum* (Klapheck et al. 1995), metal ions probably only activate it, although *de novo* synthesis may also occur (Cobbett 2000).

3.7.1.1 Induction of phytochelatins by arsenate

Arsenate and arsenite both induce the synthesis of PCs (Grill et al. 1987; Schmöger et al. 2000; Hartley-Whitaker et al. 2001b; Schat et al. 2002). Induction by arsenate is rapid in nutrient solution-grown *Silene vulgaris* (Sneller et al. 1999), or in cell suspension cultures of this species (Schmöger et al. 2000). Vacuolar storage of an As-PC complex has been proposed (Sneller et al. 1999; Schmöger et al. 2000; Hartley-Whitaker et al. 2001b), although the full picture of the localization of As-PC complex is still evolving (Meharg and Hartley-Whitaker 2002). Evidence has been reported that in *Holcus lanatus,* a portion of the total As may be stored in some form other than As-PC (Hartley-Whitaker et al. 2001b). Indeed, in *Brassica juncea*, arsenate and arsenite are stored in both the roots and shoots as a trivalent As-*tris*-thiolate complex (As bound to three thiolate groups), and in the roots also as trivalent As-*tris*-glutathione. The thiolate originates most probably from glutathione or PCs (Pickering et al. 2000), suggesting that in this species, PCs may serve as an intermediary rather than end product.

3.7.1.2 Induction of phytochelatins by lead

Plant roots may accumulate high amounts of Pb without apparent toxic effects, but the response and sensitivity varies with species (Wierzbicka and Antosiewicz 1993; Piechalack et al. 2002) and with the age of the plant (Tomaszewska et al. 1996). Induction of PCs by Pb is one of the responses of plants (Grill et al. 1987; Mehra et al. 1995; Piechalak et al. 2002). Pb exhibits several coordination numbers when binding with PCs, the number depending on the length of the PC chain; PCs with longer chains bind Pb more strongly than those with shorter chain length. Glutathione has been suggested to transfer Pb to a PC molecule (Mehra et al. 1995). Yet, in root cultures of *Rubia tinctorium,* strong induction of PCs occurred by Pb, but it was not bound to the induced PCs (Maitani et al. 1996).

The induction of PC and h-PC synthesis by Pb varies among legumes (Piechalak et al. 2002). Comparison of the root TIs and PC and h-PC concentration in *Vicia, Pisum* and *Phaseolus* showed that *V. faba* had the highest tolerance, but lowest PC concentrations, and the PCs disappeared after 72 h, while *P. sativum* showed medium tolerance, slightly delayed induction, but high PC and h-PC concentrations (Piechalak et al. 2002). Similar to the diverse roles suggested for PCs under arsenate exposure (Pickering et al. 2000; Schmöger et al. 2000; Hartley-Whitaker et al. 2001a, b), their role with regard to Pb may differ between species (Piechalak et al. 2002).

3.7.1.3 Induction of phytochelatins by zinc

Zn is a weak inducer of PCs (e.g. Grill et al. 1987), although the $PsMT_A$ gene, which has been identified in the roots of *P. sativum*, regulates the synthesis of MT-like proteins (Evans et al. 1990; Robinson et al. 1992). In this species, Zn is inferior to Cu and Cd as an inducer of this synthesis (Robinson et al. 1992; Klapheck et al. 1995). Zn has been shown to induce non-PC thiols, which in a number of early works were mistaken for PCs, due to the assessment method used (Harmens et al. 1993). Other response mechanisms, such as complexation with organic acids, have been shown to be more prevalent and effective mechanisms for inactivating Zn than are PCs (Ernst et al. 1992; Wang et al. 1992; Harmens et al. 1993; Ebbs et al. 2002; Schat et al. 2002).

3.7.2 Organic acids

Despite their role in the rhizosphere (Jones et al. 1996; McCully 1999; Ryan et al. 2001), the amount of organic acids has been shown to increase in plants or in callus cultures exposed to metals, particularly, Cd, Zn and Pb. Organic acids have been implicated in avoidance, deactivation, cellular compartmentalization and intraplant translocation of metallic elements (Ernst 1976; White et al. 1981; Ernst et al. 1992; Wang et al. 1992; Harmens et al. 1994; Mazen and El Maghraby 1997/98). The specific properties of organic acids relating to metal tolerance of plants appear to be dependent on the species and metal, because reported evidence as to their primary role in metal tolerance is contradictory (Godbold et al. 1984; Harmens et al. 1994; Chardonnens et al. 1999; Sarret et al. 2002).

The 'Zn-malate-shuttle' (Fig. 3 in Ernst et al. 1992) is thought to transport Zn to the vacuole, malate being liberated in exchange for oxalate or citrate, and shuttled back to the cytoplasm. Yet, recent evidence on Zn-tolerant and hyperaccumulator *Arabidopsis halleri* suggests that in the aerial portions of this species Zn is complexed in the vacuole mainly with malate, whereas in the roots of plants of this species grown in contaminated soil, it occurred as Zn malate, citrate and phosphate (Sarret et al. 2002). In the roots and leaves of zinc-exposed *Silene vulgaris*, the oxalate level is higher than that of malate, citrate, maleate or succinate, and this acid has been proposed to play a significant role in the vacuolar, but not in the cytoplasmic binding of Zn (Wang et al. 1992; Harmens et al. 1994). The diversity in the roles of organic acids is further illustrated by the fact that in hydroponically grown *P. sativum,* succinate decreased the translocation of Zn to the shoot, due to the formation of a Zn-succinate complex (Doncheva et al. 2001). Moreover, in *Cardaminopsis halleri*, *S. cucubalis* and *Minuartia verna* grown in soils contaminated with Zn and Cu, precipitation of Zn silicate in the cytoplasm has been suggested to function as a measure linked to its transport to the vacuole and to Zn tolerance (Neumann and zur Nieden 2001).

The root/shoot transport of zinc has been proposed to occur as malate and citrate complexes (White et al. 1981). Malate and citrate are found, but not oxalate, in the xylem fluid in *S. vulgaris* (Harmens et al. 1994), *Lycopersicum esculentum* and *Glycine max* (White et al. 1981). However, in *Thlaspi caerulescens*, Zn is mainly transported in the xylem as hydrated cations, and thus, in this species, chelation with citrate plays a less significant role (Salt et al. 1999).

Pb also has been detected in the transpiration fluid in chelated form (Tanton and Crowdy 1971). Knowledge is more limited of the relationship between organic acids and Pb, although a positive relationship in some species has been established. For example, in *Oryza sativa* (Yang et al. 2000) and *Eichhornia* (Mazen and El Maghraby 1997/98), synthesis of oxalate represents a significant response mechanism that enhances tolerance to Pb.

3.7.3 Polyphosphates

As shown in a study of a number of crop plants, phytate globoids are involved in the detoxification of some metals, although not in *Pisum arvense* (van Steveninck et al. 1994). Previously, similar globoids had been detected in the roots of *Deschampsia caespitosa* (van Steveninck et al. 1987) and in the fronds of *Lemna minor* in which Zn was deposited in small cytoplasmic vacuolar globules containing Zn of up to 20% concentration and also Mg and K. In *Lemna,* these globoids contained some Ca, probably derived from the calcium oxalate crystals present in the fronds (van Steveninck et al. 1990). In *Plectonema boryanum* (Cyanophyceae), polyphosphates have also been suggested to function in the detoxification of Pb and some other metals (Jensen et al. 1982).

3.7.4 Proteins

At a certain Zn concentration range (0.02-200 μmol litre^{-1}) the amount of apoplastic polypeptides in the shoots of *Hordeum vulgare* seedlings increase. The strongest increase is in molecular-weight groups of 16, 23, 27 and 28 kDa (Brune et al. 1994). Ni is an even stronger inducer. The regulation of induction occurs at the transcriptional or translational level, but the precise role of these proteins is not known yet (Blinda et al. 1997). Pathogenesis-related (PR) proteins have also been reported to be induced by heavy metals in a metal-specific way, such that in the leaf tissue of *Nicotiana tabacum* a low (0.1 mM) Zn concentration is a strong inducer, but Co and Cu do not induce this protein at all (Hensel et al. 1999).

Heat shock proteins occur in all living organisms in response to high temperature and other types of stress (Vierling 1990), including heavy metals (reviews: Hall 2002; Siedlecka and Krupa 2002), and may protect proteins as ‘molecular chaperons´ (Vierling 1990), and aid in the repair process (Hall 2002). The protein ubiquitin, found in damaged tissues, may be involved in the marking of these proteins (Vierling 1990; Hall 2002). Ubiquitination and subsequent degradation of transporter proteins have been suggested as mechanisms against Zn toxicity (Gitan and Eide 2000).

3.7.5 Cell division

A bioassay on *Tradescanthia*, *Allium* and *Vicia* species showed that the severity of cellular-level impact of As differed among plant species and was dependent on the external concentration and species of As (Steinkellner et al. 1998). As interferes with cell division by disturbing the organization of microtubuli and subsequent formation of the mitotic spindle. As may also inhibit DNA repair enzymes (Steinkellner et al. 1998; Panda and Panda 2002).

In a group that includes As, Pb and Cd, Pb, at elevated concentrations, is the second most potentially harmful element to cell division occurring in plants (Steinkellner et al. 1998) and disrupts it in many species (Wozny and Jerczynska 1991; Liu et al. 1994; Eun et al. 2000). Even a low concentration (10^{-7} M) of organic or inorganic Pb reduces the mitotic index (Radecki et al. 1989; Wozny and Jerczynska 1991). Polynucleated cells and micronuclei are common; effects of Pb on mitosis resemble those of colchicine (Wierzbicka 1988; Liu et al. 1994; Eun et al. 2000).

Of the mineral nutrients, low Zn concentration (0.2 μg litre^{-1}) decreases the mitotic index and extends by 132% the cell-doubling time of meristematic cells in nontolerant *Festuca rubra* (Powell et al. 1986). Elevated concentrations of Zn are, however, not strongly genotoxic (Steinkellner et al. 1998; Gómez-Arroyo et al. 2001).

3.7.6 Cell walls

The exclusion ability of plant roots is limited (Ernst 1976; Baker 1987; Greger 1999), but exclusion by means of dictyosome vesicles to the cell walls does occur (e.g. Malone et al. 1974). Anions, such as arsenate, usually do not move apoplastically (Clarkson 1996). The role played by various charged groups in cell walls and the associated expansins in cell-extension is under discussion (Ernst et al. 1992; Cosgrove 1999).

Pb is associated with cell walls, e.g. in the roots of *Lupinus luteus* (Przymusinski and Wozny 1985), *P. sativum* (Päivöke 1983a; Sieghardt 1984), *Raphanus sativus* (Lane and Martin 1977), and *Zea mays* (Tung and Temple 1996), and sites hosting Pb deposits show irregular thickening (Malone et al. 1974). The meristematic root zones are rich in Pb (Sieghardt 1984; Tung and Temple 1996), whereas the interior of the root cap, and the quiescence centre are devoid of it (Wierzbicka 1987; Tung and Temple 1996). The endodermis functions as a barrier between cortex and stele (van Fleet 1961). Pb is deposited in the endodermal cell walls of *P. sativum* and causes lignification (Päivöke 1983a). Protoxylem (Päivöke 1983a; Sieghardt 1984), protophloem (Sieghardt 1984; Wierzbicka 1987) and pericyle (Tung and Temple 1996) also contain Pb deposits.

Zn has been detected in deposites in the cell walls and intercellular space of many plant species growing in Zn-enriched soils (Ernst 1974; 1997a; Neumann et al. 1997; Salt et al. 1999) often as Zn-silicates. For example, in heavy metal-tolerant *Cardaminopsis halleri,* the outer epidermal cell walls of leaves possess deposits containing Si, Zn, Cu and Sn (Neumann and zur Nieden 2001). Zn is found in *Thlaspi caerulescens*, particularly, in the inner cortex, next to the endodermis and in plasmodesmata (Vazquez et al. 1992).

3.7.7 Suberization and lignification

A common effect of arsenate, Pb and Zn on *P. sativum* is enhanced lignification (Päivöke 1983a, b), which also occurs in other species and in response to different types of stress (Punz and Sieghardt 1993; Snowden et al. 1995; Hagemeyer and Breckle 1996). Increased lignification of forage crops reduces their digestibility and nutritive value (Boudet 2000). Low availability of P, nitrate and Fe enhances phenylpropanoid synthesis (Dixon and Paiva 1995); e.g. in *Zea mays* Mg deficiency enhances suberization in root hypodermis and endodermis (Pozuelo et al. 1984). Inhibition of carbon dioxide fixation also enhances phenylpropanoid synthesis, likely via some N-mediated signalling mechanism (Matt et al. 2002).

