

## Effect of pH and zinc stress on micropore system of rye roots

A. Szatanik-Kloc

Institute of Agrophysics, Polish Academy of Sciences, Doświadczalna 4, 20-290 Lublin, Poland

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**A b s t r a c t.** After zinc stress the total micropore volume decreased remarkably while the average micropore radius increased remarkably for the rye roots. Pore size distribution functions of the roots after the additional zinc application showed the decrease of the small micropore fraction from *ca* 2 to 10 nm and the increase of the large micropore from *ca* 22 to 50 nm. The root surface pores were fractal. After the stress pore fractal dimension increased. The changes of the microporosity observed in the roots surface can be related to the high content of zinc in the cell wall and/or due to the shortage of  $\text{Ca}^{+2}$  the intercellular spaces particularly in the tissues of seminal cortex of the studied roots might have grown.

**K e y w o r d s:** rye roots, micropore, zinc stress, pH, fractal dimension

### INTRODUCTION

Plant roots are highly porous. It has been demonstrated that the porous structure exists in the fibre – vascular bundles of the roots in the secondary xylem which transports water and mineral salts from the roots to the shoot (active transport) and in the phloem which transports products of the photosynthesis (Evert and Eichhorn, 2006). The active transport precedes the passive transport in which water and ions are transported through apoplasts (cell walls and intercellular spaces) of the cells that build the epidermis and the primary cortex of the root. The porous structure of the apoplasts of these tissues has been shown (Carpita, 1982; Carpita *et al.*, 1979; Fleischer *et al.*, 1999). Pores of such dimensions can also be measured by *eg* osmotic permeability (Carpita *et al.*, 1979) or microscopy. Such pores are important for ionic and molecular transport processes (Clarkson, 1991; Canny, 1995).

The adsorption – desorption methods as describing water vapour condensation process occurring in the pores us to estimate various pore parameters as: volume, distribution of sizes, average radii and/or fractal dimensions. Using water vapour desorption data pores from ~1 to a few tens of nano-

meters in radius can be detected and characterized. However, it remains unclear whether the water desorption technique provides results compatible with other methods. The differences may arise, among others, from various water status of the investigated root sample. The geometry of biological materials strongly depends on moisture (swelling, irreversible shrinking after drying *etc.*). Also in the indirect experimental methods *eg* osmotic permeability or water vapour desorption) the results depend strongly on the assumptions applied in calculations. The geometric structure of the surface or the pore system may be highly irregular. The geometrical shape of many natural objects is self-similar at different scales of observation, which can be characterized by the fractal dimension, *D* (Pfeifer and Obert, 1989; Sokołowska, 2009). In this paper, a fractal model of polymolecular adsorption-desorption was used to test how self – similarity of root surfaces was altered by Zn toxicity (detailed in the Appendix). Zinc is absorbed by the plants as the microelement and is used in the activation of many enzymes. Zinc phytotoxicity is observed when the concentration of this metal in the soil solution is high (Hejazi *et al.*, 2011; Włodarczyk *et al.*, 2012). The increase of the mobility of the Zn ions is influenced among others by pH and the proportion of the ions  $\text{Ca}^{+2}/\text{Zn}^{+2}$  in the soil solution. The acid reaction of the surface favors the increase of the Zn concentration especially in the roots of plants and at the same time, it limits the calcium accessibility which may cause the maceration of the root tissue. I was found out that the pore properties and the fractal dimension evaluated using water vapour desorption isotherms markedly change under the toxic influence of  $\text{Al}^{+3}$  ions for tillering and shooting stage plant roots of wheat and roots of seedlings (Józefaciuk and Szatanik-Kloc, 2001; Szatanik-Kloc and Józefaciuk, 2007).

The aim of the study was to show the effect of the phytotoxicity of the zinc concentration and pH on the pore properties of rye roots.

