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1 **Quantification of aluminium induced changes in wheat root architecture by X-ray**
2 **micro-computed tomography**

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8 **Key words:** Root system architecture, Aluminium toxicity, *Triticum aestivum* L., x-ray Micro
9 Computed Tomography

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26 **Abstract**

27 Root architectural traits are of fundamental importance for plant performance especially under
28 unfavourable soil conditions. This study examined the effect of aluminium (Al) toxicity in
29 different growing media (nutrient solutions and soil) on root architecture of two wheat
30 (*Triticum aestivum* L.) cultivars with different Al tolerances.

31 Seedlings were grown in acid and limed soil and in two contrasting nutrient solutions. Root
32 systems of soil grown plants were scanned using x-ray Micro Computed Tomography (μ CT)
33 while that of nutrient solution grown plants were assessed using WinRhizo, 3 and 5 days after
34 planting (DAP), respectively.

35 Al caused significant reduction of all examined root traits (number of seminal roots; root
36 length; length of the longest seminal root; root surface area; and root volume). Growth in acid
37 soil caused significant reduction in root length, length of the longest seminal root and root
38 surface area at 5 DAP. Soil grown plants produced larger root system compared to plants
39 grown in nutrient solutions. Al toxicity induced differences of root traits were also found
40 between different nutrient solutions.

41 Beside the well-known reduction of root length, Al toxicity had a profound effect on other
42 root architectural traits. x-ray μ CT has revealed root architectural changes under specific
43 conditions of acid, Al toxic soil. Differences obtained in Al induced effects on root
44 architecture between different nutrient solutions as well as between different growing systems
45 emphasize the need for further study of root architecture especially under specific conditions
46 of Al toxicity in acid soils.

47

48 **Introduction**

49 Aluminium (Al) is a major limiting factor of crop productivity in acid soils (Kochian
50 1995). Acid (pH <5.5) soils exhibiting Al toxicity comprise up to 30% – 40% of the world's

51 arable land, and it is estimated that over 50% of world's potentially arable land is acidic (von
52 Uexküll and Mutert 1995). Solubilisation of Al oxides and hydroxides is enhanced by low pH,
53 and the predominant form of Al in the acid soils (pH <5.0) is Al³⁺ (Delhaize and Ryan 1995).
54 The most easily recognized symptom of Al³⁺ toxicity is the inhibition of root growth
55 (Delhaize and Ryan 1995). Therefore, measurement of the root growth in solution culture
56 assays has been used for screening Al tolerant genotypes (Samac and Tesfaye 2003).
57 Nevertheless, in only a few cases has Al tolerance observed in solution cultures been
58 correlated with Al tolerance in acid soils (Samac and Tesfaye 2003). Discrepancies in
59 genotype rankings regarding Al tolerance have been attributed to different factors which
60 affect effective Al concentration in nutrient solutions, and in addition can reduce repeatability
61 of the results. Typically researchers used simple nutrient solutions with low ionic strength and
62 wide range of Al concentrations. However, Gregory and Hinsinger (1999) highlighted that
63 research on roots needs to involve complex growth medium such as soil, opposed to
64 commonly used hydroponics, gels and sand culture. Furthermore, most research performed in
65 nutrient solutions has focused on the root apex, which is the most sensitive site of root to Al
66 toxicity, while the whole root architecture has gained less attention.

67 Although Al tolerance in wheat appears to be controlled by a single dominant gene
68 (Delhaize et al. 1993a; Riede and Anderson 1996), many root traits are under polygenic
69 control and expression of these genes is influenced by mutual interactions of roots with the
70 abiotic and biotic soil environment (McCully 1999). The importance of root architecture for
71 plant growth and performance, especially under environmental stress has recently gained
72 more attention (e.g. Lynch 1995; López-Bucio et al. 2003). Non-invasive techniques such as
73 x-ray Micro Computed Tomography (μ CT) provide an opportunity to examine 3-D root
74 architecture (Tracy et al. 2010) non-destructively in the opaque matrix of soil.