Lignin biosynthesis is part of the normal life cycle of plants and requires cooperation between symplast and apoplast; monomers are synthesized in the cytoplasm and polymerization occurs in the apoplasm (Lewis and Yamamoto 1990; Boudet 2000). Biosynthesis is under transcriptional regulation (Dixon and Paiva 1995). Deamination of phenylalanine (Whetten and Sederoff 1995; Lecourieux et al. 2002) and tyrosine (Lewis and Yamamoto 1990) initiate the biosynthesis of phenylpropanoids. Exposure of *Triticum aestivum* to Al, Cd, Zn and some other heavy metals, as well as lowering the availability of Ca, also enhances the expression of *wali* genes encoding phenylalanine ammonia-lyase (PAL; Snowden et al. 1995). Since lignin and suberin biosyntheses are induced by a number of factors, the rise in PAL activity is a nonspecific response.

Several other enzymes are also involved, such as peroxidases, cinnamyl alcohol dehydrogenase (CAD) and cinnamoyl CoA reductase (CCR), but to quantitatively relate the activities of all these enzymes with the degree of lignification is difficult (Lewis and Yamamoto

1990; Boudet 2000). CAD is coded by a single gene and stress activates it (Boudet 2000). The final stages of lignin biosynthesis are likely to involve β-glucosidases (Whetten and Sederoff 1995), peroxidases and laccases (Goldberg et al. 1985; Lewis and Yamamoto 1990; Quiroga et al. 2000). Oxygen radicals are geared to natural lignin biosynthesis (Whetten and Sederoff 1995; Boudet 2000), but they also appear in response to stress, including heavy metal exposure and nutrient deficiencies (Dietz et al. 1999).

3.7.8 Membrane functions

Metal ions may alter biomembrane structures (Kasamo and Sakakibara 1995), e.g. causing severe damage to chloroplast membrane structures (Simola 1977; Sresty and Madhava Rao 1999) and inducing regions of opened bilayer (Cumming and Taylor 1990). The permeability decreases with increasing density of membrane phospholipids, changes that usually indicate oxidative stress (review: Rama Devi and Prasad 1999).

3.7.9 Oxidative stress

High malondialdehyde concentration is an indication of lipid peroxidation and oxidative stress (Minotti and Aust 1987; Gora and Clijsters 1989; review: Rama Devi and Prasad 1999). Malondialdehyde is formed as a result of lipoxygenase activity; in 15-day-old *Phaseolus vulgaris*, exposure to phytotoxic levels of Zn and Cu caused induction of lipoxygenase activity and resulted in the accumulation of malondialdehyde (Gora and Clijsters 1989). On the other hand, in liposomes of rats the degree of phospholipid peroxidation *in vitro* was attributed to the relationship between the molar ratio of citrate and ferrous Fe, in other words, to the ratio between oxidized and reduced Fe (Minotti and Aust 1987).

In addition to *P. vulgaris* (Gora and Clijsters 1989; Chaoui et al. 1997), high concentration of Zn causes oxidative stress, e.g. in *Brassica juncea* (Prasad et al. 1999) and Pb in *P. sativum* (Malecka et al. 2002). In *Holcus lanatus* exposed to arsenate, lipid peroxidation occurs in the roots of nontolerant, but not in those of tolerant clones, because the latter were able to reduce the intake of arsenate, to form arsenate-phytochelatin complexes and to quench the reactive oxygen species effectively (Hartley-Whitaker et al. 2001a).

Unbalanced production of free radicals and active oxygen species causes oxidative stress (reviews: Dietz et al. 1999; Alscher et al. 2002). Antioxidant enzymes are induced, including peroxidases, superoxide dismutases (SODs) and catalase (Dietz et al. 1999; Alscher et al. 2002), although high element concentrations may exceed the quenching capacities of these enzymes (Garnczarska and Ratajczak 2000). Hydrogen peroxide plays a central role in stress signal transduction and in the initiation of a cascade of metabolic responses to oxidative stress (review: Pastori and Foyer 2002). The SODs produce H_2O_2 which is inactivated in a cycle involving ascorbate, glutathione, ascorbate peroxidase and glutathione reductase (Pastori and Foyer 2002). Ascorbate is present in plant cells in considerable quantity (Dietz et al. 1999; Pastori and Foyer 2002), and it contributes, together with glutathione, to the cytoplasmic buffer capacity, and probably plays a role in cell growth (review: Smirnoff and Wheeler 2000; Veljovic-Jovanovic et al. 2001).

4. MATERIALS AND METHODS

This section supplements information given in the original papers I-IV, includes information on preliminary assessments and justifies the choices made.

4.1 Seed material and duration of cultures (I-IV)

After testing the cv. ‘Early Onward’ of *P.*

sativum and discussions with the seed supplier (Siemen Oy), cv. 'Phenomen' ('Fenomen', 'Ilmiö') was believed to best represent field cultivars of this species. Factors affecting the choice of culture periods have been explained (I-IV). Preliminary tests showed that this cultivar flowered within three weeks (21 days). Harvesting for dry hay, forage or silage of intercropped pea coincides with flowering (Valle 1960; Järvi 1984). The 3-12-day cultures corresponded to active hydrolysis and remobilization of cotyledon reserves (Bain and Mercer 1966).

4.2 Soil mixture (I-IV)

After growth chamber trials, a greenhouse environment was concluded to best serve the aim of the study. Combinations of garden soil, fertilized and limed peat, compost, granite sand and quartz sand were tested. Cv. 'Phenomen' grew homogenously in the chosen mixture of peat, granite sand and quartz sand (4:3:3 w/w), which also represented the major contributors to soils in Finland (Sillanpää 1982; Simonen 1992). The soil was unsterilized, and neither soil microflora were specified nor inoculation of *Rhizobium leguminosarum* done. The occasional nodules, however, indicated the presence of natural rhizobia (I-III). Preliminary efforts at inoculating the soil mixture of the pots with a few millilitres of water suspension of pure culture of *R. leguminosarum* (HT3) showed that only a limited number of nodules were formed. The soil mixture was rich in P and N (Table 1/I), the latter of which factors may explain the poor nodulation (Voisin et al. 2002). As the aim of the work was to study the overall N status, determination of the total N content was performed (I-III).

The study comprised two nonessential elements and one micronutrient, 4-5 soil concentrations each (Tables 1a-b); a large quantity of soil mixture was required, and therefore, it was prepared in several replicate lots. For the calculation of soil arsenate, Pb and Zn concentrations, the soil content of pots, filled visually to a level equal to that of the pots used in the study, was permitted to air-dry for a few days, kept overnight at 105 $\pm$ 2 °C, cooled in the oven (6 h), and weighed. Drying time of 2 x 24 h plus 6 h cooling yielded similar dry weights. The larger pots, used in 12-day cultures, contained 755 $\pm$ 11 g dwt (n = 23), and the medium-sized pots, used in 21-day cultures, 426 $\pm$ 5 g dwt (n = 70). The dwts were used in the calculation of soil concentrations of arsenate, Pb and Zn instead of air-dried weights to avoid inaccuracy caused by seasonal variation in air and soil moisture content. Another set of samples was permitted air-dry at room temperature for soil analysis.

Total soil As content was determined with neutron activation by the Technical Research Centre of Finland (VTT) Reactor Laboratory, in an air-dried soil mixture (I). Viljavuuspalvelu Oy carried out the remaining soil analyses with standard methods in air-dried ground and sieved ($\leq$ 2 mm) soil samples (Table 1/I).

4.3 Artificial contamination of soil (I-IV)

Sodium arsenate (Na_2HAsO_4 x $7H_2O$, p.a. Merck), lead acetate [$(CH_3COO)_2$ Pb x $3H_2O$, p.a. Merck], and zinc acetate [$(CH_3COO)_2$ Zn x $2H_2O$, p.a. Merck] were added to pots as explained (I-IV), the soil mixture of which was premoistened with ion-exchanged water. After reaching the final arsenate, Pb or Zn concentrations, the pots were permitted to stand for a few days, or until moderately moist, before sowing, since *P. sativum* tolerates wet soils poorly (Pate 1977a; Järvi 1984); the elements could also briefly stabilize. Irrigation was performed with ion-exchanged water by sprinkling manually through the top of the pots.

In the 21-day experiments, which were started first, the required stock solution volumes were adjusted to yield full milligrams of the studied elements or salt

Table 1 a-b. Artificial contamination of potted soil mixture: stock solutions, volumes of stock solutions used, element concentrations, and total contents as per total content of element salt and element per pot, and per kilogram dry soil.

(a) 3- to 12-day culture:

Arsenic	Concentration of stock solution	ml of stock solution	arsenate mg pot^{-1}	arsenate mg kg^{-1} soil	As mg pot^{-1}	As mg kg^{-1} soil	arsenate or total As concentration
	1 mM	30	9.4	12.5	2.25	3.0	40 µmol kg^{-1} soil
	1 mM	50	15.6	20.8	3.75	5.0	67 µmol kg^{-1} soil[a]
	1 mM	80	25	33.3	6.0	8.0	107 µmol kg^{-1} soil
Lead		ml of stock solution	Pb acetate mg pot^{-1}	Pb acetate mg kg^{-1} soil	Pb mg pot^{-1}	Pb mg kg^{-1} soil	Pb acetate or total Pb concentration
	10 mM	150	570	754	311	412	2.0 mmol kg^{-1} soil
	25 mM	120	1140	1509	622	824	4.0 mmol kg^{-1} soil
Zinc		ml of stock solution	Zn acetate mg pot^{-1}	Zn acetate mg kg^{-1} soil	Zn mg pot^{-1}	Zn mg kg^{-1} soil	Zn acetate or total Zn concentration
	100 mM	40	878	1163	262	346	5.3 mmol kg^{-1} soil
	100 mM	70	1536	2035	458	606	9.3 mmol kg^{-1} soil

Table 1 continued

(b) 21-day culture:

Arsenic	Concentration of stock solution	ml of stock solution	arsenate mg pot^{-1}	arsenate mg kg^{-1} soil	As mg pot^{-1}	As mg kg^{-1}soil	arsenate or total As concentration
	1 mM	10	3.2	7.5	0.75	1.8	24 μmol kg^{-1} soil
	1 mM	30	9.4	22.1	2.25	5.3	71 μmol kg^{-1} soil[a]
	1 mM	50	16.6	36.7	3.75	8.8	118 μmol kg^{-1} soil
	1 mM	100	31.2	73.3	7.5	17.6	235 μmol kg^{-1} soil
Lead		ml of stock solution	Pb acetate mg pot^{-1}	Pb acetate mg kg^{-1} soil	Pb mg pot^{-1}	Pb mg kg^{-1} soil	Pb acetate or total Pb concentration
	10 mM	20.3	77	181	41	100	0.5 mmol kg^{-1} soil
	10 mM	46.3	176	412	95	225	1.1 mmol kg^{-1} soil
	10 mM	110	413	970	225	530	2.6 mmol kg^{-1} soil
	25 mM (10 mM)	110 (275)	1042	2447	570	1338	6.5 mmol kg^{-1} soil
	25 mM (10 mM)	160.2 (400.6)	1520	3568	830	1948	9.4 mmol kg^{-1} soil
Zinc		ml of stock solution	Zn acetate mg pot^{-1}	Zn acetate mg kg^{-1} soil	Zn mg pot^{-1}	Zn mg kg^{-1} soil	Zn acetate or total Zn concentration
	5 g litre^{-1}	26.8	134	316	40	94	1.4 mmol kg^{-1} soil
	5 g litre^{-1}	60.4	302	711	90	211	3.2 mmol kg^{-1} soil
	5 g litre^{-1}	80.5	403	948	120	282	4.3 mmol kg^{-1} soil
	10 g litre^{-1}	82.3	822	1936	245	576	8.8 mmol kg^{-1} soil
	10 g litre^{-1}	167.9	1679	3950	500	1175	18.0 mmol kg^{-1} soil

[a] rounded in the text into 70 μmol kg^{-1}

per pot, while in the 12-day experiments, which were started later, an easier approach using full millilitre volumes was adopted. 'Total arsenate, Pb or Zn (content) concentration per pot' was the working concept. The exact soil concentrations were calculated later based on the average soil dwt per pot (Tables 1a-b). In the text, for unity, 'mol kg^{-1}' is used. For easier comparison with the literature, other units may be mentioned. The choice of arsenate, Pb or Zn concentration ranges in the 3-12- and 21-day cultures has been explained (I-III).