#### MATERIALS AND METHODS

The plants of winter rye variety Rostockie were grown in the nutrient solution prepared according to Hoagland (Starck, 2007), at pH7 with 16/8 h and 23 /18°C (day/night) regime induced with sodium 400 W lamps, in 5 dm<sup>3</sup> vessels containing 40 plants each, in three replicates. The water level was controlled and adjusted every day. The solutions were renewed every 7 days (Szatanik-Kloc *et al.*, 2009). The plants were stressed at pH4.5 during 14 days with different zinc concentrations (20, 200 and 400 mg dm<sup>-3</sup>) at shooting stage. Zinc was added as ZnCl<sub>2</sub>. Plants grown continuously at pH7 without Zn<sup>+2</sup> addition were taken as controls. During the stress the solution pH was measured and adjusted to the value of 4.5 every 24 h. After 14 days the plant roots were harvested, rinsed three times in subsequent 50 ml aliquots of 0.01 mol dm<sup>-3</sup> HCl, five times in distilled water, air dried and collected for further analyses. HCl washing was applied to replace the surface exchangeable ions by protons which was necessary to standardize the conditions of the further experiment. Zinc was not detected by ICP AES in the final HCl washing solution for the sample of roots grown at the extreme zinc concentration that proves to some extent the correctness of the washing procedure. In order to preserve the structure of the tissues the roots were frozen in the liquid nitrogen and then lyophilized (Lyofilizator ALPHA 1-4 LSC firmy CHRIST-Germany).

The microporosity and its changes occurring in the epidermis and cortex were characterized by the water vapour adsorption-desorption method. Water vapour adsorption-desorption isotherms were measured using the vacuum chamber method at a constant temperature kept at 298± 0.1 K. An adsorption isotherm is a function relating the amount of absorbed gas (vapour),  $a$  (mg g<sup>-1</sup>), to its equilibrium pressure,  $p$  (Pa), during the pressure increase at a constant temperature. A desorption isotherm is a function during the pressure decrease. The root samples (*ca* 0.3 g air dry roots) placed in the weighing vessels were closed in a vacuum chamber over the sulfuric acid solutions of stepwise decreasing concentrations (increase of the relative water vapour pressure  $p/p_0$ ); thus the adsorption curve was registered. Next the acid concentration was increased (the decrease of the relative water vapour pressure  $p/p_0$ ) to measure desorption curve. The mass of the root plus the mass of water adsorbed on the root at a given pressure,  $a(p/p_0)$ , was measured by weighing after 48 h of equilibration. After finishing the adsorption-desorption measurements the dry mass of the roots,  $m_r$ , was determined by weighing the samples after 24 h drying at a temperature of 378 K (Szatanik-Kloc and Józefaciuk, 2007). Pore characteristics were calculated using experimental desorption data from 0.35 <  $p/p_0$  < 0.95 range *ie* for pore radii

range of *ca*  $l < r < 50$  nm. Below  $p/p_0 = 0.35$  value the adsorption-desorption process concerns monolayer. It is here assumed that there is no capillary condensation in the pores. The isotherms were measured in three replicates. The variations in replicated data did not exceed 5%. Due to the rather small number of data, no special statistical analysis were performed and only average values of pore parameters and 95% confidence intervals were calculated. The estimation technique is detailed in the Appendix.

#### RESULTS AND DISCUSSION

Desorption isotherms for the studied roots are presented in Fig. 1. The isotherms, for control (pH7) and for pH4.5 (samples without Zn) samples were practically identical for roots of the plants. This indicates that influence of the hydrogen ions (at pH4.5) on adsorption properties was insignificant. Protons alone did not alter the roots physicochemical makeup. The amount of the adsorbed water vapour decreased with the increase of zinc stress concentration. The amount of the desorbed water vapour ( $p/p_0$  range: 0.35-0.95) in the roots stressed at the additional Zn application decreased.

The pore size distribution functions of the studied roots calculated on the basis of these isotherms are presented in the Fig. 2. Most micropores have radii from *ca* 5 and 22 nm ( $\log(r) \sim 0.5$  and  $\sim 1.22$ ) dominate of the studied roots. It is difficult to attribute micropores of given radii to particular structural features of roots (surface tissue) anatomical components. However, some (more speculative at present) comparisons may be drawn: The cell-wall microfibrils are between 3 and 30 nm in diameter so the micropores formed within the microfibrils net have radii of a few nanometers. Also micropores of about 2 nm radius are reported to occur in the (radish and sycamore) apoplast (Carpita, 1982; Clarkson, 1991; Szejnkowska, 2000). The large micropores may correspond to intercellular spaces.