75 The aim of this study was to quantify Al induced changes in root architecture of two
76 wheat cultivars that differ in Al tolerance (Al tolerant Sivka and Al sensitive Scout 66) grown
77 in different growing systems (nutrient solutions and soil), and to compare the usefulness of
78 two methods (WinRhizo and x-ray μ CT) for assessing Al induced changes in the root
79 architecture.

80

81 **Materials and Methods**

82 *Plant material and growing conditions*

83 Seeds of Al tolerant wheat cultivar Sivka were obtained from the University of Zagreb,
84 Faculty of Agriculture, Department of Plant Breeding, Genetics, and Biometrics
85 (Svetošimunska cesta 25, 10000 Zagreb, Croatia), and Al sensitive cultivar Scout 66 from the
86 Crop Research Institute, Gene Bank Department (Drnovská 507, 161 06 Praha 6 – Ruzyně,
87 Czech Republic). Seeds were surface sterilized in 2.5% sodium hypochlorite, thoroughly
88 rinsed with distilled water and soaked for 6 h hours in distilled water. All seeds were
89 germinated for 64 h on filter paper soaked with 0.2 mM CaCl_2 at 23/18 °C with a 16/8 h,
90 day/night regime.

91

92 *Soil-based experiment*

93 Soil samples (silty loam, luvisol) were collected from the Ap horizon of an arable field
94 near Gospić, Croatia (44°32'45''N, 15°22'28''E). Soil samples were air dried and sieved to <2
95 mm diameter. Selected physical and chemical characteristics of the soil are shown in Table 1.
96 To get soils with different pH half of the soil samples were limed using 1.0 g CaCO_3 kg^{-1} .
97 Prepared soil samples were moistened to field capacity and incubated for 2 months at room
98 temperature. After the incubation period soil pH was 5.8, and Al saturation was 3.32%.
99 Before planting, soil samples were sieved through <1.0 mm diameter mesh and were placed

100 into 50 mm diameter and 100 mm high plastic columns to achieve a bulk density of 1.0 g cm⁻³. The soil was watered and maintained at a volumetric water content of 15% and kept in
 101 ³. The soil was watered and maintained at a volumetric water content of 15% and kept in
 102 growth chambers during the seed germination period (64 h). Four uniformly developed
 103 seedlings per cultivar were selected for growth (one plant per column). Germinated seeds
 104 were placed in 1 mm diameter, 2 mm deep holes drilled in the soil columns. The seeds were
 105 placed in the hole with the radical downwards before being covered with soil. Plants were
 106 grown in a growth chamber with 16/8 h, 23/18 °C day/night regime and 75% relative
 107 humidity.

108

109 Table 1. Physical and chemical properties of the soil used in the study.

Sand ^a	Silt ^a	Clay ^a	pH ^b	C _{org} ^c	N ^d	P ^f	ECEC ^g	Ca	Mg	K	Na	Al	Al ^h
————%————			H ₂ O	%	mg kg ⁻¹	————cmol(+) kg ⁻¹ ————						sat (%)	
8.0	72.3	19.7	4.6	2.9	0.4	12	4.46	1.4	0.54	0.62	0.05	1.85	41.5

110 ^aSoil particle size distribution was determined by pipette-method with sieving and sedimentation.
 111 ^bpH potentiometrically.
 112 ^cOrganic carbon content (C_{org}) determination after dry combustion.
 113 ^dTotal nitrogen by modified Kjeldahl method.
 114 ^fPhosphorus by ammonium lactate method.
 115 ^gEffective cation exchange capacity (ECEC = Ca + Mg + K + Na + Al) and base saturation level were
 116 determined in barium chloride extracts; Determination of exchangeable acidity in barium chloride extracts.
 117 ^hAl sat – Al saturation = 100 x (exchangeable Al)/(ECEC).
 118

119 *Nutrient solution experiment*

120 The experiment was prepared as a randomized block design with ten replicate plants of
 121 each cultivar per treatment. Plants were grown on an opaque plastic mesh in two different
 122 nutrient solutions which were previously used in experiments related to Al toxicity. The first
 123 nutrient solution (NSR) was used previously by Rengel and Jurkić (1992; 1993) and the
 124 second nutrient solution (NSD) was used by Delhaize et al. (1993a; 1993b). Treatments were
 125 represented as control nutrient solutions, pH 4.0, without aluminium (NSR0 and NSD0,
 126 respectively), and nutrient solutions with aluminium (supplied as AlCl₃), pH 4.0 (NSR1,

