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Studies on Terrestrial Herbaceous Plants Tolerance to Excess Heavy Metals: Methodological Approach

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Abstract. Plant tolerance to heavy metals is a scientific issue attracting significant attention due to the possible use of tolerant plants for phytoremediation purposes as well as due to the fact that the molecular mechanisms of this phenomenon are not clear enough. Despite of the increasing volume of research on the problem, the available information in many cases is incomplete and/or difficult to compare with other studies because of the significant differences in the experimental designs, range of used metal concentrations, exposure time, etc. In this review-paper both the advantages and limitations of the used experimental designs as well as the methods for evaluation of heavy metal tolerance are briefly discussed.

Key words: heavy metals, plant tolerance, experimental design, methods

Introduction

The contamination of the environment by heavy metals (HM) represents a real ecological problem. In Bulgaria the soils contaminated by HM cover an area of about 200 000 da (GRANCHAROV & POPOVA, 2003). The sustainable use of these soils can be achieved by developing various remediation phytotechnologies as well as adaptive agriculture practices (VASSILEV *et al*., 2005a). For this purpose, it is necessary to have a detailed understanding of the interaction between the HM and the plants, which includes the mechanisms of 1) the uptake and the distribution of HM by the plants, 2) the metal phytotoxicity, and (3) the plant's tolerance towards the excess of metal ions in the environment.

In general, the plant's tolerance towards HM represents the ability of particular plants or populations to thrive under conditions that are characterized by having excess of metal ions, which have toxic effect for other plants (MACNAIR *et al*., 2000). The first research on the plants' tolerance towards HM ever made dates back to the beginning of the 20th century, when it was established that two populations of the species *Silene dioica* have different tolerance towards the excess of Cu (ERNST *et al*., 1992). Thanks to the development of experimental biology, our understanding of the plant tolerance towards HM is gradually enriched. At present day, the assumption is that the tolerance is based on two different strategies: 1) to avoid the entry of excess HM

into the plants, and 2) to achieve effective intra-cellular detoxification. The main tolerance-related mechanisms are already well-known, the most important of which include 1) the reduced uptake and/or accelerated excretion of HM by the cells, 2) the metal detoxification and compartmentalization, 3) the control of the metal-induced oxidative stress, etc. (VASSILEV & NIKOLOVA, 2010).

The scientific interest with respect to the plant tolerance towards HM has become considerably larger in recent years. On one hand, this is due to possible usage of tolerant plants for phytoremediation of soils contaminated by HM (KULAKOV *et al.*, 2009), and on the other, the interest is a result of the possible wider usage of the plants as model objects for eco-toxicological studies (HOCK & ELSTNER, 2005). The number of research papers related to the identification of plants that have high tolerance and hyper-accumulative abilities towards HM are constantly increasing (SCHULZE *et al.*, 2005).

At the same time the gathered information is not always accurate, objective or comparable to other results. The main reasons for this include the considerable differences in the utilized experimental designs, the metal concentrations, the exposure time, etc. For the reasons mentioned above, there is a need for critical analysis of the available information, which would identify the strengths and weaknesses of the different types of tests, and would also describe the established methods that are used to determine the plant tolerance towards HM.

Types of research

The research on the plant tolerance towards HM is carried out on the basis of different experimental designs, which can be generally classified as either *in vitro* or *in vivo*. The *in vivo* research is carried out through hydroponic, pot, or field tests, where every type of test has its advantages and disadvantages.

When employing the *in vitro* framework, the cellular organelles, groups of cells and leaf blades, are being incubated into solutions of different concentrations of HM. Under these conditions, the expositions are shorter, the applied concentrations of HM are larger, and the observed effects are more direct. The *in vitro* framework is being used to draw the characteristics of the potential effects of HM on the plant cells.

Under the *in vivo* framework the plants are being cultivated in conditions marked by excess of the necessary HM (Cu, Zn, Mn), or by the presence of other HM (Cd, Pb, As), where the latter have been biologically inactive. This framework is closer to the natural growing conditions; however, when employing the latter, it is difficult to differentiate the direct effects of HM from the indirect ones, as far as the separate physiological processes are concerned. In recent years, research has predominantly been based on the *in vivo* framework.