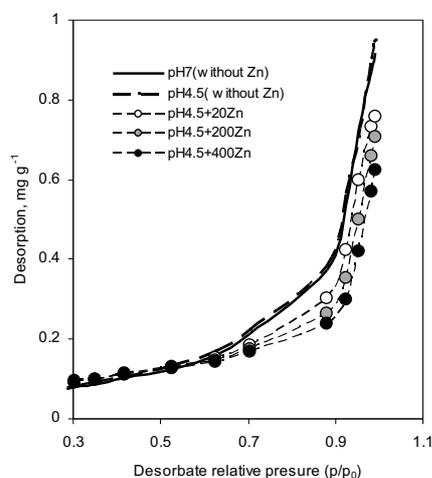
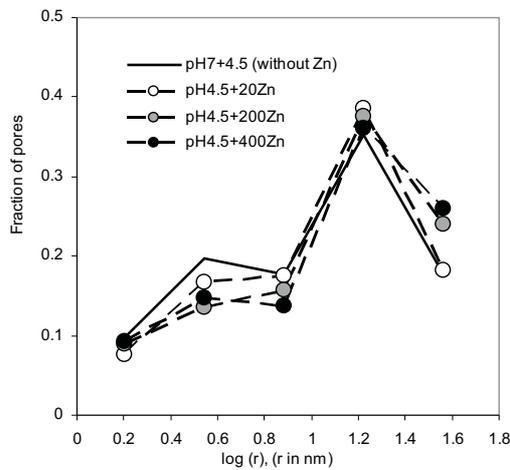


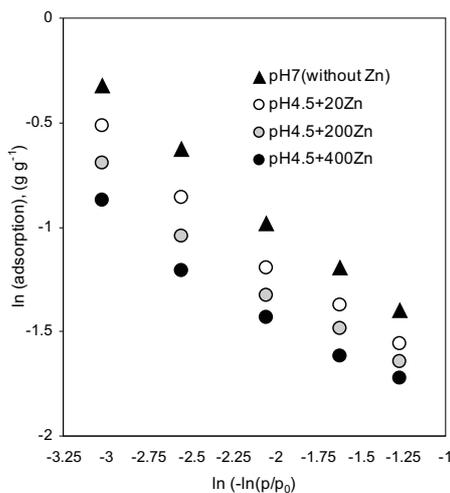
Fig. 1. Water vapour desorption isotherms of the studied rye roots.

The fraction of smaller size micropores and medium size micropores (micropores between *ca* 2-10 nm radii –  $\log(r)$  between 0.2-0.9) decreased after the Zn-stress. The fraction of large micropores (micropores between *ca* 22-50 nm radii –  $\log(r)$  between 1.22-1.56) in the rye roots increased after zinc treatment.

Figure 3 presents the  $\ln$ - $\ln$  plots of adsorption versus adsorption potential for the studied roots. In these plots the linear character can be distinguished in the range *ca*-3< $\ln(A)$ <-1 (which corresponds to equivalent micropore radii of ~3 to ~25 nm) which indicates that the micropores in the rye



**Fig. 2.** Micropore size distribution functions for the studied rye roots. Experimental data are divided into five logarithmically equal ranges: pore fraction whit radii I - <1 - 2.2> nm ( $\log 0.2$ ), II - <2.2 - 4.8> nm ( $\log 0.54$ ), III - <4.8 - 10.5> nm ( $\log 0.88$ ), IV - <10.5 - 22.9> nm ( $\log 1.22$ ), V - <22.9 - 50> nm ( $\log 1.56$ ).



**Fig. 3.** Logarithmic ( $\ln$ - $\ln$ ) plots of desorption (a) versus desorption potential,  $\ln(p/p_0)$ , for the studied rye roots.

roots may be fractal. The fractal dimension was taken as the slope of the straight line within the range  $\ln(A)$  for the coefficient of correlation  $R^2 \geq 0.97$  could be found.

