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IMPLEMENTATION OF A KINETIC MODEL FOR EVALUATION OF LEAF ION LEAKAGE FROM SUNFLOWER (*HELIANTHUS ANNUUS*) PLANTS SUBJECTED TO HIGH ZINC AND LEAD CONCENTRATIONS

Kocheva K.*[*], *M. Chavdarova, E. Gesheva, S. Doncheva, G. Georgiev

Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev str., Bldg. 21, 1113 Sofia, Bulgaria

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Summary: In the present study, the effect of excess Zn and Pb ions added to the nutrient solution of hydroponically grown sunflower plants was studied in order to evaluate their impact on the properties of leaf tissue by employing an electrolyte leakage kinetics model. Based on the behavior of model parameters, it was demonstrated that treatment with excess Zn had a stronger effect on leaf leakage kinetics than Pb. This could be attributed to the higher mobility of Zn ions which were more readily transported towards the leaves in comparison to Pb. Ion fluxes through different subcellular compartments, namely apoplast (including cell wall) and symplast (comprising the vacuole and cell membranes) were used to explain the changes in ion leakage kinetics as reflecting the functional status of cellular membranes.

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Key words: Electrolyte leakage; Pb; metal toxicity; sunflower; Zn.

INTRODUCTION

Metal toxicity is a common abiotic factor experienced by land plants worldwide. In contrast to organic pollutants, heavy metals cannot be biologically or chemically degraded, and thus may accumulate locally or be transported over long distances (Gonzalez-Mendoza et al., 2009). The effect of toxic concentrations of metals on plant metabolism is of particular importance for growing crop plants in contaminated areas (Verbruggen et al., 2009). Sunflower is a major crop which is also known for its ability to accumulate toxic concentrations of different elements from the substrate (Krystofova et al., 2009). There is an increasing interest in heavy metal detoxification in plants and assessing the fluxes of harmful ions across cell membranes is especially important

[*]Corresponding author: konstvk@abv.bg

(Hall, 2002; Reichman, 2002; Rivelli et al., 2012).

Measurement of the amount of electrolyte leakage from plant tissues is a long-standing method for evaluation of membrane functional status as affected by various types of abiotic stress, growth and developmental processes, and genotypic variation (Prášil and Zámečník, 1998; Bajji et al., 2002; Farooq and Azam, 2006). In contrast to most of the available techniques for estimation of ion leakage which measure conductivity in a single point, Whitlow et al. (1992) proposed repeated quantification of this parameter thus establishing a time course of tissue leakiness. In this regard, the recently developed kinetic approach offered the advantage of accurately distinguishing ion fluxes through different subcellular compartments, namely apoplast and symplast, due to their diverse electrolyte permeability (Kocheva et al., 2005). Lately, an improvement has been proposed by introducing the rates of ion fluxes as promising parameters for evaluation of drought stress impact on plant status (Kocheva et al., 2014). However, the kinetic model has not been implemented for evaluation of the effect of toxic ions on the rates of ion leakage through plant cells. Therefore, the present work was aimed to assess the impact of excess Zn and Pb on membrane properties based on the behavior of kinetic model parameters.

MATERIALS AND METHODS

Cultivated sunflower (*H. annuus* cv. 1114) seeds were superficially sterilized with 4% $NaClO_4$ and germinated for 3 days on wet filter paper in a thermostat in the dark. Plants were grown for 4 weeks in a climatic chamber at 23/17°C day/night temperature, 14 h photoperiod, irradiance of 250 μmol m^{-2} s^{-1} and 70% relative humidity on constantly aerated nutrient solution with minerals concentrations as follows: 200 μM $Ca(NO_3)_2.4H_2O$, 100 μM $MgSO_4$, 400 μM KNO_3, 300 μM NH_4NO_3, 10 μM Fe-EDTA, 5 μM NaH_2PO_4, 8 μM H_3BO_3, 5 μM $MnSO_4.H_2O$, 0.16 μM $CuSO_4.5H_2O$, 0.38 μM $ZnSO_4.7H_2O$, 0.06 μM $(NH_4)_6Mo_7O_{24}.H_2O$ (pH 4.3). Twenty-days-old plants were grown for 6 days on nutrient solution additionally supplied with 0.4 mM $ZnSO_4.7H_2O$ or 0.5 mM $Pb(NO_3)_2$ respectively for the two stress treatments. Controls were grown on basic nutrient solution.