127 NSD1, respectively). Ionic activities and Al speciation in nutrient solutions were calculated
 128 by GEOCHEM-EZ (Shaff et al. 2010) and are shown in Table 2. Based on the calculations,
 129 the free activities of Al³⁺ were 0.0 (in NSR0 and NSD0) and 72.0 μM L⁻¹ (in NSR1 and
 130 NSD1). Nutrient solutions were continuously aerated, daily replenished and the pH was
 131 adjusted with 0.1 M HCl. Plants were grown in a growth chamber with 16/8 h, 23/18 °C
 132 day/night regime and 75% relative humidity.

133

134 Table 2. Chemical composition and ion activities of nutrient solutions calculated by
 135 GEOCHEM-EZ.

Nutrient solution	NSR0	NSR1	NSD0	NSD1
pH	4.0	4.0	4.0	4.0
Ionic strength	0.02063	0.02117	0.00283	0.00329
Nutrient	Free concentration in nutrient solution mM L ⁻¹			
NO ₃	10.0	10.0	1.75	1.75
NH ₄	0.5	0.5	0.25	0.25
K	1.99	1.99	0.5	0.5
Ca	3.61	3.63	0.5	0.494
Mg	1.84	1.85	0.124	0.124
SO ₄	1.68	1.63	0.128	0.107
PO ₄	-	-	*	*
Fe	-	-	0.009 E-03	*
B(OH) ₄	-	-	*	*
Mn	-	-	1.97 E-03	1.97 E-03
Zn	-	-	0.344 E-03	0.34 E-03
Cu	-	-	0.195 E-03	0.196 E-03
Cl	0.126	0.565	0.11	0.37
Al	-	0.072	-	0.072
Al complex with SO ₄	-	0.072	-	0.00074
Al complex with OH	-	0.004	-	0.0115

136 *Notes.* * Almost the entire nutrient is in complexes. E-03 concentrations are in μM L⁻¹.

137

138 **Root Imaging**

139 For the x-ray μCT scanning, the columns with live plants were scanned on the third and
 140 fifth day after planting (DAP) using a Phoenix Nanotom[®] (GE Measurement & Control

141 Solutions, Wunstorf, Germany) x-ray μ CT scanner set at 100 kV and 210 μ A, with a 0.2-mm
142 copper filter and voxel resolution was set at 50 μ m. For each column, 1200 image projections
143 were collected over a 30-min period. Image slices were reconstructed into 3D volumes using
144 software Datos|x with beam-hardening reduction algorithms applied and then visualised and
145 analysed in VGStudioMax[®] 2.0 (Volume Graphics GmbH, Heidelberg, Germany). Roots
146 were segmented from the obtained images using the *Region Growing* selection tool following
147 the method of Tracy et al. (2012). Segmented root systems were used for quantitative
148 determination of number of seminal roots, root length, length of the longest seminal root, root
149 surface area and root volume.

150 After the final μ CT scan at 5 DAP, roots were extracted from the soil and carefully
151 washed and scanned using Epson Perfection V700 photo scanner and WinRhizo[®] software
152 (WinRhizo 2009 Reg., Regent Instruments Canada Inc.). Root measurements of the plants
153 grown in nutrient solutions were conducted at 3 and 5 DAP, using Epson Perfection V700
154 photo scanner and WinRhizo software.

155

156 ***Statistical analysis***

157 Data were analysed using the SAS[®] 9.2 statistical package (SAS Institutes, Cary, NC).
158 For the comparison of the scanning techniques (x-ray μ CT versus WinRhizo) results of the
159 root traits (number of seminal roots, root length, the length of the longest seminal root, root
160 surface area and root volume) of soil grown plants that were obtained at 5 DAP were
161 compared using ANOVA, followed by the use of Tukey's honestly significant difference
162 (HSD) test. For comparisons of different nutrient solutions (NSR0, NSR1, NSD0, and NSD1),
163 soil treatments (acid versus limed soil), and growing systems, results of the root traits were
164 analysed using repeated measures (Mixed Model Repeated Measures, Littell et al. 1996).