The applied effects of the *in vivo* research can have chronic or acute characteristics. In the former case the plants suffer from the negative effects of the HM, but remain alive, whereas in the latter, they die. The tests involving concentrations that cause acute phytotoxicity are still predominant, however, this approach is subject to growing levels of criticism (MILONE *et al.*, 2003) since the used concentrations are often unrealistically high and do not let the plants' defensive mechanisms to manifest themselves.

Types of experimental designs

Hydroponic experiments

In the hydroponic experiments the plants are grown on nutritious solutions, while controlling the environment's parameters (light, temperature, relative air humidity, photoperiod, mineral nutrition). The main advantage of these tests is that they are accurately reproducible (Fig. 1A). One of the disadvantages is the inability for a mycorrhiza to develop, which in natural conditions is important for the absorption of HM and for the plants' tolerance towards the latter.

The absorption and phytotoxicity of HM are most accurately measured in hydroponic conditions. In these conditions it is of particular importance to maintain the predefined concentration of the studied HM. This is achieved to a degree by changing often the solutions, and by controlling the levels of pH. This way, one limits the possibility of having a part of the studied HM transformed into a form that is impossible to be absorbed by the plants.

In the traditional hydroponic solutions, the element Fe is being introduced as a chelate of ethylene-diamine-tetraacetic acid (Fe-EDTA) in order to prevent its being transformed into a non-absorbable form. When introduced into the root cells, the complex becomes dissociated and the free Fe penetrates into the protoplasm (CHANEY *et al.,* 1972). The presence of EDTA in the hydroponic solutions can, however, create problems for some tests involving HM (CHANEY, 1993). For instance, the presence of EDTA in the solution hinders the sampling of frameworks involving Zn phytotoxicity. Compared to the Fe, the Zn has a higher constant for connecting to the EDTA, as a result of which it can replace it from the complex. This in turn reduces the number of free Zn ions in the solution, and furthermore, it causes a Fe deficit, since the replaced Fe has been transformed into a non-absorbable form. The sample frameworks that are characterized by an excess of Pb or Cd are also problematic for research. In hydroponic conditions, the Pb practically becomes entirely precipitated. In order to ensure the absorption of Pb by the plants, some researchers mix the Pb with EDTA beforehand (GEEBELEN *et al.,* 2002). In this case, about 70% of the Pb is being absorbed and transported into the plants in complex state (SARRET *et al.,* 2001), which does not reflect the real situation in the contaminated soils. As far as sample frameworks involving Cd are concerned, when used in realistic concentrations (1-2 μM) they create the possibility for the reduction of the number of free Cd ions in the solution.

In order to avoid the above-mentioned shortcomings of the traditional hydroponic solutions, it is recommended to use the so-called "chelate-buffered solution". The main way in which they differ from the traditional solutions, is that they create sample frameworks, which are based on "ion activities", and not on ion concentrations. The concentrations of the ions in the soil solution are generally much lower than those in the hydroponic solutions. However, because of the dynamic ion balance between the solid and the liquid phase in the soil, the ion activities in the soil solution are very well buffered. The situation is different in the hydroponic solutions, especially in relation to the macro elements. The selection of the chelating compound in the chelate-buffered solutions depends on the studied HM, on the type of stress (toxicity, deficit), and on the type of plant. The selected chelating compound is usually added in excess, generally in the range of 20 to 100 μM more than the sum of the macro elements in the solutions. The ion activities of the HM could be determined with the help of specialized computer software, such as for instance GEOCHEM-PC (PARKER *et al.,* 1995).

Pot experiments

In these experiments the plants are being grown in soil or other inert substrata such as sand, perlite, vermiculite, etc. in greenhouses. As a rule of thumb, the duration of these tests is longer than that of the hydroponic ones, and could encompass the entire vegetation.

The main advantage of the soil tests is their proximity to the natural conditions and the possibility for the development of mycorrhiza. Their main disadvantage, on the other hand, is the strong dependency of the observed effects upon the properties of the used soil (Fig. 1B). Sample designs are often being used with artificially contaminated soils (metal-spiking studies), in which case their properties have a considerable effect on the mobility,

absorption and, consequently, the effects of the HM on the plants.

A critical juncture when working with soils is the way in which they are treated with HM. Usually, the contamination is being carried out with salts of the examined HM, which are easily soluble. From a technical point of view, the HM can be introduced into the soil in a relatively homogenous way by means of fine sprinkles of the water solutions of the compounds onto a thin layer of dry soil. After treating the soil, it needs to be left aside for several months, in order to reach a balance between the separate pools of HM in it (VASSILEV *et al.*, 1998). When inducing complex contamination with several HM, it is necessary to monitor the level of the salts, in order to avoid any side effects, related to possible soil salinity.