For determination of electrolyte leakage 7 leaf pieces (1 cm^2 in diameter) were cut from different plants separately for each treatment. All tissues were briefly washed with distilled water to remove the solution from injured cells and were then submerged in 20 ml of distilled water for 24 h at constant room temperature (20°C). During this incubation period conductivity of the solutions was measured at multiple time points with a conductometer (Elwro 5721, Poland). For obtaining the total electrolyte content, tissues were killed by boiling for 30 min at 100°C and conductivity of the solutions was read for the last time. Results were expressed as a relative conductivity ratio κ/κ_{max} where κ is conductivity of samples at a particular moment and κ_{max} is total electrolyte content. Thus a multiple-point kinetics curve was obtained. Fitting of experimental data was performed by the Exponential Associate function of Origin 5.0 software:

$$\kappa/\kappa_{max}=C_o(t)\approx A_1(1-e^{-t/t1})+A_2(1-e^{-t/t2})+C_o^o$$

From this kinetics, four main parameters were derived describing ion efflux from the leaves: amplitude A_1 and time constant t_1 of the first (prompt) phase, as well as amplitude A_2 and time constant t_2 of the second (slow) phase. A_1 and A_2 are dimensionless coefficients related to the volumes of apoplast (V_e) and symplast (V_i) and to ion concentration in them. These two parameters reflect the maximal capacity of each compartment for donating ions to the overall efflux. Time constants t_1 and t_2 represent the rate of efflux from the two compartments and are defined as:

$$1/t_1=(1+\alpha)(P_wA_w/V_e)$$

$$1/t_2=(1+\beta)(P_mA_m/V_i)$$

where $\alpha = V_e/V_o$ and $\beta = V_i/V_o$ are the ratios of apoplast and symplast volumes respectively to the volume of the external solution V_o; P_w and P_m are permeabilities of apoplast (wall) and symplast (membrane); A_w and A_m are the corresponding surfaces of walls and membranes (Kocheva et al., 2014). Higher values of the time constants indicate slower leakage rate.

RESULTS AND DISCUSSION

Of the two studied treatments, Zn had a more significant effect on the biometric parameters than Pb as compared to control plants (Table 1). It most strongly affected roots fresh and dry biomass as well as root and shoot length. Pb-treated plants showed a higher reduction in root and shoot length in comparison to untreated plants. They exhibited a slighter reduction in fresh and dry biomass of roots and shoots than the respective parameters in Zn-treated plants as compared to controls.

The kinetic approach derived from multiple time-points recordings of conductivity offered the ability to accurately distinguish ion fluxes from different subcellular compartments thus elucidating diffusion processes between apoplast and symplast (Kocheva et al., 2005). Here, we have applied the elaborated diffusion model for interpretation of leakage kinetics from leaves of sunflower plants subjected to Pb or Zn treatment in order to evaluate the effect of metal toxicity on membrane competence. Best fit of experimental data was accomplished by the exponential associate function (Fig. 1) and estimated values of the four variable parameters are given in Table 2.

It was previously shown that exposure of *Helianthus* plants to excess Pb for longer periods caused an increase in electrolyte leakage from the leaves of treated plants in comparison to controls (Doncheva et al., 2013). Our present results indicated that short-term Pb treatment had impact

Table 1. Effect of Pb and Zn treatment of sunflower plants with respect to length, fresh weight and dry weight of roots and shoots. Data are means ± SD (n=5). Means with the same small letter are not significantly different at P <0.05.

	Length [cm]		Fresh weight [g]		Dry weight [g]	
	Root	Shoot	Root	Shoot	Root	Shoot
Control	28±2.0^a	25.4±1.7^a	2.6±0.2^a	2.9±0.1^a	0.143±0.009^a	0.224±0.009^a
Pb treated	22±0.5^b	17.5±0.6^b	2.3±0.2^a	2.1±0.2^b	0.120±0.005^b	0.172±0.005^b
Zn treated	27±1.2^a	16.0±0.2^c	1.3±0.1^b	1.9±0.1^b	0.069±0.006^c	0.161±0.006^c