4.4 Sowing and sequencing of cultures (I-IV)

Long periods of imbibition lower the germination rate in *P. sativum* (Crawford 1977; Simon 1984). Seeds were soaked about 2 h in distilled water and sown without sterilization (I-IV). The number of plants per pot, parallel plants and replicate cultures in each type of experiment has been explained (I-IV). The 21-day cultures were sequenced due to the high number of pots per each replicate culture (Appendix 1). For calculations, three control sets were chosen, based on the timing that best coincided with the harvesting of plants grown with arsenate, Pb or Zn, and the same control values were used (I-III; Appendix 1).

4.5 Greenhouse conditions (I-IV)

The greenhouse conditions were uncontrolled. The gardener recorded daily maximum and minimum temperatures, which during the 21-day-cultures ranged between 27-33, and 17-21 °C, respectively (I-III). Natural light was supplemented with a set of ordinary fluorescent lamps, adjusted above the cultures to a 16/8 h light/dark period. Light intensity at the level of plants was recorded (Li-COR, Model Li-185A photometer) in the course of the two seasons, from mid-April to the end of August (about 20 recordings per period), during which the 21-day cultures were grown (I). The 3- to 12-day cultures ran throughout the year, whenever time allowed.

4.6 Assessment of responses to arsenate, lead and zinc (I-IV)

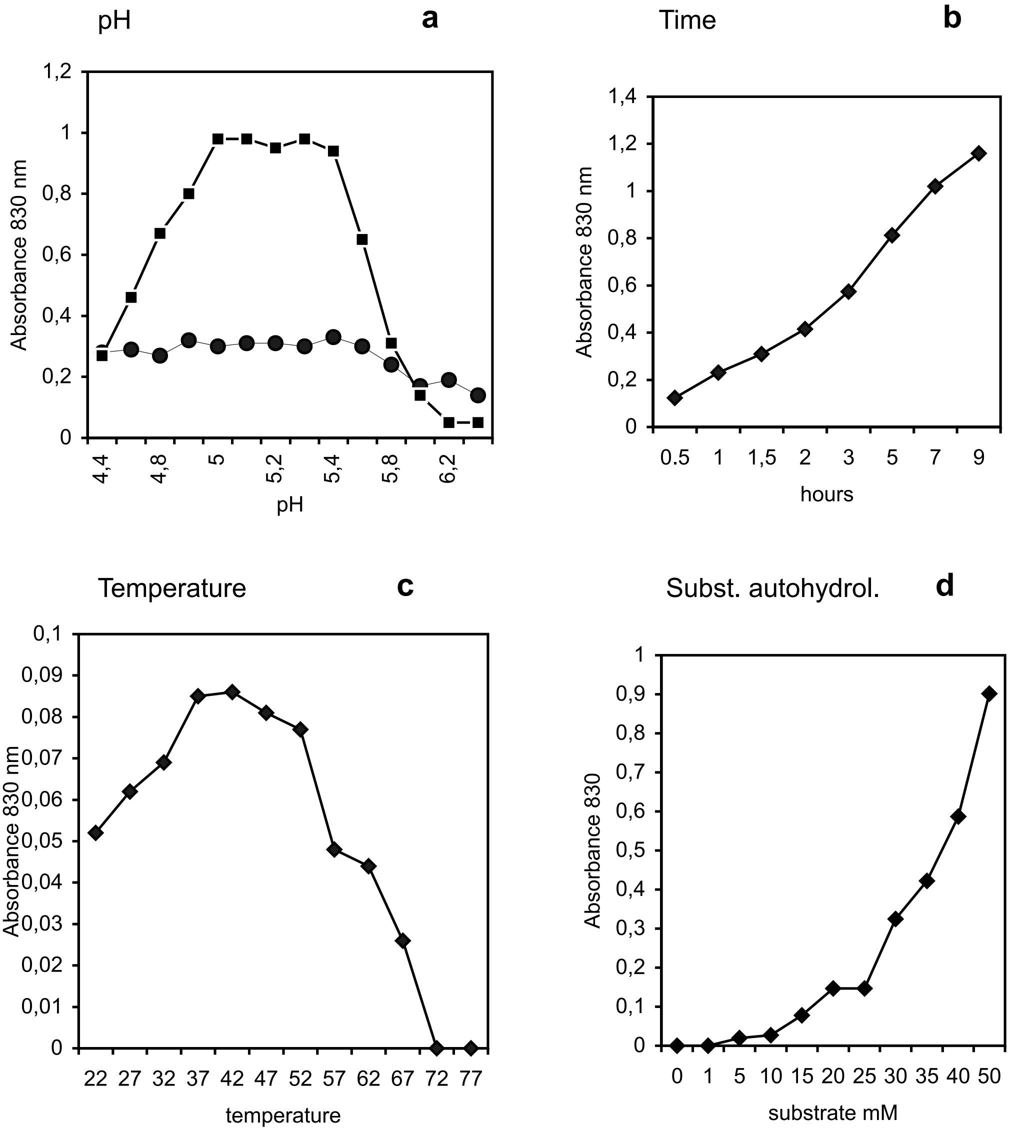
4.6.1 Growth and development (I-IV)

Growth was assessed in terms of length of the shoots and roots, and fresh weights and dwts of the cotyledons, roots and shoots (with flowers and buds) of the 3- to 12- and 21-day-old *P. sativum* as explained (I-IV). The fresh weights and dwts were determined in aluminium foilwraps, the latter after drying overnight at 103 ± 2 °C. The fairly high drying temperature was chosen in order to stop all metabolic processes for good storability of the dry samples. It was recognized, however, that some loss of dry matter might occur at higher than 80 °C temperatures (e.g. Campbell and Plank 1998). The root dwt-based TI for Pb and 10% shoot yield (dwt) reducing soil Pb concentrations were determined (II).

4.6.2 *In vivo* and *in vitro* phytase activity of cotyledons (I-III)

The full protocol of phytase assays has been explained (I). *In vivo* and *in vitro* total (I-III) and specific phytase activities (II; III) of the cotyledons were assayed at days 3, 6, 9 and 12. The choice of 30 cotyledon pairs per assay was believed to better describe the responses to the elements studied than activity per cotyledon pair. The partial purification of the cotyledon extract with fractional ammonium sulphate precipitation and dialysis has been described (I). Optimum assay conditions were determined first (I; Figs. 1a-e):

pH, reaction time and temperature: The crude cotyledon extract showed little response to pH, whereas the partially purified cotyledon extract had maxima at pH



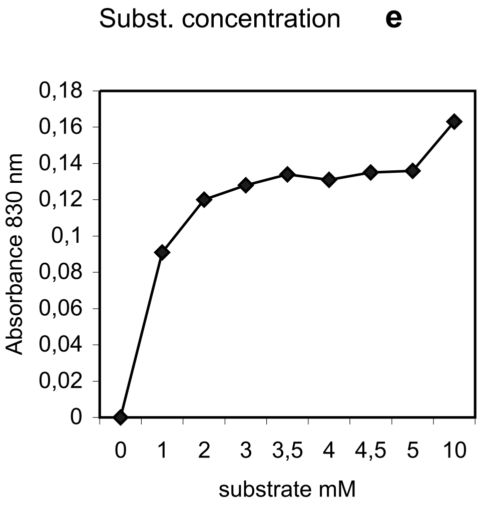


FIG. 1a-e. Characterization of assay conditions of phytase activity. In (a) dots, crude extract; squares, partially purified extract

Table 2. Assay conditions of plant phytases: pH and temperature.

Species and cultivar (cv.)	Plant organ	pH	Temperature	Reference
Pisum sativum cv. 'Alaska'	cotyledons	5.1	35-40 °C	Guardiola and Sutcliffe 1971
P. sativum cv. 'Alaska'	cotyledons	5.0	35 °C	Garcia-Luis and Guardiola 1974
P. sativum cv. 'Dwarf Gray' cv. 'Early Alaska'	cotyledos	5.2	37 °C	Chen and Pan 1977
P. sativum cv. 'Early Alaska'	dry seed powder	5.0, 8.0	37 °C	Scott 1991
P. sativum	pea flour	5.0, 5.5, 7.0, 8.0	37-55 °C	Fredrikson et al. 2001
Phaseolus aureus	germinating seeds	7.5	37 °C	Maiti et al. 1974; Mandal and Biswas 1970
P. vulgaris (many cultivars)	dry seed flour	5.0, 8.0	37 °C	Scott 1991
Vicia faba cv. 'Minor'	cotyledons	5.0	50 °C	Eskin and Johnson 1987
Glycine max	cotyledons	4.5-5.0	58 °C	Hegeman and Grabau 2001
G. max	cotyledons	4.5-4.8	55 °C	Gibson and Ullah 1990
Lactuca sativa cv. 'Grand Rapids'	seeds	5.0, 5.2, 7.0	37 °C	Mayer 1958
Brassica campestris cv. 'Toria'	seeds	5.2	50 °C	Mahajan and Dua 1997
Hordeum vulgare	seeds	5.0, 6.0	58 °C	Greiner et al. 2000
Triticum aestivum	seeds	5.2	37 °C	Courtois et al. 1952
T. aestivum	wholemeal flour	5.2	55 °C	Peers 1953
Secale cereale	flour	5.2	37 °C	Anagnostopoulos and Lino 1957
Oryza sativa cv. 'Rupsail'	seeds endosperm	4.0 9.0	37 °C	Mukherji et al. 1971
Lilium longiflorum Thunb.	pollen	5.0 8.0	45-50 °C 55-60°C	Scott and Loewus 1986
Aspergillus ficuum	secreted by fungus	5.0 (2.5)	55-58 °C	Gibson and Ullah 1990

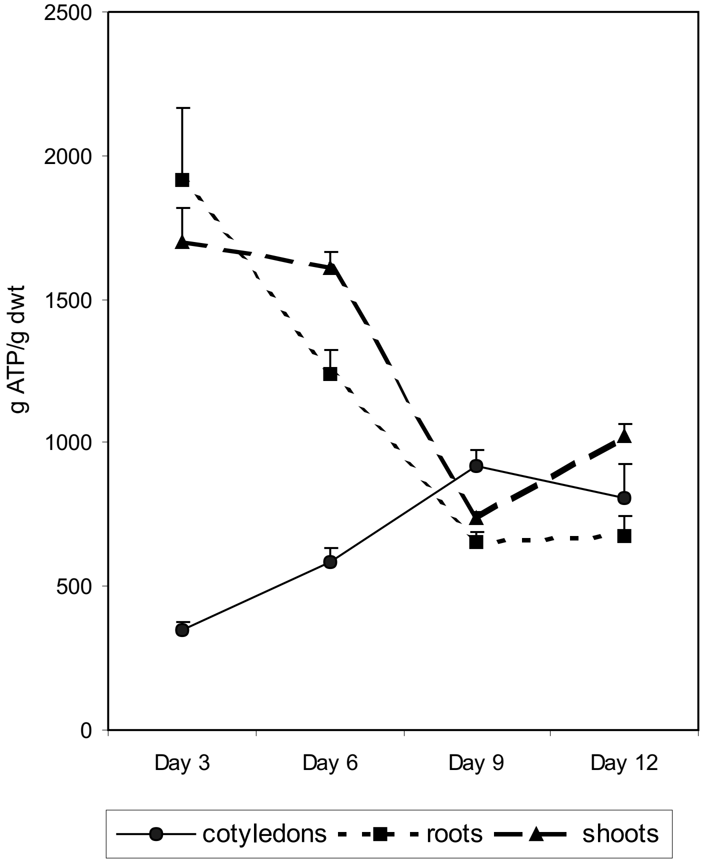


FIG. 2. Development of ATP concentration in *P. sativum* control seedlings during 12 days.

5.0 and 5.2-5.4 (I; Fig. 1a). Phytases with pH optimum around 5.0-5.2 have been shown to hydrolyse seed phytate in *P. sativum* and other species (Table 2). *P. sativum* cv. 'Early Alaska' also contains an alkaline phytase, but similar to *Medicago sativa*, the activity is slightly higher at pH 5.0 than at 8.0 (Scott 1991); a pH value of 5.0 was used (I-III). Phytase activity of seeds rises for a long period of time (e.g. Mayer 1958); a range from 30 min to 9 h was tested and 2 h chosen (I; Fig. 1b). Both the acid (Chen and Pan 1977) and alkaline (Scott 1991) phytases of *P. sativum* have temperature optima around 36-37 °C (Table 2), coinciding with the 36 °C of cv. 'Phenomen' used here (I; Fig. 1c).