165

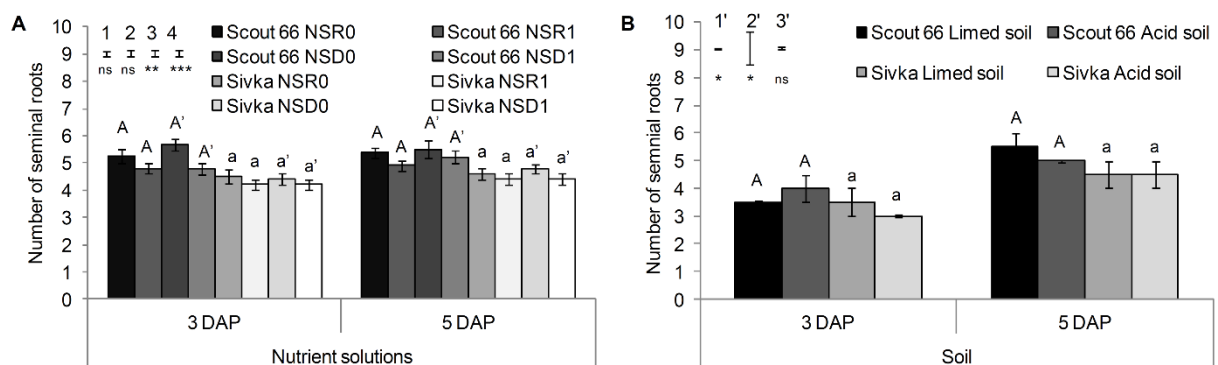
166 **Results**

167 ***Effect of aluminium toxicity and soil acidity on root traits***

168 Root traits of Al tolerant (Sivka) and Al sensitive (Scout 66) wheat cultivars grown in
 169 different nutrient solutions with toxic concentrations of Al and in control solutions (without
 170 Al) and in acid and limed soil are shown in Figure 1,2,3,4, and 5.

171 The number of seminal roots was consistently larger for Scout 66 compared to Sivka
 172 across nutrient solutions ($P < 0.001$) and soil treatments ($P < 0.05$). In both nutrient solutions
 173 (NSD and NSR) and at both measurement times (3 and 5 DAP) Al treatments reduced ($P <$
 174 0.01) the number of seminal roots (from 5.01 in Al treatment solutions to 4.61 in control
 175 solutions) (Figure 1A). In soil the number of seminal roots increased over time, from 3.5 (3
 176 DAP) to 4.88 (5 DAP) ($P < 0.05$) (Figure 1B).