Numerical values of the average micropore radius, total micropore volumes and fractal dimensions of the studied roots are shown in Table 1. As it is seen, the average micropore radius and fractal dimensions increased with the increase of zinc ions concentration. The decrease of total micropore volume is observed after high concentrations of zinc ions.

The statistically significant differences between the control roots and the incubated ones in the additional zinc application (20, 200, 400  $\text{mg dm}^{-3}$  of the solution) were observed for the total volume and the average micropore radius (Table 2). The statistically significant increase of the fractal

**Table 1.** Average radius ( $r_{av}$ ), micropore volume ( $v_{tot}$ ) and fractal dimension ( $D$ ) for the studied rye roots (average values  $\pm 95\%$  confidence intervals)

Parameters	pH7, 4.5*	pH4.5+20Zn	pH4.5+200Zn	pH4.5+400Zn
$r_{av}$ (nm)	12.9 $\pm$ 0.2	15.4 $\pm$ 0.02	17.1 $\pm$ 0.2	17.6 $\pm$ 0.3
$v_{tot}$ ( $\text{mm}^3 \text{g}^{-1}$ )	752.5 $\pm$ 2.2	572.5 $\pm$ 0.4	494.5 $\pm$ 0.2	401.0 $\pm$ 0.8
$D$	2.38 $\pm$ 0.02	2.4 $\pm$ 0.03	2.46 $\pm$ 0.04	2.52 $\pm$ 0.05

\*No statistically significant differences were for control (pH7) and for grown at pH4.5 without  $\text{Zn}^{+2}$ .

**Table 2.** Statistical analysis of obtained results (Student-t-Two tailed, Type 2 (homoscedastic)), variances of the samples not significantly different from themselves – the average not significantly different for  $P > 0.05$

Reaction	pH7, 4.5*	pH4.5+20Zn	pH4.5+200Zn	pH4.5+400Zn
		$r_{av}$		
pH7+4.5	1*	0	0	0
pH4.5+20Zn	0**	1	0	0
pH4.5+200Zn	0	0	1	1
pH4.5+400Zn	0	0	1	1
		$v_{tot}$		
pH7+4.5	1	0	0	0
pH4.5+20Zn	0	1	0	0
pH4.5+200Zn	0	0	1	0
pH4.5+400Zn	0	0	0	1
		$D$		
pH7+4.5	1	1	0	0
pH4.5+20Zn	1	1	0	0
pH4.5+200Zn	0	0	1	0
pH4.5+400Zn	0	0	1	1

\*1 – truth, \*\*0 – false.

dimension was noted for the incubated roots at the zinc concentration of 200 and 400 mg dm<sup>-3</sup> of the solution (Table 2). The increase of the fractal dimensions attests to the geometrical differentiation of the roots porous surface under the used stressor – zinc. Zinc plays an important role in the plant metabolism. It activates many enzymes in the plants such as ascarbionic anhydrase, alcohol dehydrogenase, dehydrogenase of reduced NAD (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate). Zinc influences processes of ribosome formation, cell membranes permeability and controls element proportions on the cell level (Kabata-Pendias and Pendias, 1999). Absence of that element retards tryptophan synthesis which has the direct influence on auxin productions and indirectly leads to limitation of plant growth rate (Starck, 2007). Both the shortage and the excess of the zinc causes the metabolic and developmental disorders. In the roots zinc accumulates itself in the external layers – in the rhizodermic and in the cells of seminal cortex (Heumann, 2002) as well as in cell walls and vacuole (Baranowska-Morek, 2003; Wójcik *et al.*, 2006). Immobilization of metals in the cell wall was proven in the case of Cu<sup>+2</sup>, Zn<sup>+2</sup>, Pb<sup>+2</sup> and Al<sup>+3</sup> ions (Siedlecka *et al.*, 2001). The cell wall can stop absorption of metals by cell from 80 to 95%. Carboxyl groups of cell wall components particularly of pectin's and hemicelluloses containing the galacturonic and glucuronic acids are responsible for this effect.