Substrate autohydrolysis, concentration and specificity: Phytic acid, *myo*-inositol 1,2,3,4,5,6-[hexa*kis*dihydrogen phosphate], undergoes significant autohydrolysis at higher concentrations (≤ 5 mM), but at the 2 mM substrate concentration used it was insignificant (I; Figs. 1d-e). The phytase extract also hydrolysed other substrates (I).

4.6.3 ATP (IV)

Since the ATP Monitoring reagent was expensive, the number of ATP analyses had to be limited. The ATP concentration of control plants was first followed from days 3 to 12, and day 9 was chosen for the wider analyses. At that point in time, the ATP concentrations of cotyledons, roots and shoots were of similar magnitude (Fig. 2). ATP was determined with the luciferin-luciferase method, based on oxidative decarboxylation of D-luciferin, coupled with the utilization of ATP and emission of detectable light (Brolin and Wettermark 1992). The reaction is similar to that in natural luminescence (Nicolas 1983). An LKB Wallac 1250 luminometer was used, in which a pure ATP solution, 10^{-5} M in Tris-Titriplex buffer (pH 7.75), was used in the standard adding technique, a preferred standard procedure (Simpson and Hammond 1991).

4.6.4 Chlorophyll a and b (I-IV)

Samples (explained in I; IV) were homogenized with mortar and pestle in an icebath in 80% acetone (0.01% Mg carbonate). Double centrifugation (4800 rpm 10 min) at +4 °C with resuspension was done. Chlorophyll a and b were determined as explained (I-IV).

4.6.5 Accumulation and partitioning of elements (I-III)

Accumulation and partitioning of As, Pb and Zn was assessed as total element contents and as fraction (%) per plant organ (I-III). Either the average dwts of plant organs were used in the calculation of the total element contents (I; II) or they were calculated on an individual plant dwt-basis (III). Soil-plant transfer was also assessed (I; II).

4.6.6 Element analysis (I-III)

Digestion method: Cold digestion with perchloric acid in sealed polypropylene bottles was the preferred method, but due to explosion risk it was not allowed. Therefore, contacts were made with a number of laboratories that performed vegetable analyses. The predigestion method developed utilized a sequence of cold and hot digestion in concentrated HNO_3 and gradually increasing the temperature up to 180 °C (II). Since the digestion acid was evaporated over a water bath and the residue collected in 0.1 M HCl diluted to 25 ml (II), the problems of SO_4^{2-} ions interfering in the atomic absorption determinations were avoided, and the HCl concentration was also kept low (0.025 M). The standard predigestion method currently used for element analysis is very similar (e.g. Campbell and Plank 1998).

Element analysis: Atomic absorption with a graphite furnace facility was considered the best method for Pb analysis. The Department of Forensic Medicine of the University of Helsinki kindly gave access to such a device. Yet, after all the analytical parameters were established and a considerable number of test runs done, budgetary constraints interrupted this cooperation as well as some other efforts. An option to have the analyses performed by an outside laboratory became a reasonable solution. In the present study As was analysed in the VTT, Reactor Laboratory with the neutron activation method (I). It was regarded as an ideal method for the determination of total As (Rosenberg and Lakomaa 1983). Arsenate interfered with the Fiske-Subbarow (1925) spectrophotometric method of total P analysis, and therefore plasma emission was chosen for analysing P in plants grown with arsenate (I). This choice was based on a small cohort of samples analysed by the VTT Chemistry Laboratory with this method. Viljavuuspalvelu Oy performed the analysis of total Pb, Zn, Ca, Mg, K, Na, P, S, Mn, Cu and Fe on either predigested or on dry plant material with the methods described (I-III). Of all the element analyses, the total N (I-III) analysed with the micro-Kjeldahl method and the share of P analyses (II; III) done with the Fiske-Subbarow (1925) method, as well as all predigestion of the plant material, were performed by the author.

4.7 Analytical accuracy (I-IV)

Contamination of samples: Due to the soil culturures used, contamination of plant samples was a possibility (I-IV). The Ti and Al contents of plant samples have been suggested as indicators of such contamination (Cherney and Robinson 1983). In a small cohort of samples analysed with plasma emission (VTT, Chemistry Laboratory), the Ti content was extremely low, from 0 (5/11 samples) to 0.002 mg kg^{-1} plant dwt (1/11 samples), as was also the Al content an average of 0.05 mg kg^{-1} plant dwt of control samples (n = 6). These checks may speak for a low contamination level (I-III). On the other hand, roots were rinsed fairly intensively to remove soil residues (I). Rinsing may cause some leaching of K and nitrate (Sonneveld and van Dijk 1982). Cleaning of glassware and utensils and the purity of reagents have been explained (I-IV). Predigested samples and double-distilled water were stored in polypropylene containers.

Crosschecks and accuracy: For predigestion samples were ground with a mortar and pestle, which probably introduced some uneven homogeneity and contributed to the standard deviations (I-III). Yet, it is recognized that individual plants differ considerably in their element contents (Lewis et al. 1992; Jones 1998b) and that seasonal variations occur (Sillanpää 1982). Vesi-Hydro Oy crosschecked a small cohort of predigested samples, but differences with the results of Viljavuuspalvelu Oy were small. The limited set of samples analysed in the VTT Chemistry Laboratory served to crosscheck the plasmaemission analyses performed by Viljavuuspalvelu Oy. The differences between these samples were within the range of differences between plant individuals (I-III; Sillanpää 1982; Jones 1998b). Reagent blanks were run in each predigestion lot. To reduce the cost of analysis, six reagent blanks were randomly chosen for analysis.

The Commission of the European Communities (BCR) standard reference material (*Olea europaeus* leaves) was available only towards the end of the analytical work (BCR 1982). A separate lot was predigested, but the instrumental analyses were performed with the rest of the samples. The average recovery for Zn was 115 ± 15% (n = 6) and for Pb 120 ± 7% (n = 6). β-Alanine was included in each lot of N analyses, with a recovery of 97 ± 1% (n = 25 x 18). The recovery in a cohort (n = 40) of ATP samples was 93 ± 2%. Since a number of elements were analysed with more than one method, these analyses could be compared; the differences were within the range observed between plant individuals (I-III; Sillanpää 1982; Jones 1998b).

4.8 Statistical analysis (I-IV)

Sample types: The types of sample have been explained in the original papers (I-IV). Root, shoot and cotyledon samples were analysed so that ultimately the results represented entire plant individuals, and were all independent samples. The sampling method enabled tracking back to records on individual plants. Samples were pooled only for neutron activation analysis (I). The assay protocols for phytase activity resulted in 6-8 dependent samples (I); in calculations, n = 6 was used for both types of activity.

Significance of differences and relationships: The significance of differences between the means of parameters were tested either with t-test (I) or by Tukey´s test (III; IV) or both (II). Analysis of variance (ANOVA) was also used (III). To assess linear relationships, the correlation coefficient (*r*) was calculated (Campbell 1967) manually (II) and by using Microsoft Office Excel, Premium and Professional 2000, Millennium Edition computer program (III; IV). For ANOVA, the data was not log-transformed (Köhl and Lösch 1999); a normal distribution and homogeneity of variance were assumed (Campbell 1967; Ranta et al. 1991). All comparisons were run on equal numbers of samples. Subjecting root and shoot dwts of replicate cultures to ANOVA showed some significant variance between the 10 control replicates of 21-day duration, but not among those three replicates used as the controls (section 4.4; Appendix 1). Variation also occurred particularly among shoot dwts of replicate cultures grown with Pb and to a lesser extent with arsenate or Zn.

5. RESULTS AND DISCUSSION

5.1 Germination and remobilization from cotyledons (I-IV)

The high concentrations of arsenate, Pb and Zn (70 µmol As, 4 mmol Pb or 9.3 mmol Zn kg^{-1} dry soil) reduced germination up to 30-40% (IV). In addition to ATPases (IV), the initial reaction in breaking of dormancy in *Glycine max* has been suggested to involve inositol 1,3,4,5,6-penta*kis*phosphate 2-kinase, which transfers the 2-position phosphate from phytate to adenosine diphosphate (ADP; Phillippy et al. 1994). This reaction is separate from that of phytase hydrolysis. In other species, hormonally controlled protein-phosphorylating kinases (Walker-Simmons 1998) and NO_3^- (McIntyre 2001) have been suggested to be involved.

Germination requires cooperation between different seed constituents (Welbaum et al. 1998). In *P. sativum*, anaerobic respiration initially provides nearly half of the ATP (Goksöyr et al. 1953; Al-Ani et al. 1985), but alcohol dehydrogenase activity declines in 2-3 days (Goksöyr et al. 1953). Apart from the possibility that As, Pb and Zn interacted with the enzymatic reactions involved in the breaking of dormancy and early glycolytic respiration (IV), disturbance in the reconstitution of mitochondria (Bain and Mercer 1966; Nawa and Asahi 1973) could have prolonged the glycolytic phase with a subsequent accumulation of ethanol, to which *P. sativum* is particularly sensitive (Crawford 1977). Germination ends by the emergence of the embryonic axis (Welbaum et al. 1998).

5.1.1 Dry matter (I-IV)

Zn retarded the loss of cotyledon dry matter (Tables 1/III; 1/IV), and at day 12, cotyledon water percentage was 13-20% below that of the controls (Table 1/III). The lowered water content of the cotyledons throughout the 12-day growth of seedlings could have affected many cellular processes (Welbaum et al. 1998), e.g. reduced *de novo* enzyme synthesis of hydrolytic enzymes in the cotyledons (Nawa and Asahi 1973; Simon 1984). Starch is one of the major reserve materials in *P. sativum*, and starch phosphorylase and β-amylase are constitutive, but α-amylase, which performs the bulk of starch hydrolysis, is synthesized *de novo* (Morohashi and Ueno 1980; Beers and Duke 1990). Abscisic acid controls starch hydrolysis (Yomo and Varner 1973). In *Vigna radiata*, *Arachis hypogea* and *Triticum aestivum,* seed phytate content and starch hydrolysis relate inversely (Sharma et al. 1978). Therefore, retarded phytate hydrolysis (Figs. 1a/I; 1a-b/II; 1a-b/III) may also have indirectly affected carbohydrate export. On the other hand, the lower rate of utilization of reserve materials in plants grown with Zn (Tables 1/III; 1/IV) can also be a reflection of reduced growth of seedlings (Tables 1/III; 1/IV), enhanced utilization of nutrients from the soil and enhanced photosynthetic capacity. It is likely that the impacts of Zn on the growth of seedlings, release of individual elements and hydrolytic processes in the cotyledons all contributed to the observed results (Tables 1/III; 1/IV).

5.1.2 I*n vivo* and *in vitro* phytase activity (I-III)

Phytic acid occurs mostly as K-, Mg- and Zn-phytate in the protein bodies of seeds (review: Raboy 1997). K is the principal element in *P. sativum* (Crean and Haisman 1963). Higher plants usually have 6-phytases and microorganisms 3-phytases (Loewus et al. 1990). Phytase activity progressively releases intermediary *myo*-inositol phosphates and inorganic phosphate, the latter of which inhibits the activity (Bianchetti and Sartirana 1967; Loewus et al. 1990). Inhibition occurs at the mRNA synthesis level in *Triticum durum* (Bianchetti and Sartirana 1967). The gene *phyA* has recently been reported and the structure of enzyme proteins identified in a few species (Hegeman and Grabau 2001; review: Brinch-Pedersen et al. 2002). The functions of the alkaline phytase are unclear (Scott 1991; Brinch-Pedersen et al. 2002), but in *Lilium longiflorum* pollen, it may be involved in Ca-linked signal transduction rather than phytate hydrolysis (Loewus et al. 1990). Ca activates phytases in *L. longiflorum* (Scott and Loewus 1986) and in the seeds of *Phaseolus vulgaris* (Scott 1991) and *Vigna radiata* (Dasgupta et al. 1996).