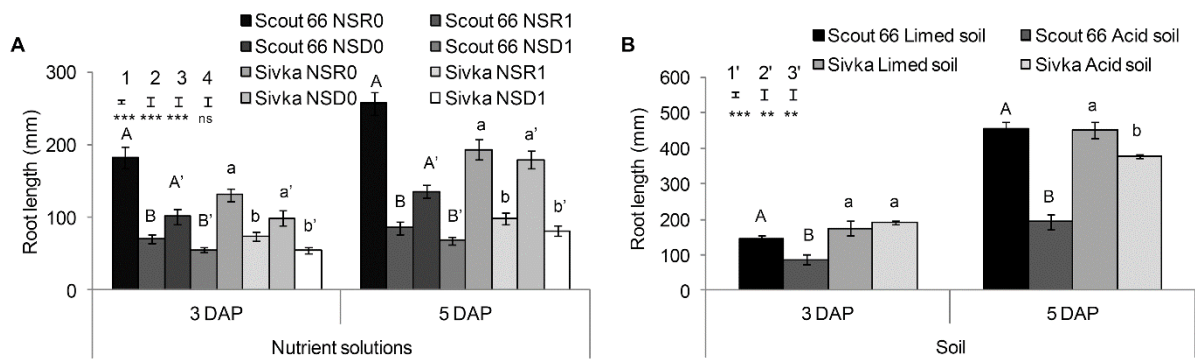
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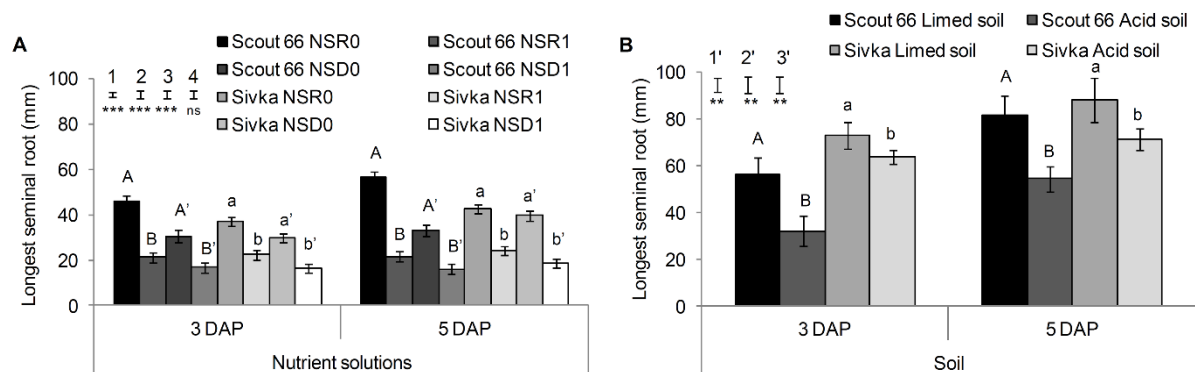
179 Figure 1. Comparison of the number of seminal roots of wheat cultivars Scout 66 and Sivka grown in Al
 180 treatment solutions (NSR1 and NSD1) and control nutrient solutions (NSR0 and NSD0) (A) and in acid and
 181 limed soil (B). For plants grown in soil roots were scanned by x-ray μ CT and measured by (VGStudioMax), and
 182 for plants grown in nutrient solutions roots were scanned and measured by WinRhizo, at 3 DAP and 5 DAP,
 183 respectively. Error bars associated with the histograms are ± 1 standard error of the mean. The vertical bars
 184 represent standard error of the difference (SED) for (1) day, (2) nutrient solution, (3) nutrient solution treatment,
 185 (4) cultivars; (1') day, (2') soil treatment, (3') cultivars. Below the vertical bars (SED) ANOVA for the main
 186 effects is presented as: *Significant at the 0.05 probability level; **Significant at the 0.01 probability level;
 187 ***Significant at the 0.001 probability level; and ns = not significant. For figure A: means with the same letter
 188 are not significantly different between nutrient solution treatments within each nutrient solution type at each
 189 measurement time; for Scout 66 (capital in NSD and capital with apostrophe in NSR, respectively) and for Sivka
 190 (small in NSD and small with apostrophe in NSR, respectively). For figure B: means with the same letter are not
 191 significantly different between soil treatments at each measurement time for Scout 66 (capital) and for Sivka
 192 (small).