The binding of zinc ions by surface carboxylic groups presented on the opposite sides of the fine micropore wall (micropore wall –COO-Zn<sup>+</sup>-COO- micropore wall) thus closing the gap. The decrease of the micropore fraction ranging from *ca* 2 to 10 nm radii can be also partly connected with the micropore gaps closing by Zn<sup>+2</sup> ions bonds. Table 3 (Szatanik-Kloc *et al.*, 2010) presents the influence of Zn-stress on the concentration of calcium in roots. High concentrations of zinc ions may cause limited sorption of calcium. The limited adsorption of calcium could indirectly reduce the cementing role of the pectinian of calcium (pectinian of calcium are the components of the middle lamella of the cell wall and are responsible for the cell junction in the root tissue). It could cause the cell wall maceration as well as the

**Table 3.** Calcium and zinc concentration in roots of the rye of the Zn-stressed, (average value from 3 replicated experiments, ± 95% confidence) data from (Szatanik-Kloc *et al.*, 2010)

Variance	Ca <sup>+2</sup> (g kg <sup>-1</sup> )	Zn <sup>+2</sup> (g kg <sup>-1</sup> )
pH7	9.4 ± 0.8	0.05 ± 0.02
pH4.5	9.1 ± 0.6	0.04 ± 0.01
pH4.5+20Zn*	6.8 ± 0.6	0.2 ± 0.006
pH4.5+200Zn*	5.8 ± 0.02	0.5 ± 0.003
pH4.5+400Zn*	5.5 ± 0.02	0.6 ± 0.004

\*Number for the elements symbol wish is Zn<sup>+2</sup> concentration in the nutrient solution.

growth of the intercellular spaces. Therefore, the significant increase of the large micropores fraction (radius > 10 nm) together with the increase of the average radius of the micropores in the incubated roots under the phytotoxic zinc concentration might be connected with the destruction of the root tissue and the increase of the distance between the cells (loosening of the tissue).

Similar of the micropore characteristics for roots grown at pH 7 and pH 4.5, independently of plant age, suggest that the root tissue properties are rather defined for a given plant and these are not sensitive to the influence of protons. So the main physicochemical effect of low pH (no Zn) environments on roots should be the protonation of the negatively charged surface functional groups with the drop in pH (decrease of the cation exchange capacity) leading to malnutrition and starvation of plants.

## CONCLUSIONS

1. The results indicate that the high concentrations zinc ions level markedly affect micropore properties of rye roots.
2. The increase of fractal dimension, average micropore radii and the relative amount of large micropores were noted under the influence of toxicity concentrations of the zinc.
3. The total micropore volume and relative amount of smaller size micropores and medium size micropores decreased under the Zn-stress.
4. Between the roots of control (pH7) and the roots incubated at pH 4.5 (without Zn<sup>+2</sup>) are no significant differences in the micropores characteristics.

## APPENDIX

The adsorption – desorption method is one of the methods of examining the structure of adsorbents. The porosity of adsorbents and its sizes and the volume of capillaries can be determined using the concept of the capillary condensation. In the case of porous adsorbents on the surface of the capillaries the adsorption for small values  $p/p_0$  is similar to the adsorption of non – porous adsorbents. At higher values of  $p/p_0$  the adsorption is multilayer. The heat of the adsorption when creating such layers is close to the heat of condensation. Therefore, in this case the adsorbate has the features similar to the liquid. The condensation of the adsorbent vapour in the capillaries of adsorbent is linked to the dependence between the vapour pressure and the curvature of the area of the liquid. Adsorption layers of the adsorbate covering the capillaries walls of the porous adsorbent have the concave surface. The saturated vapour pressure over such surface is lower than over the flat surface. Hence, we observe the earlier vapour condensation in the capillaries before reaching the level of the pressure of saturated vapour over the flat surface (Kruk and Jaroniec, 2001; Ościk, 1982). The porous structure of natural adsorbents is heterogenic, therefore, its pores do not fill simultaneously. In the process

of adsorption (when increasing  $p/p_0$ ) as the capillaries fill up both the hemispherical and cylindrical menisci are created, while in the process of adsorption only the hemispherical meniscus occurs. It leads to the phenomenon called hysteresis 'loop' of the capillary condensation. In order to characterize the porosity of natural adsorbents we use the desorption path of isotherm (one type of meniscus). The desorption path of isotherm can be used to measure the effective size of capillaries assuming they are cylindrical (Ościągła, 1982).