Arsenate reduced (after day 6) *in vivo* total phytase activity of the cotyledons of *P. sativum* cv. 'Phenomen' (Fig. 1a/I). The type of inhibition is competitive in *Brassica campestris* (Mahajan and Dua 1997). In this work, 4 mmol Pb kg^{-1} soil concentration enhanced the specific *in vivo* activity from day 6 to 9 (Fig. 1b). Precipitation of Pb phosphate may have removed the feedback regulator (phosphate), as found in *L. longiflorum* pollen (Loewus et al. 1990). Other metals (Mg, Mn, Co, Fe) also activate phytase (Reddy et al. 1989).

Zn inhibits phytases in many species (Table 1 in Loewus et al. 1990; Mahajan and Dua 1997). Here the *in vivo* total and specific activities declined from day 9 on (Figs. 1a-b/III), but the protein content of phytase extract remained high (III). Limited substrate availability may have contributed (III), caused by the stability of the Zn-phytate-protein complex (Brown et al. 1961; Cheryan 1980). The correlation between shoot Zn and N concentrations and the *in vivo* phytase activity (III) may point to some shoot-borne signal mechanism, which has been suggested to control the hydrolysis of reserve materials in other species (Loewus and Loewus 1983). *In vitro* phytase graphs (Figs. 1b/I; 1c/II; 1c-d/III) resembled those reported on cv. 'Bonneville' of *P. sativum* exposed to Cr (Dua and Sawhney 1991). Age-related conformational changes do occur, which alter access to the active site of phytase *in vitro* (Dasgupta et al. 1996; Ha et al. 2000). Probably more than one mechanism contributed in the present investigation.

5.1.3 Remobilization of mineral elements (I-III)

The linear relationship between export of major elements and cotyledon dry matter in *P. sativum* (Guardiola and Sutcliffe 1972) was broken by arsenate, Pb and Zn (Tables 4b,5/I; 3,4/II; 1,2/III). A characteristic common to all three elements was that remobilization of Fe was unaffected and that of Mn enhanced (Table 3). In *P. sativum* seeds, Fe occurs in the seed coat, and in the embryo it is deposited as ferritin (Marentes and Grusak 1998). Fe and Ca are usually not associated with phytate, although phosphate in ferritin may be associated with phytic acid (Raboy 1997).

Arsenate and Pb reduced the export of Zn (Table 3). The embryo is rich in Zn which is easily mobilized from the seeds during germination (Longnecker and Robson 1993). Except Mn, Pb tended to reduce and Zn, with the exception of K, to enhance the export of mineral elements from the cotyledons (Table 3). In noting the unaffected concentration of cotyledon P (Table 1/III), the rate of phytate hydrolysis may not have caused the recorded reduced K export under Zn exposure (Table 2/III). It has rather been linked to protein synthesis in the axis (III), which is susceptible to interference by Zn (Vallee and Auld 1990; Berg and Shi 1996).

Development of cotyledon reserves

Table 3. Cotyledon mineral element concentrations of *P. sativum*, exposed to arsenate or Pb for 9 days, or to Zn for 12 days, in the greenhouse.[a]

	Day 9[b]			Day 12[c]	
Element	Control	As 70 µmol kg^{-1}	Pb 4 mmol kg^{-1}	Control	Zn 9.3 mmol kg^{-1}
Ca mg g^{-1}	5.3±0.3	4.3±0.9	6.0±1.2	12.0 ± 1.4^{a}	1.7 ± 0.03^{b}
Mg mg g^{-1}	3.3±0.5	2.4±0.4	5.8±0.1*	7.8 ± 1.2^{a}	2.2 ± 0.2^{b}
K mg g^{-1}	6.0±0.5	6.8±0.8	9.4±2.1	5.7 ± 0.8^{a}	11.4 ± 0.7^{b}
Na mg g^{-1}	0.7±0.06	1.1±0.2	4.1±1.0**	1.9 ± 0.4^{a}	1.0 ± 0.2^{a}
Cu µg g^{-1}	18.2±3.0	14.6±1.5	19.0±1.6	23.8 ± 2.9^{a}	13.2 ± 0.6^{b}
Mn µg g^{-1}	41.8±2.5	27.2±4.2*	21.4±2.6***	70.0 ± 10.0^{a}	18.0 ± 2.7^{b}
Zn µg g^{-1}	38.8±2.0	45.2±2.9*	54.2±4.8*	64.0 ± 17.4^{a}	2600 ± 235^{b}
Fe µg g^{-1}	73.8±8.5	66.6±16.6	107.6±26.3	59.0 ± 13.3^{a}	65.2 ± 5.1^{a}

[a] At day 9, control values and values regarding arsenate (As) are derived from Table 5/I and those of Pb from table 4/II. At day 12, the control values and those of Zn are derived from Table 1/III and values of other elements from Table 2/III. At day 9 cotyledons were analysed without the seed coat and at day 12 with the seed coat.

[b] Significance levels by t-test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

[c] In Tukey's test, figures marked with the same superscript do not differ at the $P < 0.05$ level.

and their export in *P. sativum* may follow similar routes. During seed development, mineral elements and organic compounds are symplastically transported between a few phloem strands of the seed coat and cotyledon parenchyma (Tegeder et al. 1999; Wolswinkel 1999), although better understanding of this transport phenomenon is still evolving (Patrick and Offler 2001). Nicotianamine has been suggested to facilitate phloem transport of Fe and some other metals (Stephan and Scholz 1993; von Wirén et al. 1999) and phloem-xylem exchange (Stephan and Scholz 1993), although its full role is under discussion (Reichman and Parker 2002). Phloem sap also contains other suitable ligands, such as organic acids, amino acids and peptides (Stephan and Scholz 1993). For example, Mn may be translocated as organic compound or free ion (Van Goor and Wiersma 1976). The element-specific response to arsenate (Tables 4b,5/I), Pb (Tables 3,4/II) and Zn (Tables 1,2/III) suggests that some element-specific mechanisms, such as phloem loading and translocation, were affected.

Nutrient demand–availability interactions are currently thought to regulate nutrient flow to developing seeds (Patrick and Offler 2001) and probably also remobilization (Loewus and Loewus 1983). The enhanced export of Mn (Table 3) and its correlation with shoot growth (III) support this view. Moreover, in cv. 'Alaska' of *P. sativum,* deshooting reduced N export, while the presence of cellular or external Ca enhanced it (III; Guardiola and Sutcliffe 1972).

5.2 ATP concentration of seedlings (IV)

ATP concentration of cotyledons and shoots of 9-day-old *P. sativum* were high at low external concentrations of arsenate, Pb and Zn (40 µmol As, 2 mmol Pb and 5.3 mmol Zn kg^{-1} soil), but were unaffected at higher (70 µmol As, 4 mmol Pb and 9.3 mmol Zn kg^{-1} soil) concentrations (Table 2/IV). Since the total ATP content of plant or tissue expresses the balance between ATP synthesis and use, these results indicate the presence of imbalance and altered adenylate energy charge, which has wide ramifications (Noctor and Foyer 2000). The negative correlations between growth and ATP concentration may imply that the three elements impacted indirectly, via the ATP, on the growth of seedlings (IV).

Enhancement of ATP production at low concentrations of metallic or metalloid elements (Table 2/IV) is known and thought to express a higher demand for it in response to increased transport activity and oxidative stress (review: Lösch and Köhl 1999). For example, in the leaves of *Glycine max* (Lee et al. 1976) and in isolated mitochondria of the shoots of *Zea mays* (Bittell et al. 1974), low concentrations of Pb (Bittel et al. 1974; Lee et al. 1976) and Zn enhanced respiration, but higher concentrations inhibited it. In *Z. mays*, Pb (0.1 mM) caused swelling of mitochondria (Bittell et al. 1974). Cytoplasmic arsenate may compensate for P in ATP (Meharg 1994) and in some phosphorylation reactions (Edmonds and Francesconi 1981), although easy reduction of arsenate to arsenite jeopardizes sulphydryl-rich proteins (Nissen and Benson 1982; Ullrich-Eberius et al. 1989).

In *P. sativum* cv. 'Bonneville', mitochondrial and extramitochondrial isocitrate dehydrogenase and malate hydrogenase, and mitochondrial α-ketoglutarate dehydrogenase and succinate dehydrogenase, are all inhibited at 0.5 mM Pb and 0.25 mM Cd (Bansal et al. 2002); Zn also inhibits isocitrate dehydrogenase in *P. sativum* (Omran and Dennis 1971). In isolated mitochondria of *Z. mays*, succinate oxidation declines by 50% at 0.1 mM or higher Pb and Zn concentrations (Bittel et al. 1974). ATP recycling may be impaired by interference in the membrane-bound H$^+$-ATPases (Lösch and Köhl 1999; Li et al. 2001), which is also reflected in the ATP synthase activity (Fillingame 1999; Tate 2001).

The surplus in ATP/chlorophyll concentration ratio (Table 2/IV) may point to the known differential susceptibilities of carbon dioxide fixation and photosynthetic light reactions. In *P. sativum* cv. 'Ilowiechi', the former is more sensitive to Pb (1-5 mM) than the latter (Parys et al. 1998), whereas in cv. 'Meteor', photosystem II is inhibited at 50 µM of Zn, but photosystem I is unaffected at 400 µM concentration of Zn (Baker et al. 1982). Imbalanced photosynthesis may generate surplus of cellular NADPH as well, although oxidative stress consumes reducing power (Smirnoff and Wheeler 2000). The fundamental regulator between ATP-producing and -utilizing pathways is inorganic phosphate (Rao 1996; Noctor and Foyer 2000). Impacts of arsenate, Pb or Zn on the acquisition or cellular availability of phosphate (Fig. 3a/II; Tables 8/I; 3/II; 1,3/III) are also likely to be reflected in ATP status (Table 2/IV).

5.3 Growth and accumulation of arsenic, lead and zinc (I-III)

5.3.1 Arsenate (I)

Seedlings (Tables 2,3/I) and adult *P. sativum* cv. 'Phenomen' (Figs. 2a-b/I) were sensitive to arsenate. This is tantamount to an earlier survey of field crops, including pea, alfalfa, different types of bean and cucumber, which showed little or no tolerance to As, whereas many cereals and root vegetables tolerated it better (Tables 5-7 and 5-8 in National Research Council of Canada, NRCC 1978). In potted cultures of *Gossypium hirsutum* and *Glycine max* the yield limiting threshold values are 4.4 µg As and 1 µg As g^{-1} plant dwt (Deuel and Swoboda 1972). In former orchard soils contaminated by Pb arsenate and containing 4.9-64 mg As kg^{-1} soil, *Trifolium repens* and *Lolium perenne* accumulated a mean of 2-2.5 µg As g^{-1} plant dwt (Merry et al. 1983). At the 70 µmol (5.3 mg) As kg^{-1} soil concentration in the present investigation, the shoot had 15 ± 0.5 µg As g^{-1} dwt (Table 7/I). As discussed, this fairly high value may reflect an easier availability of As from freshly contaminated soil and the greenhouse environment (I). Nevertheless, soil micro-flora readily transforms arsenate into other species, some of which may be more toxic than arsenate (Meharg and Hartley-Whitaker 2002). The soil phosphate concentration was also fairly high (Table 1/I; Urvas 1995), and elevated phosphate availability has been reported to either reduce (Hurd-Karrer 1939; Meharg and Macnair 1992a) or raise plant As content (Small and McCants 1962).

Relatively more As accumulated from lower than from higher soil concentrations (Tables 4a,7/I), although As also adheres tightly to the root surface (Carbonell-Barrachina et al. 2000), which may be reflected in the As concentration of the roots. Under P-deficient conditions, methylated forms have been identified in the roots, stems, and leaves of *P. sativum* cv. 'Sugarsnap' (Nissen and Benson 1982). In *Brassica juncea,* arsenate and arsenite have been reported to translocate as uncomplexed oxyions (Pickering et al. 2000). Accumulation of As in roots (Tables 4a,7/I) is common in many species (e.g. Anastasia and Kender 1973; Weaver et al. 1984). Phytochelatins and homo-phytochelatins (section 3.7.1) are rapidly induced by arsenate and have been thought to contribute to the sequestration (Grill et al. 1986; Schmöger et al. 2000; Schat et al. 2002).