When testing with inert substrata (sand, perlite, etc.) the HM is being changed in regular intervals, while ensuring that the excess volume of the solution is leaked from the containers. When changing the solutions, the substrata first needs to be washed with distilled water, in order to prevent a possible overdose of HM.

When working with sand cultures, the used sands needs to soak for 24 hrs in a solution with 20% of hydrochloric acid, in order to have all the salts dissolved. The sand then needs to be well washed with distilled water. The advantage of the tests involving substrata over those involving soils is the inherent possibility to separate and measure the mass of roots. Furthermore, the sand cultures can also host the development of mycorrhiza, unless they have been sterilized.

The container soil tests involve the application of mineral fertilization and maintenance of particular water regime. It is recommended to introduce the necessary mineral elements in the soil in the beginning of the tests, because if they are introduced in parts, this could lead to changes in the reaction (pH), and consequently to the mobility and absorption of the HM by the plants during the vegetation period. The water regime has to be maintained by pouring water into the pots until they become a certain weight, the latter being calculated in relation to pre-determined soil humidity.

A B

Fig. 1. General view of the experimental design involving heavy metals in the case of (A) hydroponic and (B) soil cultivation of the plants (Koleva and Vassilev, original)

Those opposed to the use of salts in sample soil tests involving HM, base their arguments on two main facts – "salt" effect and the effect of the "plateau", both of which exert strong influence on the derived results (BASTA *et al.*, 2005). The salt effect reflects the higher mobility and consequently the plants' access to the HM when the latter have been added as salts into the soil, in comparison to their being accessible in soils contaminated by industrial activity. The

effect of the plateau is characterized by a lower absorption of HM by the plants when the latter are grown on soils contaminated by industrial activity, as opposed to being contaminated by salts of HM. Usually, the absorption of HM in the soils contaminated by salts has a direct dependency upon the common concentration, whereas in the industrially contaminated soils the dependency is expressed by a curve, which reaches saturation, i.e. the absorption decreases as the concentrations of HM in the soil increase.

The mentioned facts favor the avoidance of the experimental frameworks that introduce salts of HM, whenever this is possible. It is better if the different levels of HM are derived on the basis of mixing non-contaminated soils with soils that are contaminated by industrial activity, or with biological sediments containing HM. It is necessary, however, that the physico-chemical characteristics of the mixed components to be as similar as possible, and that they lead initially to the same reaction (pH).

In certain cases, in order to avoid the dependency of the biological effects of HM on the properties of the specific soil, the so-called 'artificial' soils are being used (a mix of quartz sand, clay and calcium carbonate) which are prepared according to established methodologies (VASSILEV *et al.,* 2005b).

Field experiments

The main advantage of the field trials is the fact that they provide information about the interaction between the plants and the HM in the soil, in the context of specific environmental factors. There are, however, some shortcomings.

The plants that grow on soils contaminated by HM can, to a certain degree, absorb HM from the air as a result of closely situated industrial sources. In such conditions, in addition to HM in the soil, air pollutants can also be phytotoxic. The compared variations in these tests are usually determined by the distance from the industrial source, as well as by the direction of the winds (IANKOV *et al.,* 2000). Despite the fact that the variations are not big, some soil and climate-related differences do nevertheless exist. It is also known that the industrially contaminated soils are characterized by heterogeneity with respect to the content of HM, both, in horizontal and vertical directions. The roots of plants generally avoid areas covered by soils contaminated by HM, as a result of which there are substantial differences between the productivity under homogenous and heterogeneous distribution of HM in the soil (PODAR *et al.,* 2004). The mentioned peculiarities of the field trials with regards soils contaminated by HM need to be taken into account when interpreting the results.

The combined use of the different experimental frameworks in research work compensates to a certain degree for their individual shortcomings and leads to objective results.

Approaches and methods for determining the tolerance of plant genotypes towards heavy metals

The tolerance towards HM is determined through various laboratory and vegetative tests (KÖHL & LÖSCH, 1999). The tests include easy-to-determine parameters such as root length, biomass of the roots or of the entire plants, fertility of the seeds, pollen development, as well as physiological parameters such as leaf gas exchange, chlorophyll fluorescence, the ability of cells to plasmolyze and others.

