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Research article

# Effects of zinc on Scots pine (*Pinus sylvestris* L.) seedlings grown in hydroculture

Yury V. Ivanov*, Alexander V. Kartashov, Alexandra I. Ivanova, Yury V. Savochkin, Vladimir V. Kuznetsov

*Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Moscow, Russia*

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ABSTRACT

The 6-week-old seedlings of Scots pine (*Pinus sylvestris* L.) showed high sensitivity to chronic exposure to zinc in hydroculture, which manifested in a significant inhibition of growth. Changes in the architecture of the root system and the suppression of its growth were shown to be the most striking effects of the toxic effect of zinc. Based on the data relating to the accumulation of zinc predominantly in the root system (by up to 35 times at 300 μM $ZnSO_4$) and to the reduction in its translocation into the aerial organs, we concluded that *P. sylvestris* is related to a group of plants that exclude zinc. The seedlings developed a manganese deficiency (revealed by a reduction in Mn content in the roots and needles of up to 3.5 times at 300 μM $ZnSO_4$) but not an iron deficiency (revealed by an increase in iron content of up to 23.7% in the roots and up to 42.3% in the needles at average). The absence of signs of oxidative stress under the effect of the zinc was detected as evidenced by the reduction in the content of malondialdehyde and 4-hydroxyalkenals in the seedling organs. The leading role of low molecular weight antioxidants in the prevention of oxidative stress in the seedling organs was suggested. Under the influence of zinc, a significant increase in the Trolox Equivalent Antioxidant Capacity of ethanol extracts of the seedling organs was found, which was caused by an increase in the total content of (+)-catechin and proanthocyanidins.



## 1. Introduction

The increase in emissions of heavy metals into the environment, as a result of intensive anthropogenic activities, has created a real threat to the productivity and stability of plant communities (Kahle, 1993; Schutzendubel and Polle, 2002; Pulford and Watson, 2003). Due to the toxic effect of heavy metals, close to non-ferrous smelters the earth turns into an industrial barrens, devoid of vegetation, whose area ranges from a few thousand to hundreds of thousands of hectares (Kozlov and Zvereva, 2007). About 75% of industrial barrens are formed on forested lands, and the largest of these are located in the northern hemispheres of Russia and Canada (Kozlov and Zvereva, 2007; Owojori and Siciliano, 2015). The area of boreal forests damaged in varying degrees by emissions from metallurgical works exceeds dozens of times the area of industrial barrens (Sukhareva and Lukina, 2014). The high level of contamination by metals of soils in these areas significantly hinders the natural regeneration of forests because of the low resistance of the seedlings compared with that of mature trees (Kahle, 1993; Pulford and Watson, 2003). The situation is exacerbated by a low mobility of heavy metals in the soil profile (Kahle, 1993; Nagajyoti et al., 2010), because of which heavily contaminated areas remain barren for several decades even after the cessation of emissions (Kozlov and Zvereva, 2007). The temperature increase currently registered in the northern hemisphere is predicted to lead in the 21st century to a transformation of the hydrological regime of the boreal forest soils (Jungqvist et al., 2014) and to an increase in the bioavailability and toxicity of heavy metals to growing vegetation (Owojori and Siciliano, 2015).

Most species of woody plants are characterized by little adaptive capacity to the high content of heavy metals in soils owing to a long reproductive cycle, preventing the natural selection of metal-

*Abbreviations:* TEAC, Trolox Equivalent Antioxidant Capacity; MDA, malondialdehyde; 4-HAE, 4-hydroxyalkenals; TBARS, thiobarbituric acid reactive substances; PA, proanthocyanidins.

* Corresponding author. Present address: Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, ul. Botanicheskaya, 35, Moscow, 127276, Russia.

*E-mail address:* ivanovinfo@mail.ru (Y.V. Ivanov).

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resistant genotypes (Kahle, 1993; Pulford and Watson, 2003). Exceptions are about 130–140 species of woody plants of the tropical climate, classified as hyperaccumulators of heavy metals (Saladin, 2015). Among the woody vegetation of temperate and subarctic climates, hyperaccumulators of heavy metals do not occur (Pulford and Watson, 2003; Saladin, 2015). The discovery of *Salix* and *Betula* ecotypes resistant to high concentrations of lead in the soil (Kahle, 1993) about 30 years ago has shifted the focus of research to fast-growing species of deciduous woody plants (Pulford and Watson, 2003). Currently, the main objects of study are the genera *Salix*, *Populus*, *Betula*, and their hybrid forms, resistant to soil contaminated with high levels of heavy metals (Unterbrunner et al., 2007; Utmazian et al., 2007; Marmiroli et al., 2011). In this context, coniferous woody plants, which are often used as biomarkers of anthropogenic impact (Kozlov and Zvereva, 2007), remain poorly investigated (Pulford and Watson, 2003; Saladin, 2015). However, on account of the alarming extent of the damage to boreal forests by heavy metals, the study of mechanisms of adaptation of coniferous plants to these heavy metals is of particular relevance.

The aim of the present examination was to investigate the toxic effects of zinc on the growth parameters, balance of essential trace elements, and processes of lipid peroxidation in the organs of Scots pine seedlings (*Pinus sylvestris* L.), as well as to establish the role of low molecular weight antioxidants in maintaining the antioxidant status of seedlings. Scots pine was chosen as the research object because of its important forest-forming role in the composition of the boreal forests of Eurasia and its high sensitivity to zinc (Ivanov et al., 2013a), which is one of the main pollutants of the heavy metals group (Nagajyoti et al., 2010).