193



195

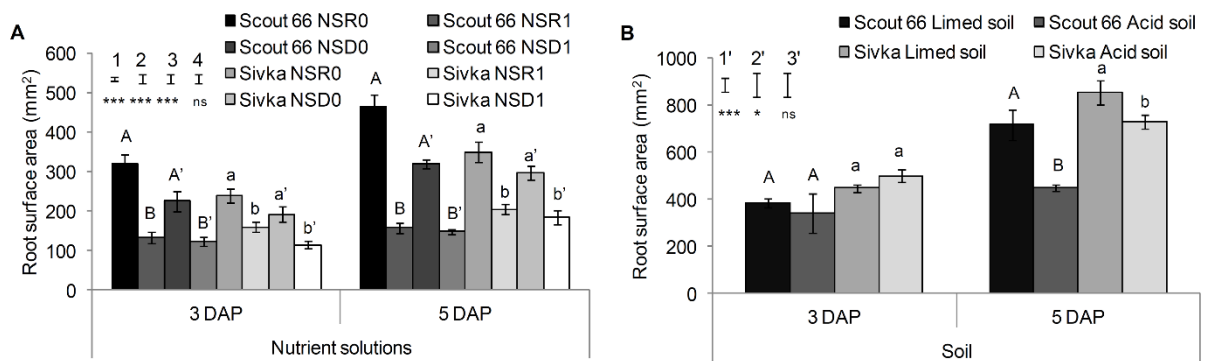
196 Figure 2. Comparison of mean root length of wheat cultivars Scout 66 and Sivka grown in Al treatment solutions
 197 (NSR1 and NSD1) and control nutrient solutions (NSR0 and NSD0) (A) and in acid and limed soil (B). For
 198 plants grown in soil roots were scanned by x-ray μ CT and measured by (VGStudioMax), and for plants grown in
 199 nutrient solutions roots were scanned and measured by WinRhizo, at 3 DAP and 5 DAP, respectively. Error bars
 200 associated with the histograms are ± 1 standard error of the mean. The vertical bars represent standard error of the
 201 difference (SED) for (1) day, (2) nutrient solution, (3) nutrient solution treatment, (4) cultivars; (1') day, (2')
 202 soil treatment, (3') cultivars. Below the vertical bars (SED) ANOVA for the main effects is presented as:
 203 *Significant at the 0.05 probability level; **Significant at the 0.01 probability level; ***Significant at the 0.001
 204 probability level; and ns = not significant. For figure A: means with the same letter are not significantly different
 205 between nutrient solution treatments within each nutrient solution type at each measurement time; for Scout 66
 206 (capital in NSD and capital with apostrophe in NSR, respectively) and for Sivka (small in NSD and small with
 207 apostrophe in NSR, respectively). For figure B: means with the same letter are not significantly different
 208 between soil treatments at each measurement time for Scout 66 (capital) and for Sivka (small).
 209



210

211 Figure 3. Comparison of length of the longest seminal root of wheat cultivars Scout 66 and Sivka grown in Al
 212 treatment solutions (NSR1 and NSD1) and control nutrient solutions (NSR0 and NSD0) (A) and in acid and
 213 limed soil (B). For plants grown in soil roots were scanned by x-ray μ CT and measured by (VGStudioMax), and
 214 for plants grown in nutrient solutions roots were scanned and measured by WinRhizo, at 3 DAP and 5 DAP,
 215 respectively. Error bars associated with the histograms are ± 1 standard error of the mean. The vertical bars
 216 represent standard error of the difference (SED) for (1) day, (2) nutrient solution, (3) nutrient solution treatment,
 217 (4) cultivars; (1') day, (2') soil treatment, (3') cultivars. Below the vertical bars (SED) ANOVA for the main
 218 effects is presented as: *Significant at the 0.05 probability level; **Significant at the 0.01 probability level;
 219 ***Significant at the 0.001 probability level; and ns = not significant. For figure A: means with the same letter
 220 are not significantly different between nutrient solution treatments within each nutrient solution type at each
 221 measurement time; for Scout 66 (capital in NSD and capital with apostrophe in NSR, respectively) and for Sivka
 222 (small in NSD and small with apostrophe in NSR, respectively). For figure B: means with the same letter are not
 223 significantly different between soil treatments at each measurement time for Scout 66 (capital) and for Sivka
 224 (small).
 225