The tolerance of the genotypes is determined either on the basis of the changes of the parameters relating to a single concentration of HM, or to a concentration interval. In the former case the tolerance index (TI) is being measured, which represents the ratio between the magnitudes of the parameter measured under conditions of higher concentration of HM vis-à-vis its relative value under controlled conditions, expressed in percentage. Table 1 contains data regarding the TI of various plant types with respect to Cd.

When determining the TI, two approaches are being used – sequential determination, and parallel determination. The sequential determination is used when it is necessary to determine the tolerance of every member of the population, mainly for genetic research and for

selection purposes. The used parameter in this case is measured in a non-destructive way, by firstly, having the individual plants cultivated in a controlled environment, and then in environment characterized by higher concentration of HM.

Table 1. Values of TI (%) measured on the basis of lengthening the roots in the nutritional solution of Rorison, containing 10 μM Cd (as per BAKER & WALKER, 1989)

Plant species	TI (%)
Festuca rubra L.	101 ± 21
Agrostis capillaries L.	83 ± 20
Holcus lanatus L.	65 ± 12
Poa annua L.	47 ±13
Lolium perenne L.	24 ± 6

The tolerance index, as mentioned above, is calculated as a fraction of the value of the parameter of the environment that has increased metal concentration, and the value in the controlled environment, multiplied by 100. The disadvantage in this approach is the dependency of the effect on changes in the growth rate of the ontogenesis, as well as the necessity to maintain constant conditions during the experimentation. In the latter case, the plants of a particular genotype are being grown in a parallel fashion – in controlled environment and in environment having an increased concentration of HM. Under this framework the dependency on ontogenetic effects is smaller, however the observed differences can be the result of not only the concentration of the HM, but also of differences in the tolerance of the separate individual plants within the population, unless cloned material is being used.

The curve "dose-response" is being described on the basis of the parallel cultivation of plants in controlled environment and in environment contaminated by HM. The latter serves as the basis for determining the so-called effective concentrations (EC) of HM. The most frequently determined concentrations are the following: (no-observed-adverse effect-concentration), which represents the highest external concentration of HM, where there isn't effect on the studied parameter; EC_{25} и EC_5 – external concentrations of HM, which lead to changes in the parameter by 25 and 50%, respectively (Table 2). The change usually leads to inhibition, but when determining enzyme activities or other dynamic variables, this could be a matter of temporary stimulation.

Table 2. NOAEC и EC_{50} values with respect to the growth of the roots and leafs of four plant types under conditions of soil contamination with Cd (mg kg^{-1} DW) (AN, 2004)

Plant species	NOAEC (roots)	NOAEC (leaves)	EC_{50} (roots)	EC_{50} (leaves)
Sorghum bicolor (L.) Moench	20	20	61	39
Cucumis sativus L.	40	40	88	102
Triticum aestivum L.	< 40	40	113	98
Zea mays L.	160	160	268	208

Root lengthening

The root test is the most popular method in the experimental research, used for determining the tolerance towards HM. It is based on the fact, that when in toxic concentrations, the HM inhibits the linear growth of the roots (WOOLHOUSE, 1983). It is

established that the length of the roots is a very sensitive indicator, because the HM have influence on, both, the cell division in the meristem zone, and on the cell elongation.

The root lengthening is a typical study of relatively short tests. In most cases, its duration does not last for more than 7-8 days. It is suitable for determining the tolerance of plant species, whose root growth is relatively quick. On the other hand, it is more suitable for determining the tolerance towards heavy metals that have a relatively quick effect on the root growth. In this aspect, Cu is more suitable than Zn or Cd. Usually, the longer the test becomes the lower the established effective concentrations, which could be a consequence of the plants' acclimation towards the specific heavy metal.

When a heavy metal is necessary for the plant growth and development (for instance Cu or Zn) it is also present in the solution of the controlled version. It is necessary that its concentration in the controlled version is optimal for the root growth, because in cases when the concentration is lower, their growth can be stimulated (WILKINS, 1978). The concentration of HM that is used for screening of the tolerance of the genotypes needs to be selected in such a way, as to ensure that the TI in the most sensitive genotypes is not higher than 0, and in the most tolerant – not lower than 100. This can be achieved by conducting preliminary experiments.