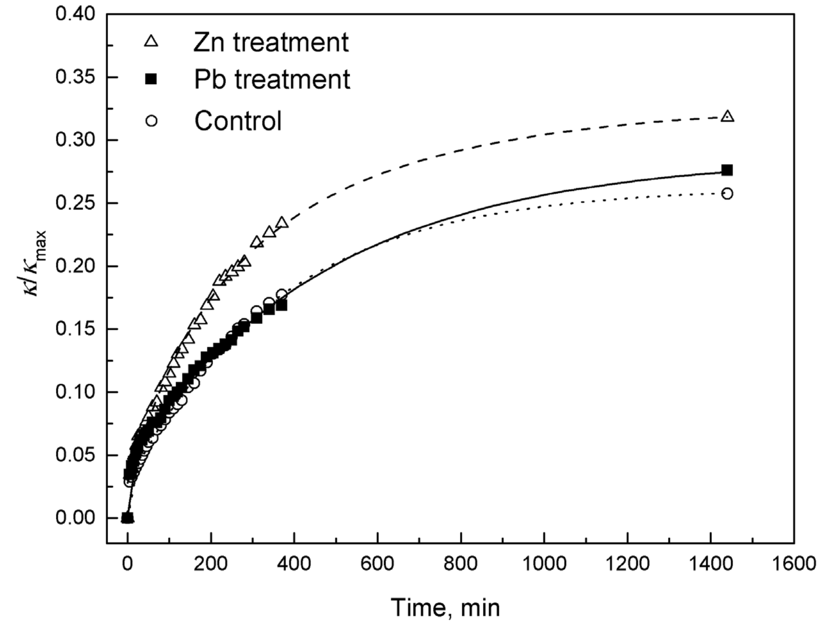

Figure 1. Kinetics of electrolyte leakage from leaves of sunflower plants treated with Zn and Pb. Samples were prepared by incubating 7 leaf pieces in 20 ml distilled water for 24 h while conductivity was measured continuously at multiple time points. Relative conductivity, κ/κ_{max} was estimated by relating conductivity at a particular time to final conductivity after killing the samples.

on the overall leakage kinetics as well (Fig. 1). It caused only a slight increase in net effluxes from apoplast (cell walls) and symplast (membranes) as assessed by higher amplitudes A_1 and A_2 of the two kinetics phases (Table 2). However, the rates of leakage through these compartments were significantly higher in comparison to controls as evidenced by the decreased time constants t_1 and t_2.

On the other hand, Zn treatment had a substantial negative effect on membrane properties and led to more evidently increased electrolyte efflux from the leaves of the stressed plants (Fig. 1). Although amplitude A_1 was only slightly higher than in controls, the two-fold decreased time constant t_1 indicated a faster leakage

Table 2. Main parameters of the diffusion model describing the kinetics of ion leakage from leaves of sunflower plants grown under optimal conditions or treated with excess Pb or Zn. Data are means ± SD. Means with the same small letter are not significantly different at P <0.05.

	Amplitude A_1 of the prompt phase	Time constant t_1 of the prompt phase	Amplitude A_2 of the slow phase	Time constant t_2 of the slow phase
Control	0.0384±0.0019^{b}	8.0±0.4^{a}	0.241±0.013^{b}	615.9±39.9^{a}
Pb treatment	0.0456±0.0027^{a}	5.5±0.2^{b}	0.287±0.019^{a}	482.3±24.4^{b}
Zn treatment	0.0395±0.0022^{b}	4.0±0.1^{c}	0.282±0.024^{a}	310.1±18.6^{c}

through the cell walls of Zn-treated than untreated plants. Regarding the slower second phase, increased amplitude A_2 in combination with the lowered time constant t_2 revealed greater and faster ion efflux through the cell membranes of Zn-treated leaves in comparison to controls.

All four parameters were able to adequately distinguish the two separate treatments (Table 2). The time constants of the two phases were significantly affected by Pb and Zn treatments and showed a prominent decrease in comparison to control values. The smaller time constants indicated a faster leakage of ions from the leaves of Pb and Zn-treated than from untreated plants. Amplitudes of the prompt phase indicated that higher amounts of electrolytes flowed out from the apoplast of Pb than from Zn-treated plants, although at a lower rate (higher t_1 value). Similar amounts of ions were released from the symplast, but the highest leakage rate was found in Zn-treated plants which could reflect greater membrane permeability, hence higher damage of membrane functions was caused by Zn than by Pb treatment.

A possible explanation for the stronger damaging effect of Zn on leaf cell membranes could be sought in the higher mobility of Zn ions towards the leaves in comparison to Pb (Rivelli et al., 2012).

The presented results revealed the capacity of the kinetic model to be readily implemented for differentiation of the impact of toxic ions on both apoplast and symplast. It could be concluded that Zn and Pb treatments had diverse effects on sunflower leaves with respect to the degree of damage on cell walls and membranes. The kinetic approach was able to distinguish between two different stresses based on its model parameters. Moreover, as we have also shown elsewhere, the type of the kinetics was not affected by stress, only the values for the model parameters were found to be changed (Kocheva et al., 2014).

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