5.3.2 Lead (II)

Low (2 mmol Pb kg^{-1}) soil Pb level was more harmful to the growth of *P. sativum* seedlings than higher (4 mmol Pb kg^{-1}) soil concentration (Table 2/II). In the 21-days exposure, growth of the shoots and roots was reduced at and above 6.5 mmol Pb kg^{-1} soil (Table 5/II), but the EC_{10} for shoots was 1.4 mmol Pb kg^{-1}. Here, the dry matter of roots was a more sensitive parameter for external Pb (Fig. 2/II) than the length, i.e. the elongation parameter (Table 5/II), and the dwt-based root TI showed an inverse dependence on soil Pb concentration. A TI of 90% occurred at 3.5 mmol Pb kg^{-1} soil, confirming the higher sensitivity of the shoots than of the roots (Fig. 2/II).

Within the range of soil Pb concentrations, which are considered acceptable in agricultural soils (II; ≤ 500 mg Pb kg^{-1} dry soil) and which occur *in situ* in cultivated or garden soils in Europe (Davies 1992; Kabata-Pendias et al. 1992), the aerial portions of *P. sativum* cv. 'Phenomen' accumulated considerable amounts of Pb. At 1.1 mmol (225 mg) Pb kg^{-1} soil and below the shoot EC_{10} soil Pb level (Fig. 2/II), the shoots had 23 ± 4 µg Pb g^{-1} dwt, which is higher than the phytotoxicity thresholds given for *Trifolium pratense* (15 µg Pb g^{-1} dwt) and *Zea mays* (12 µg Pb g^{-1} dwt), but lower than that of *Secale cereale* (60 µg Pb g^{-1} dwt; Bergmann 1992). The fairly high nutrient level of the soil mixture (Table 1/I) probably reduced Pb availability. In field exposure, an NPK fertilizer reduced Pb concentration of several crops (Trüby and Raba 1991), similar to the situation found in corrective fertilization of polluted forest soils (section 3.2.3; Derome and Saarsalmi 1999). The potential impact of Pb acetate, artificial contamination of soils and greenhouse conditions have been discussed (II).

A medium soil Pb concentration (6.5 mmol kg^{-1} soil) yielded the highest fraction (14%) in the shoot (II), although the shoot top contained much less Pb than the average occurring in the shoot (II). Pb translocates in the xylem in chelated form (Tanton and Crowdy 1971), and in *Picea rubens* Pb has been shown even to remobilize from roots and move acropetally (Donnelly et al. 1990). Roots accumulated high amounts of Pb (II), which is common in many species (Ernst 1974; Cseh 2002;

Krupa et al. 2002), and in view of the lower sensitivity of the root dry matter than that of the shoots, effective inactivation mechanisms must have been in place (Fig. 2/II). Interactions between Pb and cell walls and apoplastic space, as well as some other possible inactivation mechanisms were discussed in section 3.7. Apart from these mechanisms, lead also tightly adsorbs onto the root surface (Hagemeyer and Breckle 1996; Tung and Temple 1996), which may influence the uptake of other elements.

5.3.3 Zinc (III)

Growth of the shoots of adult *P. sativum* was sensitive to an enhanced concentration of Zn, and the dwts were reduced at a moderate (3.2 mmol kg^{-1} soil) Zn concentration (Table 3/III). The fact that there was little dependence of dwts on soil Zn level may imply activation of less Zn-sensitive metabolic mechanisms. In control plants, growth correlated with Na, Cu, Mn and Fe, whereas in Zn-exposed plants, K and Ca displayed a high number of correlations (III; Appendix 2). The Ca-Zn relationship is antagonistic; e.g. in *Triticum turgidum* Ca inhibits Zn uptake (Hart et al. 1998), whereas K usually correlates with many macronutrients (Markert 1997).

The shoot Zn concentrations of adult plants of *P. sativum* cv. 'Phenomen', grown at 3.2 and 8.8 mmol Zn kg^{-1} soil (Table 3/III), were of similar magnitude as those reported in *P. sativum* grown in soil with neutral pH, but at a coinciding soil Zn range (Table 1 in Chaney 1993). In cv. 'Phenomen', shoot internal Zn concentrations stagnated at first and then showed a 5-fold increase when the soil Zn concentration rose from 8.8 to 18.0 mmol Zn kg^{-1} (Table 3/III). This 'plateau' phenomenon has been attributed to some *in-planta* factors and the abrupt increase to some damage or overloading of cellular barriers (e.g. discussion in Hamon et al. 1999). As the fraction of shoot Zn declined in an inversely linear manner with soil Zn level, while at the same time the total shoot Zn content was directly correlated with soil Zn, the results support the argument that some plant internal factor(s) other than overloading of the translocating systems may be involved (Fig. 2b/III). In *P. sativum* cv. 'Citrine', succinate causes a partitioning pattern of Zn (Doncheva et al. 2001) similar to the one observed here in cv. 'Phenomen' (Fig. 2b/III).

Enhanced synthesis of oxalate and involvement of Mn were discussed, and Mn was suggested to contribute to the partitioning pattern of Zn (III). This view is further supported by the fact that the Zn-Mn parallel change occurred in both Zn- and Pb-exposed plants (Table 4). In a potted greenhouse study of *Trifolium subterraneum*, Mn did not affect Zn uptake, but it reduced that of Pb (McKenzie 1978). The Mn-Zn relationship is, however, further complicated by the results on seedlings of *P. sativum* cv. 'Phenomen', since Zn accumulated in the cotyledons, while at the same time the export of Mn was enhanced from the cotyledons (Fig. 2a/III; Table 3). On the other hand, this result supports the view that under Zn exposure, the need for Mn in the growing axis appeared to have been enhanced. Under arsenate and Pb exposure, export of Zn was retarded from the cotyledons, whereas that of Mn was enhanced (Table 3).

5.4 Chlorophyll concentrations (I-IV)

Chlorophyll a and b concentrations were increased, but their ratio decreased at moderate soil concentrations of arsenate after 21 days of exposure (Table 6/I), whereas after 9 days of exposure, arsenate significantly affected neither the chlorophyll concentrations nor their ratio (Table 2/IV). The situation was reversed with regard to 9 and 21 days of exposure to enhanced soil concentrations of Zn: in the former case, the chlorophyll a and b concentrations were reduced (Table 2/IV), whereas in the latter case, the chlorophyll concentrations or their ratios were not significantly affected (III).

A change in the chlorophyll a/b ratio observed in the 21-day-old plants grown with arsenate (Table 6/I) may indicate alterations in photosystem II (Young et al. 1996; Mysliwa-Kurdziel et al. 2002), inhibition of chlorophyll a to b conversion or rapid chlorophyll a degradation (Mysliwa-Kurdziel et al. 2002). In the 9-day-old seedlings exposed to elevated soil concentrations of Zn, the ATP concentration was high in the shoots (Table 2/IV), which may have been a contributing factor to the low chlorophyll concentration, since enhanced ATP availability may have activated the ATP-powered proteins involved in chlorophyll catabolism (Matile et al. 1999). Since the chlorophyll a/b ratio did not change in the Zn-exposed seedlings (Table 2/IV), it may also indicate that chlorophyll synthesis was affected at an early stage. Senescence of the lower internodes of adult, 21-day-old *P. sativum* cv. 'Phenomen' was enhanced (I-III), which may be geared to the observed remobilization of elements from the lower internodes to shoot tops (II; III). On the other hand, senescence has also been suggested to indicate the degradation of membrane structures of chloroplasts, i.e. toxicity (discussed in Krupa and Baszyński 1995).

Remobilization of Mg to the shoot tops probably contributed to the results that Pb (II) and Zn (III) hardly affected the chlorophyll concentrations in the 21-day-old plants. Since the shoot Zn concentrations of seedlings (Table 1/III) were at or above toxicity levels (III) and nearly double the levels found in the adult plants (Table 3/III), the high shoot Zn concentration is likely to have contributed to the reduction of chlorophyll concentrations in the seedlings (Table 2/IV). Loss of chlorophyll may serve as one of the indicators of inadequate detoxification of metals (Ernst et al. 2000). Thus, under longer exposure time (21 days), cellular sequestration processes of Zn probably developed, with a subsequent reduction in the shoot Zn concentration (III) from the level of that recorded in the seedlings (Table 1/III), which allowed the recovery of chlorophyll concentrations in the 21-day-old plants (III). The result that seedlings recover from Zn toxicity symptoms, so that these symptoms are absent in adult plants, has been reported (e.g. Hoagland et al. 1936).

5.5 Concentrations and relationships of mineral elements (I-III)

The element concentrations of control plants were within sufficient/normal values (Bergmann 1992; Marschner 1995), except that of P, which was higher but which probably can be attributed to the favourable soluble P concentration of the potting mixture (Table 1/I). The mean N and Mg levels of the potting mixture were also higher, but the Fe and Mn concentrations were lower (Table 1/I) than the average found in Finnish agricultural soils. The conductivity value (Table 1/I), reflecting the water-soluble elements, was higher than the average found in organic soils in Finland (Sillanpää 1982; Urvas 1995). When comparing the Zn concentrations of control plants (I-III), it should be noted that those included in I (Table 8/I) and II (Table 6/II) refer to analyses using the plasma emission method with samples derived from two replicate cultures (n = 5). Those included in III were analysed with the flame atomic absorption method (Table 3/III) with samples also derived from two replicate cultures (n = 6), but the replicate cultures were only partially the same, and all plant individuals were differet in the two sets of analyses. Another contributing factor was that different aliquots of soil mixture were also used (explained in section 4.2), which introduced another factor of variability. There was considerable variation between plant individuals within each culture and between the two cultures. As explained, the differences were, however, within the range observed between cultures and individual plants within each culture (section 4.7; Sillanpää 1982; Jones 1998b).

The mineral element status of *P. sativum* cv. ‘Phenomen’ at the seedling (Tables 4b,5/I; 3,4/II; 1,2/III) and flowering (Figs. 3a-b/II; Tables 8/I; 6/II; 3,4/III) stages of development was altered by arsenate, Pb and Zn. Reductions occurred in N, P, K and Fe concentrations, whereas those of Mg and Na rose. Root Mn concentrations also tended to rise, but the changes were statistically insignificant (Tables 8/I; 6/II; 4/III).

In the following, element uptake mechanisms and interactions between elements and growth parameters are discussed, but due to the wide scope of the issues involved, the following discussion is restricted to the interactions occurring mainly at the uptake and *in-planta* levels. It is, however, recognized that soil and greenhouse conditions, while affecting the availability of arsenate, Pb or Zn, also affect other elements, and that interactions in the rhizosphere are extremely important (Sillanpää 1982). Moreover, interactions between elements while ‘in transit’ are largely still undetermined.

5.5.1 Nitrogen (I-III)

The high soluble N concentration of the soil mixture (Table 1/I) explains the observed rarity of the root nodules (I-III). Since the pH of the soil was acid (Table 1/I), the plant N content mainly represents nitrate acquisition. In adult *P. sativum* cv. ‘Phenomen’ the N concentrations dropped at low soil Pb or Zn levels (0.5 and 1.4 mmol kg^{-1}, respectively), and thereafter, hardly responded to rising soil concentrations of Pb or Zn, yet the root/shoot partitioning was unaffected (Fig. 3a/II; Table 3/III). The involvement of interaction with root Mg and H$^+$-ATPases was suggested as a potential mechanism for Zn to reduce N acquisition (III), and it may also pertain to Pb and arsenate. This suggestion was made based on increased Mg concentration (Tables 8/I; 6/II) and correlations (III; Appendix 2). Elsewhere, such a mechanism has been suggested to be a significant contributor to reduced N acquisition under exposure to toxic concentrations of elements (review: Klobus et al. 2002). Had a direct inhibition of the high- or low-affinity nitrate transporter systems (review: Williams and Miller 2001) occurred, a soil Pb or Zn concentration-dependent decrease in the total plant N could have been expected, but it did not occur (Fig. 3a/II; Table 3/III). Other aspects include low plant P status (Fig. 3a/II; Tables 8/I; 3/III), which in *Phaseolus vulgaris* reduces nitrate uptake due to ATP-deficiency (Gniazdowska et al. 1998; Gniazdowska and Rychter 2001), as well as low carbon dioxide fixation and senescence (I-III), which inactivate nitrate reductase (NR; Pate 1977b; Matt et al. 2002).