In most cases, the growth of the longest root is measured, unlike cases involving dicotyledonous plants, where the length of the main root is measured (VASSILEV *et al.*, 2005c). Certain authors, however, measure the growth of all roots (ŠTEFANOVIČOVÁ *et al.*, 2000). In order to determine the growth, in the beginning of the test the tips of the roots are marked with a permanent marker or are being submerged into a suspension of active carbon. Since the roots grow apically, the growth (usually in mm) is measured in the end of the test as the difference between the mark and the new length.

Plant biomass increase

The duration of this test is longer (several weeks or months) and is always carried out using the parallel method, due to the unavoidable influence of ontogenetic and ecological factors. Both, hydroponic and substrata-container tests can be used for the test. The fresh or dry mass of the surface roots or of the entire plants is being used as criteria for the determination of the TI in the end of the examination period, both, in the controlled environment and in the environment having an increased concentration of (VASSILEV *et al.*, 2007). Some authors use the relative growth rate of the entire plants as a parameter (ERNST *et al.*, 1992). The latter is being determined according to the formula described below, on the basis of the plant's dry mass in the beginning (DW_1) and in the end (DW_2) of the examination period (ΔT in days):

$$RGR = (ln\ DW_2 - ln\ DW_1) / \Delta T$$

The main advantage of using the weight of the biomass as an indicator is its integral character and easy measurement. On the other hand, its precise determination a long period of time, and its accuracy is not always high enough.

Plasmolytic test

The tolerance criterion in this test is the ability of the cells of different genotypes to plasmolyze after a period of 24 to 48 hours of being in solutions that have different concentrations of HM. Usually, bits of epidermis or tissue are being put in the solutions, where the latter would have 3-4 layers of cells, and the plasmolysis is being induced by 1M of sucrose. The cell membrane's integrity, and respectively, the vitality of the cells, is being calculated as a percentage of plasmolyzed cells. This method can be used for comparative research on the phytotoxicity of different HM.

Other methods

The test of seed germination can also be added in addition to the above-mentioned methods. In general, this method is not sensitive enough towards excess of HM (BAKER & WALKER, 1989), but continues to be used for different types of toxic tests (AN, 2004). The HM concentrations, which inhibit the seed

germination, are usually much higher than those inhibiting the growth of the sprouts or of the young plants.

Recently, in order to increase the sensitivity of the tolerance tests, a number of functional parameters have also been included (Table 3). In this respect, the non-destructive physiological analyses are of most interest, such as the leaf gas exchange and the chlorophyll fluorescence (VASSILEV, 2002), as well as the activity of antioxidant enzymes (CLIJSTERS *et al.*, 1999; VASSILEV, 2003). The combination of growth and functional indicators best characterizes the tolerance of the plant genotype towards HM (VANGRONSVELD & CLIJSTERS, 1992).

Table 3. Ecotoxicological values characterizing the tolerance of barley (*Hordeum vulgare* L.) plants towards the toxic influence of Cd (as per VASSILEV, 2003). A – net photosynthetic rate; E – transpiration rate; stomatal conductivity; Chl.a – chlorophyll "a"; GPOD – root peroxidase activity; RGR – relative growth rate; DW and FW, dry and fresh weight.

Parameter	Regression equation	EC_{25}	R^2
A (µmol CO_2 m^{-2} s^{-1})	Y = -0.0009*X+0.245	72	0.75
E (mmol H_2O m^{-2} s^{-1})	Y = -0.005*X+1.98	99	0.87
q_s (mol m^{-2} s^{-1})	Y = -0.0008*X+0.122	38	0.90
Chl.a (mg g^{-1} DW)	Y = -0.03*X+7.83	64	0.86
GPOD (U g FW)	Y = 1.46*X+188.83	40	0.86
RGR (mg g^{-1} day^{-1})	Y = 0-0.53*X+32.17	13	0.95

Conclusion

The problem relating to plant tolerance towards HM has a number of theoretical and practical aspects. The accurate determination of the species and genotype tolerance towards particular HM is an important issue, which concerns the phytotechnologies used for the sustainable use of contaminated soils, as well as the ecotoxicology. There is increasing research on the topic, however, due to considerable differences in the experimental designs, the results are not straight forward or objective enough. On the other hand, the main methods used for determining the plant tolerance towards HM are based primarily on biometric measurements, which are labor-intensive and moderately accurate. To increase the reliability of the results it is necessary that the research includes sensitive functional indicators - enzymatic activities, non-destructive photosynthetic parameters, and the different experimental designs need to be combined.

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