Relationship between the pressure of water vapor (in adsorption process) and the pore radius  $r$ , is described by the equation of W. Thomson (Kelvin), which after transformation is as follows:

$$RT \ln(p/p_0) = -2V_w \gamma_w \cos \alpha / r(p), \quad (1)$$

where:  $V_w$  ( $\text{m}^3 \text{mol}^{-1}$ ) is the molar volume of adsorbed (water vapour),  $\gamma_w$  ( $\text{J m}^{-2}$ ) is the water surface tension,  $\alpha$  (deg) is water-solid contact angle (assumed usually to be 0 for well wettable surfaces). The pore radii calculated this way should be considered as equivalent radii. It is likely that actual pores are larger because a layer of adsorbed water covers their walls during condensation process. The thickness of this layer should be added to the equivalent pore radius to obtain more realistic value. To avoid assumptions on this thickness, one usually relies on the equivalent pore radii.

The volume of the condensed water in the pores at a given pressure,  $v(p/p_0)$  ( $\text{m}^3$ ), can be treated as a sum of pore volumes,  $v_i(r_i)$ , of the radii  $r_i \leq r(p/p_0)$ ,

$$v(p/p_0) = \sum_{i=1}^n v_i(r_i) \quad (2)$$

Dividing the above equation by the total pore volume,  $v_t$ , the scaled desorption isotherm,  $\Xi(p/p_0) = \Xi[r(p/p_0) \Xi(p/p_0)]$ , can be treated as a sum of fractions of particular pores,  $f(r_i)$ :

$$\Xi(p/p_0) = v(p/p_0) / v_t = \sum_{i=1}^n v_i(r_i) / v_t = \sum_{i=1}^n f(r_i) = 1, \quad (3)$$

and the pore fraction in a given range of pore sizes can be calculated as:

$$f(r_{iav}) = [\Xi(r_{i+1}) - \Xi(r_i)], \quad (4)$$

where:  $r_{iav}$  denotes the arithmetic mean of  $r_{i+1}$  and  $r_i$ . Knowing the latter values, one easily construct a pore size distribution function *ie* pore fraction versus pore radius dependence. Because pore radii cover a broad size range, the pore size distribution functions were expressed on a logarithmic scale. It is important to note that the total pore volume,  $v_t$ , in Eq. (3) was taken as  $v(p/p_0 = 0.95)$  minus  $v(p/p_0 = 0.35)$ .

The average pore radii,  $r_{av}$ , in the measuring range can be calculated as:

$$r_{av} = \sum_{i=1}^n r_i f(r_i). \quad (5)$$

Natural objects display the fractal behavior in a limited range of scales (Neimark, 1990; Pfeifer and Obert, 1889). For many porous systems, the fractal behaviour has been represented as a power law relationship between a perimeter  $P$  (surface area, porosity) and the radius  $r$ :

$$P \propto r^D, \quad (6)$$

where:  $D$  is the fractal dimension and  $r$  here represents the scale of measurements (magnification). The value of  $D$  falls in the range from 2 to 3 and expresses the degree of complexity of the surface and/or the pore structure. The lower limiting value of 2 corresponds to a perfectly smooth surface, whereas the upper limiting value of 3 relates to the maximum allowed surface complexity, approaching three-dimensional space. For example, if the fractal dimension of an object is 2.4, then the digit 2 means that this object is a 'hill-valley' type plane and the number 0.4 informs how 'effectively' this plane fills three-dimensional space. For fractal structures, the log-log plot of Eq. (6) should be linear with the slope proportional to  $D$ .

The single isotherm data can be used for calculation of the fractal dimension. If one assumes the fractal pore-volume distribution, the adsorption equation can be written as:

$$\ln(a) = \text{constant} + (D-3) \ln(A) \quad (7)$$

where:  $a$  is the amount adsorbed and  $A$  is the adsorption potential  $A = RT \ln(p_0/p)$ . Note that  $A$  is proportional to  $r^{-1}$  (Eq. (1)). The fractal dimension can be evaluated from the slope of Eq. (7). This equation is derived for polymolecular adsorption range *ie* for values of  $A$  lower than 0.

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