In *P. sativum,* nitrate reduction may occur in the roots or leaves. Amides, asparagine and glutamine account for about 70-80% of N in the xylem sap of this species, and homoserine and allantoic acid have also been indentified. Yet, cultivar-specific differences occur between *Pisum* cultivars (Pate 1977b). High root N concentration in *P. sativum* seedlings grown with arsenate or Pb (Tables 4b/I; 3/II), and low shoot concentration in those grown with Zn (Table 1/III), may point to inhibition of NR activity or reduced translocation of nitrate/assimilation products. NR is inhibited *in vivo* in the leaves and roots of *P. sativum* exposed to arsenate or Pb (Sahulka and Lisá 1980; Sinha et al. 1988). Yet, in *Vigna radiata*, 2 mM Pb inhibits NR in the roots, but enhances it in the leaves (Singh et al. 1997/98). Zn inhibits NR in the leaves of *Triticum aestivum* in a reversible manner (Luna et al. 2000). Since K is the counterion in nitrate translocation, the low K status in Pb- and Zn-exposed adult *P. sativum* (Fig. 3b/II; Table 3/III) may have influenced the translocation (Jeschke and Hartung 2001).

5.5.2 Phosphorus (I-III)

Soil P availability is usually low and plants have developed mechanisms to improve it (Dinkelaker et al. 1995; Raghothama 1999). *P. sativum* is particularly efficient in utilizing poorly soluble P compounds (Makasheva 1986), which may relate to the association of this species with arbuscular mycorrhizal (AM) fungus (Balestrini et al. 1999). In another legume, *Anthyllis cytisoides*, association with AM enhanced the uptake of P from the soil (Díaz et al. 1996).

High- and low-affinity phosphate uptake systems occur (Liu et al. 1998; Chrispeels et al. 1999) and, similar to that seen in nitrate, the uptake occurs as cotransport utilizing proton motive energy (Raghothama 1999). These transporters are regulated by plant P status (Liu et al. 1998; Raghothama 1999).

In adult *P. sativum* cv. 'Phenomen', the root and shoot P concentrations declined in a linear manner with increasing soil Pb (Fig. 3a/II). Zn also reduced the root P content (Table 3/III), and arsenate that of the shoots (Table 8/I). Arsenate competes for transporters, and even if phosphate is the preferred substrate (Ullrich-Eberius et al. 1989; Meharg and Macnair 1992a), higher soil arsenate concentrations are likely to have influenced the uptake activity. The impact of Pb and Zn on P uptake may also be linked to their interaction with inorganic phosphate in the rhizosphere (Loneragan and Webb 1993) or at the plasma membrane level (Lösch and Köhl 1999). It has been suggested that Pb and Zn (0.1-1g kg^{-1} soil) may interfere in the absorption of P from the soil or in the mycorrhizal fungus-plant transfer mechanisms, rather than in colonization of the roots by mycorrhizal fungus (Díaz et al. 1996). As discussed in section 3.2.3, Pb forms poorly soluble phosphates, and thus, at elevated soil concentrations it may also restrict the availability of P by this mechanism. Since P is a central substrate in plant metabolism, including respiration and photosynthesis (Rao 1996; Raghothama 1999), low plant P status is likely to have had a cascade effect on other elements.

5.5.3 Potassium (I-III)

Shoot K concentration of *P. sativum* was inversely correlated with soil Pb concentration (Fig. 3b/II), whereas Zn reduced root and shoot K concentration at low but not at higher ($\leq$ 8.8 mmol Zn kg^{-1}) soil concentrations (Table 3/III). As discussed, Pb may have altered the partitioning and acquisition of K (II), the impact being dependent on soil Pb level (Fig. 3b/II). The fact that Pb has also been found deposited in the stelar tissues (section 3.7.6) of *P. sativum* (Päivöke 1983a; Sieghardt 1984), renders it feasible to suggest that Pb may be in the position of affecting partitioning mechanisms, e.g. xylem and phloem loading. In hydroponically grown *Triticum aestivum* interaction between Pb, K and Ca status has been established, in which low external Pb concentration increased the uptake of K, but the extent of the impact was dependent on the K status of plants (Trivedi and Erdei 1992). Similarly, interaction between Ca, K and Zn has been described (Chaudhry and Loneragan 1972). The use of different channel-type transporters facilitates the acquisition and intraplant translocation of K but interaction between Pb and these proteins has not been described (reviews: Chrispeels et al. 1999; Czempinski et al 1999; Mäser et al. 2001).

Oxidative stress induced by arsenate, Pb and Zn may cause peroxidation of membrane lipids (Bhattacharjee 1997/98; Chaoui et al. 1997; Hartley-Whitaker et al. 2001a), although Zn is thought to protect membranes (Brown et al. 1993). But as shown in *Triticum aestivum* leaves, higher concentrations (above 0.5 mM) of Zn cause efflux of K, which is usually a sign of membrane damage (Luna et al. 2000). Since the decline in K content of *P. sativum* (Table 3/III) did not respond to Zn concentration, membrane leakage may not have been the cause. Notably, Zn-K interaction also occurred in the cotyledons (Table 2/III), in which connection the impacts on protein synthesis and transport mechanisms were discussed (section 5.1.3).

5.5.4 Calcium (I-III)

Arsenate, Pb or Zn did not affect the Ca concentration of the roots or shoots of adult plants (Fig. 3b; Tables 8/I; 3/III). Yet, the remobilization of Ca from the cotyledons was enhanced by Zn (Table 2/III), which was partly attributed to disintegration of the seed coat (III). Ca and K emerged as those macronutrients showing frequent parallel change patterns shared with two or all three of the elements under investigation (Table 4), and under Zn-exposure, these elements also correlated with growth parameters (III; Appendix 2). The suggestion that Ca may have an enhanced function under exposure to toxic concentrations of arsenate, Pb or Zn, is in line with its central role as a structural element, in maintenance of protein conformation stability, enzyme catalyst, phosphorylation reactions and in cellular signal transduction (Sze et al. 2000; White and Ridout, 2001; Lecourieux et al. 2002). For example, PAL activity responds to Ca signalling (Lecourieux et al. 2002). This enzyme is of interest, because all three elements under investigation enhance suberization and lignification in *P. sativum* (Päivöke 1983 a, b).

5.5.5 Magnesium (I-III)

Root Mg concentration in *P. sativum* rose under arsenate and Pb exposure (Tables 8/I; 6/II), while as discussed, a negative Mg/Zn correlation occurred under Zn exposure (III; Appendix 2). In *Arabidopsis*, a Mg^{2+} transporter family of 10 members functions in Mg acquisition and intraplant translocation (Li et al. 2001). Extrapolation to the present investigation (III; Tables 8/I; 6/II) may imply that Pb and arsenate activated the expression of these transporters, while Zn also competed with Mg (III; Appendix 2). However, Zn enhanced (Table 2/III) but Pb decreased (Table 4/II) the export of Mg from the cotyledons, whereas in both Pb- and Zn-exposed plants it accumulated in the shoot tops in adult plants (II; III). The differences in responses from different locations may indicate contributions of impacts on individual members of the Mg^{2+} transporter family operating in various *in-planta* locations and functions.

The Mg/Mn, Mg/Cu and Mg/Fe correlations (III; Appendix 2) likely express the capacities of the Mg^{2+} transporters, which in *Arabidopsis* traffic also Ni^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+} and Cu^{2+} (Li et al. 2001). The result that in *P. sativum*, the majority of ratios of Mg to other elements rose in plants grown with Pb (Tables 7a-b/II), the accumulation of Mg in the shoot top of Pb- and Zn-treated plants (II; III) and the correlations with chlorophyll contents under Zn exposure (III) all point to the probability that alteration in plants´ Mg status may represent a common response to soil arsenate, Pb and Zn, and that this response may contribute to mechanism(s) protecting plants from the impacts of elevated concentrations of these elements. Increase in Mg concentration under Pb and Zn exposure also occurs in other species (Biddappa et al. 1987; Table 1 in Krupa et al. 2002). Yet, enhanced Mg uptake *per se* may reduce that of K and Ca (review: Fageria 2001). The soil mixture was rich in Mg (Table 1/I), which probably facilitated its availability.

5.5.6 Sodium (I-III)

Pb increased the root Na concentration in adult *P. sativum* (Table 6/II), reduced the K/Na ratio in the roots and shoots by about 50% (Tables 7a-b/II), and decreased the remobilization of Na from the cotyledons to levels less than those seen in the control seedlings (Table 3). The use of Na arsenate probably increased the availability of Na and was reflected in the shoot Na concentration of the adult plants (Table 8/

I). However, elevated Na concentrations occur in wild plants growing in contaminated sites (Maurice and Lagerkvist 2000; Steinnes et al. 2000).

Na is a major element in soil solution, is physiologically similar to K, but nonessential to plants, and yet is taken up and is beneficial to some plants (Marschner 1995). Enhanced Na uptake may be a compensation mechanism (Maathuis et al. 1996; Buschmann et al. 2000) for low K status (Fig. 3b/II; Table 3/III); as discussed, Na in *P. sativum* can, to a certain extent, replace K (III). Elevated cellular Na^+ concentration may, however, inhibit K^+ uptake (Shi et al. 2002).

The role of high-affinity K^+ uptake transporter (HKT1) in the uptake of Na^+ has not been fully determined, although there is evidence that the HKT1 transporter facilitates low-affinity Na^+ uptake under conditions of toxic Na concentrations when K^+ uptake is absent (c. f. Yeo 1998). AtHKT1 from *Arabidopsis*, homologous with HKT1, has been shown to selectively facilitate Na^+ uptake in oocytes of *Xenopus laevis,* but in contrast to HKT1, it was not influenced by external K^+ concentration (Uozumi et al. 2000). Ca-selective and -nonselective cation channels can mediate Na^+ acquisition (Buschmann et al. 2000; Demidchik and Tester 2002), the latter showing higher selectivity for K^+ than for Na^+ (review: Demidchik et al. 2002). In bread wheat *Triticum aestivum* and durum wheat *T. turgidum*, correlation has been established between high K^+/Na^+ ratio and salt tolerance, and it was shown that the discriminatory feature in favour of K^+ under these circumstances was controlled by a single locus of one chromosome (Dubcovsky et al. 1996).

In *Linum usitatissimum,* the Na-Ca ratio increases during secondary wall differentiation and lignification (Ripoll et al. 1993), suggesting that lignification raises the Na requirement of this species. A common response of *P. sativum* cv. ‘Dippe Maj’ to elevated concentrations of arsenate, Pb or Zn is enhanced lignification (Päivöke 1983a, b). In the present study, increase in the Na-Ca ratio occurred in the shoots of adult *P. sativum* cv. ‘Phenomen’ grown in elevated concentrations of arsenate (I) and in the roots of those grown in higher concentration of Pb (Table 7b/II), whereas in plants exposed to an elevated concentration of Zn, Na correlated with many elements, and a number of correlations were inversed compared with the controls (III; Appendix 2). These observations point to the possibility that the role played by Na could have been altered by arsenate, Pb and Zn (I-III). Results obtained by other researchers that externally supplied Na may mitigate the toxic effects e.g. of Pb, were previously discussed (II).

5.5.7 Sulphur (I-II)

Under Pb exposure, the ratios of total S to the majority of other elements declined in the shoots (Tables 7a-b/II), but in the shoots of plants grown with higher soil concentrations of arsenate, the S concentration in the shoots was increased (Table 8/I). S is closely linked with glutathione metabolism, including the synthesis of phytochelatins and with a number of oxidation-reduction reactions (review: Leustek and Saito 1999). Results on the impact of arsenate or Pb on S concentration were preliminary (Tables 8/I; 6/II; 7a-b/II), but suggest that the metabolism of S is influenced by elevated soil concentrations of arsenate and Pb. Reduced availability of S could be expected to affect e.g. the synthesis of phytochelatins and the ability of *P. sativum* to sequester arsenate or Pb.

5.5.8 Zinc (I-III)

Arsenate and Pb interacted with Zn: arsenate caused an increase in roots´ Zn concentration in adult *P. sativum* (Table 8/I), but in the roots of Pb-exposed plants the increase was insignificant (Table 6/II), whereas in the seedlings the remobilization of Zn from the cotyledons was reduced by both arsenate (Table 5/I) and Pb

(Table 4/II). As discussed (section 3.5), Zn uptake and homeostasis are intricately intertwined, particularly with those of Mg, Mn, Cu and Fe. Zn, as a micronutrient, plays a central role in a large number of metabolic pathways and functions, including protein synthesis, carbohydrate metabolism, membrane integrity, auxin metabolism, gene expression and enzyme activities (Vallee and Auld 1990; Brown et al. 1993; Berg and Shi 1996; Auld 2001). One common denominator among Zn, arsenate and Pb is interaction with inorganic phosphate. The Zn-P relationship is complex and is dependent on soil conditions, nutrient balance and plant species, and differs even among cultivars of a single species. This relationship may not be fully understood as yet (Loneragan and Webb 1993; Zhu et al. 2001).