## 2. Materials and methods

### 2.1. Experimental design

The seeds of Scots pine (*P. sylvestris* L.), provided by the Training and Experimental Forestry Enterprise of the Bryansk State Technological University of Engineering (Bryansk, Russia), were germinated in a hydroculture of $ZnSO_4$ [concentrations of 1.26 (control), 50, 150 and 300 μM] on individual substrates of sterile cotton wool in polypropylene cartridges for 15 days. At the end of this period, the germination rates of the seeds were determined (Ivanov et al., 2011). After releasing of the seed coat and expanding cotyledons, the seedlings were transferred to a culture medium of the following composition: 2.0 mM $NH_4NO_3$; 1.5 mM $KH_2PO_4$; 1.0 mM $CaCl_2$; 0.5 mM $MgSO_4$; 0.1 mM $Na_2SO_4$; 55 μM $H_3BO_3$; 5 μM $MnSO_4$; 0.32 μM $CuSO_4$; 0.1 μM $Na_2MoO_4$; 0.02 μM $Co(NO_3)_2$; 1.0 μM KI; 9.5 μM $FeSO_4$; 9.5 μM $Na_2$-EDTA; pH = 4.5 (Ivanov et al., 2013a, 2013b) with concentrations of zinc [1.26 (control), 50, 150 and 300 μM]. Cultivation of seedlings was carried out in a climatic chamber, at an air temperature of 23 ± 2 °C during daytime and 18 ± 2 °C at night, with a 16-h photoperiod using fluorescent lamps OSRAM L36W/765 (150 ± 30 μmol $m^{-2}$ $s^{-1}$) for 6 weeks after mass germination of seeds. Replacement of nutrient solution was undertaken twice a week.

### 2.2. Determining the morphometric parameters of the pine seedlings

The biomass of the seedlings was determined using an analytical balance (AB54-S Mettler Toledo, Switzerland) with an accuracy of 0.1 mg after washing the root systems of the seedlings with distilled water and blotting them on a filter paper.

To determine the linear dimensions of the seedlings, they were laid out one by one on the glass of a flatbed scanner Epson Perfection V500 Photo (Epson, Japan) and scanned at a resolution of 800 dpi. MapInfo Professional v. 9.5 software (Pitney Bowes Software, USA) was used to measure the lengths of the seedling organs (primary root, hypocotyl, cotyledons, needles) and the distance from the tip of the main root to the first lateral root to an accuracy of 0.01 mm, and to count the number of first-order lateral roots and needles of the seedlings.

### 2.3. Determining the content of zinc, manganese, and iron ions in the seedling organs

To determine the content of zinc, manganese and iron ions in the organs, the 4–6 seedlings were grouped together, and their roots were washed in a 20 mM aqueous solution of $Na_2$-EDTA for 5 min to remove the ions adsorbed on the surface. Next, the roots were thoroughly rinsed in distilled water and blotted on filter paper. Thereafter, the seedlings were dissected into the root system, hypocotyl, cotyledons and needles, placed in envelopes of ashless paper, and dried at 80 °C for three days until reaching constant weight. After that, the samples were digested in solution of concentrated $HNO_3$ and $HClO_4$ (2:1 v/v) for 24 h at room temperature and then incubated in dry block thermostat (TDB-A-400, BioSan, Latvia) sequentially at 150 °C for 1.5 h, and at 180 °C for 2 h. At the end of the procedure, in order to decompose any residual acids, 37% $H_2O_2$ was added to the cooled samples until clarification and cessation of foaming.

The content of zinc, manganese and iron in the plant organs was determined using an atomic absorption spectrophotometer Formula FM 400 (LABIST, Russia) and applying hollow-cathode lamps (Hamamatsu Photonics, Japan).

The coefficients of biological absorption (the ratio of the element content in the root system of the seedlings to the content in the nutrient solution) and translocation ratios (the ratio of the element content in the needles to the content in the root system) of the elements of the mineral nutrition studied were expressed per fresh weight of the seedlings.

### 2.4. Evaluating the level of lipid peroxidation in the membranes of the seedling organs

To determine the number of secondary products of lipid peroxidation in the membranes that react with thiobarbituric acid (thiobarbituric acid reactive substances (TBARS)), we used the method proposed by Heath and Packer (1968), which was based on the formation of a coloured complex with maximum absorbance at 532 nm, and the modification described by Taulavuori et al. (2001). To construct the calibration curve, 1,1,3,3-tetraethoxypropane (Aldrich, CAS Number 122-31-6) was used.

The content of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HAE) was determined spectrophotometrically, with a maximum optical absorption at 586 nm, by measuring the product that formed during the reaction with a selective reagent 1-methyl-2-phenylindole (Aldrich, CAS Number 3558-24-5), in accordance with the method described by Gerard-Monnier et al. (1998) and modified by Johnston et al. (2007). To construct the calibration curve, 1,1,3,3-tetraethoxypropane was used.

### 2.5. Analysis of low molecular weight antioxidants

Determination of low-molecular antioxidant capacity (Trolox Equivalent Antioxidant Capacity (TEAC)) was performed spectrophotometrically (at 734 nm) according to the method described by Re et al. (1999) by reacting ethanol extracts of the plant material with an ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt] (Sigma, CAS Number 30931-67-0) radical cation decolourization assay. The total phenolics in the seedling