5.5.9 Iron and copper (I-III)

Only Zn interacted with Cu and Fe in the present study (I-III): it enhanced the remobilization of Cu from the cotyledons (Table 2/III), but reduced the total Fe content and its concentration in adult plants (III) so that the Fe concentration in shoots sank to a significantly lower level than that of the control plants (Table 4/III). In pot-grown *Triticum aestivum* cv. 'Apu', Fe/Zn correlation is one of the three significant correlations of Fe, occurring both in the soil and *in-planta* (Sillanpää 1982). It also occurred at the root/shoot level in control *P. sativum* cv. 'Phenomen', but was absent in plants grown with Zn (Appendix 2).

In Strategy I plants, Fe^{3+} is reduced to Fe^{2+} before entering the root cytoplasm (Marschner 1995), and Fe and Cu share the reductase enzyme in *P. sativum* (Welch et al. 1993). The gene and protein of this enzyme have recently been described (Waters et al. 2002). This reductase is activated by low plant Fe status (Cohen et al. 1998; Grusak 2000) and the activity appears to be controlled by shoot-borne signals, which ethylene may enhance (Schmidt et al. 2000). Since Cu concentration was unaltered in adult plants, the low total Fe content and concentration (Table 4/III) may not be attributed to inhibition of

Table 4. Shared parallel change patterns in *P. sativum,* exposed for 21 days to 70 μmol As, 6.5 mmol Pb or 8.8 mmol Zn kg^{-1} dry soil.[a]

Element pair	Occurrence	Element pair	Occurrence
K-Cu	Pb: shoots, roots	Na-K	Pb: roots
	Zn: shoots		Zn: shoots
K-Fe	Pb: shoots	Zn-Mn	Pb: roots
	Zn: roots		Zn: shoots, roots
Ca-Na	As: roots	Cu-Fe	As: roots
	Zn: shoots, roots		Pb: shoots
Ca-Cu	Pb: shoots		Zn: roots
	Zn: roots, shoots	Fe-P	As: roots
Ca-Mn	Pb: shoots		Zn: shoots
	Zn: shoots, roots	Mn-Na	Pb: roots
Ca-Fe	Pb: shoots, roots		Zn: shoots, roots
	Zn: shoots, roots		

[a] Information derived from papers I (As), II (Pb) and III (Zn).

the reductase. The Cu-Fe parallel change, recorded in plants exposed to all three elements under this investigation, is likely linked to the activity of the shared reductase (Table 4).

In *Arabidopsis,* iron transporter (IRT1) facilitates Fe uptake via the high-affinity uptake system (Connolly et al. 2002; Vert et al. 2002). In *P. sativum* cv. 'Sparkle', a homologue protein is induced under low plant Fe level (Cohen et al. 1998). A Zn concentration of 0.5 mM in nutrient solution inhibits the synthesis of this protein in *Arabidopsis thaliana* wild ecotype Columbia gl-1 (Connolly et al. 2002). A similer mechanism may have contributed to the present results, because the total Fe content of whole plants was low, without the partitioning having been altered (Table 4/III). Yet, the soil Fe concentration was also below the mean for agricultural soils in Finland (Table 1/I; Urvas 1995). Elevated soil Zn concentration may also have influenced the availability of Fe, e.g. by affecting the soil mycorrhizal fungi known to contribute to Fe solubilisation in the rhizosphere (c. f. Ernst et al. 2000).

The P-Fe parallel change (Table 4) is interesting, because it is taken as an indicator of Fe^{3+}/Fe^{2+} balance (discussed in Olsen 1972). The inverse P/Fe correlation in Zn-exposed plants, may imply increased Zn toxicity (III; Appendix 2). In pot-grown *T. aestivum* cv. 'Apu' a significant Fe/Mn correlation occurs (Sillanpää 1982). A similar relationship was also established in the roots and shoots of control plants in the present study, but in plants grown with Zn it was absent (Appendix 2).

5.5.10 Manganese (I-III)

In the present study, the rapid remobilization of Mn from the cotyledons is likely an expression of higher demand for it in the growing axis (Tables 5/I; 4/II; 2/III). In adult plants, an increasing trend in root Mn concentration was discernible (Tables 8/I; 6/II; 4/III), and the partitioning of it more towards the roots in arsenate- and Pb-exposed plants occurred (Table 8/I; 6/II). These results suggest that all three elements under investigation affected the metabolism of Mn.

Recent evidence in *Saccharomyces cerevisiae* shows that the maintenance of *in-planta* homeostasis of Mn^{2+} involves an ATPase-type Ca^{2+} pump associated with the endoplasmic reticulum, and apart from Mn^{2+} it is also capable of transporting Ca^{2+} and Zn^{2+} (Sze et al. 2000; Wu et al. 2002). In adult *P. sativum* cv. 'Phenomen', a close Mn-Ca relationship occurred: parallel change in the shoots under Pb exposure and in the roots and shoots of Zn-treated plants (III; Table 7a/II), and a positive Mn/Ca correlation in the roots of the latter (III; Appendix 2). A Ca-Mn relationship has also been reported in other species, such that addition of Ca, and also P, reduces the toxicity of Mn in plants (review: Foy 1983). A Mn/P correlation also occurred at the root/shoot level in both the controls and Zn-exposed plants (III; Appendix 2). A rise in Mn, Zn and Cd concentrations of *P. sativum* cv. 'Sparkle' under low Fe status (Welch et al. 1993) has been attributed to the capacities of the iron transporter (IRT1) to take up all these elements (Guerinot 2000). The alterations in the correlation patterns of Fe and Mn discussed above also point in this direction (section 5.5.9).

The number of correlations of Mn with other elements rose, particularly at the root/shoot level in plants grown with Zn (III; Appendix 2), whereas in Pb-treated plants, apart from Ca, Mn-N, Mn-Na, Mn-Zn and Mn-Fe parallel changes occurred (Tables 7a-b/II). The wide spectrum of relationships is understandable, since Mn is involved in many aspects of plant metabolism, including oxidations, decarboxylations, various biosynthetic pathways, and the response systems towards elevated element concentrations and oxidative stress (Mostowska 1996; Luk and Culotta 2001; Alscher et al. 2002). The correlations in Zn-exposed plants (III; Appendix 2) and in those grown with arsenate (Päivöke and Simola 2002), together with the results listed above, are all supportive of the possibility that in *P. sativum* cv. 'Phenomen', Mn is linked with a response mechanism common to arsenate, Pb and Zn. Notably, also Mn-Na parallel change occurred in both Pb- and Zn-exposed plants (Table 4). The possibility of some cooperative mechanism of action between Na and Mn being induced or being activated under the influence of elevated soil concentrations of Pb or Zn would be of interest for further investigation.

6. CONCLUSIONS

The major hypotheses were that enhanced external concentrations of arsenate, Pb and Zn imbalance the mineral element status of *P. sativum* cv. 'Phenomen' (I-III), and that this happens at or below the maximum soil Pb concentration considered acceptable in agricultural soils, 500 mg Pb kg^{-1} (II) or below soil and *in-planta* Zn concentrations considered phytotoxic, 300 mg Zn kg^{-1} soil (WHO 2001b) or 100-1400 mg Zn kg^{-1} (III; Kabata-Pendias and Pendias 2001) and 500 mg Zn kg^{-1} plant dwt (III). The recommended upper soil Zn concentration limits vary greatly, depending on the country concerned and also on the soil conditions (e.g. Table 6 in Kabata-Pendias and Pendias 2001). A significant mechanism of action in this species was hypothesized to be indirect, and one of these mechanisms included disturbance in *in-planta* ATP concentration (I-IV). The results supported these hypotheses (I-IV), both in the seedlings (3-12 days) and flowering (21 days) plants, which however displayed both coinciding and dissimilar responses. Change in the mineral element status could be observed in terms of concentrations, total contents and in interelement ratios and correlations between elements (Figs. 3a-b/II; Tables 4b,5,8/I; 3,4,6,7a-b/II; 1-4/III; Appendix 2)

Under a 21-day exposure, the growth of the shoots was more sensitive to the applied elements than that of the roots (Figs. 2a-b/I; 2/II; Tables 5/II; 3/III), which supported the hypothesis of indirect mechanisms (e.g. Punz and Sieghardt 1993). Further support came from the direct correlation between *in vivo* phytase activity and seedling growth (III); e.g. impact of Zn on growth (Tables 1/III; 1/IV) contributed to the decline in enzyme activity (Figs. 1a-b/III). Additional support came from the inverse correlations between the ATP concentrations and growth of seedlings (IV).

The present study confirmed the necessity for evaluating the accumulation of toxic concentrations of elements in crop plants on an individual element and species (cultivar) basis, since even low soil concentrations were harmful (e.g. Figs. 3a-b/II). Under Pb exposure, the medium soil level (6.5 mmol Pb kg^{-1} soil) yielded a higher fraction and total Pb content in the shoots than did the higher soil Pb concentration (II).

Under Pb exposure, the growth response was rather concentration-dependent (Fig. 2/II), but under Zn exposure growth and soil Zn level did not correlate (Table 3/III; Appendix 2). This pattern also repeated itself with regard to P (Fig. 3a/II; Table 3/III) and K (Fig. 3b/II; Table 3/III) concentrations of adult plants, and may point to different mechanisms by which Pb and Zn affected the acquisition of these elements. Yet, plant N concentrations responded to Pb and Zn in a fairly similar manner (Fig. 3a/II; Table 3/III), which may suggest towards involvement of some mutual mechanisms.

Since the export of mineral elements from the cotyledons was imbalanced in an element-specific way (Tables 4b,5/I; 3,4/II; 1,2/III), the elements under study possibly interfered with the processes that mediate or regulate the element-specific mechanisms facilitating remobilization from the cotyledons. The result that all three elements under investigation enhanced the export of Mn out of cotyledons may refer to a higher need for this element in the axis under these circumstances, or that Mn uptake and supply by the roots could have been decreased by arsenate, Pb and Zn (Table 3). The export of Fe from the cotyledons was unaffected by any of the three elements under study (Table 3), but in adult plants Zn reduced the total Fe content of the entire plants (Table 4/III), suggesting that Fe acquisition by the roots was likely to have been affected in the adult plants.

The results with flowering plants suggested that Mg, Ca, K, Na and Mn play significant roles in responses of *P. sativum* cv. 'Phenomen' to arsenate, Pb and Zn (Fig. 3b/II; Tables 8/I; 6,7a-b/II; 3,4/III; Appendix 2). All three elements increased plant Mg concentrations, which may aid in understanding the minor effects of Pb and Zn on the chlorophyll concentrations of adult plants (II; III). However, in the seedlings Zn reduced the chlorophyll concentrations (Table 2/IV). Competition between Zn and Mg was identified in adult plants (III; Appendix 2), and such competition offers potential mechanisms for impact, including those mediating element uptake and cross-membrane trafficking of elements. K and Ca displayed frequent parallel change and correlations (Table 4; Appendix 2). The fact that Zn inversed a number of correlations (III; Appendix 2), and that Pb treatment increased root Na concentration (Table 6/II), pointed towards altered roles for this element. Similarly, Zn treatment altered the correlation patterns of Mn (III; Appendix 2), which element also showed parallel change with Zn in both Pb- and Zn-exposed plants and with Mg in those grown with arsenate (I; Table 4). There was an increasing trend in the concentration of Mn in the roots in all three elements under study (Tables 8/I; 6/II; 4/III), and all three promoted the export of this element from the cotyledons (Table 3), all of which results may lend support for the view that the role of Mn may have been enhanced or altered by arsenate, Pb and Zn.

Results were obtained under greenhouse conditions, which frequently cause more pronounced responses than those observed under field conditions (e.g. Chaney 1993). The mycorrhizal aspect was not investigated, which as discussed above is a significant factor influencing nutrient availability and uptake under field conditions. Moreover, due to the N-rich soil mixture, nodulation was rare or absent (I-III), and the aspect of symbiotic N fixation was outside the scope of this work. However, it is believed that these results point out significant trends and directions, which have relevance and also need focusing in the field. The present study suggested that even though consideration needs to be given to each crop, and probably cultivar and individual element, there are a number of responses in *P. sativum* cv. 'Phenomen' that were shared by arsenate, Pb and Zn.

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