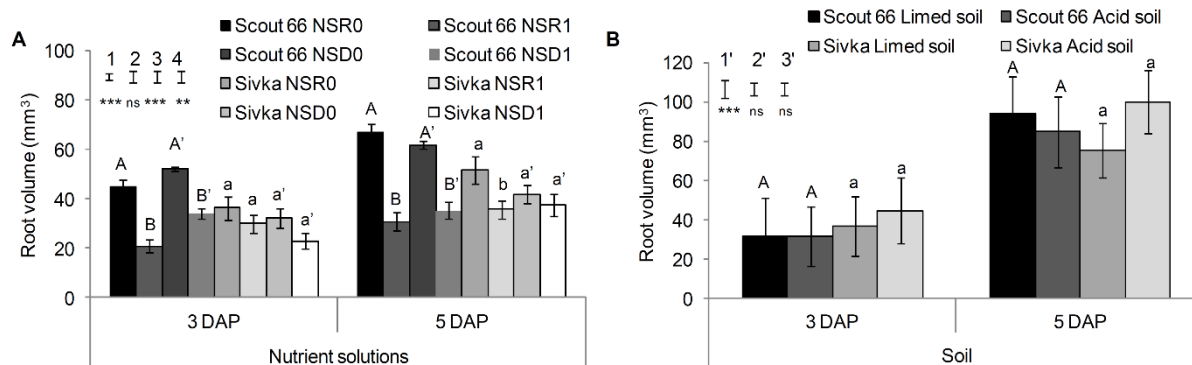
226 In nutrient solutions Al treatments reduced ($P < 0.001$) root length of both cultivars (Scout 66
 227 and Sivka), grown in both nutrient solutions (NSD and NSR) and at both measurement times
 228 (3 DAP and 5 DAP). In addition, for all cultivar \times nutrient solution \times treatment combinations,
 229 root length increased with time ($P < 0.05$) except for Scout 66 grown in NSR1 ($P > 0.05$)
 230 (70.64 mm and 85.65 mm, 3 and 5 DAP, respectively) and in NSD1 ($P > 0.05$) (55.6 mm and
 231 68.47 mm, 3 DAP and 5 DAP, respectively) (Figure 2A). Root length of plants grown in soil
 232 was affected by cultivar \times treatment \times measurement time interaction ($P < 0.05$). Reduction of
 233 root length of Scout 66 grown in acid soil was evident at both measurement time (i.e. 87.5
 234 mm vs. 146.23 mm at 3 DAP, $P < 0.05$ and 454.37, mm vs. 194.7 mm 5 DAP, $P < 0.001$, in
 235 acid vs. limed soil, respectively). A significant reduction in root length of Sivka grown in acid
 236 soil was recorded at 5 DAP (376.86 mm in acid vs. 453.24 mm in limed soil, $P < 0.05$)
 237 (Figure 2B).



238
 239 Figure 4. Comparison of root surface area of wheat cultivars Scout 66 and Sivka grown in Al treatment solutions
 240 (NSR1 and NSD1) and control nutrient solutions (NSR0 and NSD0) (A) and in acid and limed soil (B). For
 241 plants grown in soil roots were scanned by x-ray μ CT and measured by (VGStudioMax), and for plants grown in
 242 nutrient solutions roots were scanned and measured by WinRhizo, at 3 DAP and 5 DAP, respectively. Error bars
 243 associated with the histograms are ± 1 standard error of the mean. The vertical bars represent standard error of the
 244 difference (SED) for (1) day, (2) nutrient solution, (3) nutrient solution treatment, (4) cultivars; (1') day, (2') soil
 245 treatment, (3') cultivars. Below the vertical bars (SED) ANOVA for the main effects is presented as:
 246 *Significant at the 0.05 probability level; **Significant at the 0.01 probability level; ***Significant at the 0.001
 247 probability level; and ns = not significant. For figure A: means with the same letter are not significantly different
 248 between nutrient solution treatments within each nutrient solution type at each measurement time; for Scout 66
 249 (capital in NSD and capital with apostrophe in NSR, respectively) and for Sivka (small in NSD and small with
 250 apostrophe in NSR, respectively). For figure B: means with the same letter are not significantly different between
 251 soil treatments at each measurement time for Scout 66 (capital) and for Sivka (small).

252

253



254

255 Figure 5. Comparison of root volume of wheat cultivars Scout 66 and Sivka grown in Al treatment solutions
 256 (NSR1 and NSD1) and control nutrient solutions (NSR0 and NSD0) (A) and in acid and limed soil (B). For
 257 plants grown in soil roots were scanned by x-ray μ CT and measured by (VGStudioMax), and for plants grown in
 258 nutrient solutions roots were scanned and measured by WinRhizo, at 3 DAP and 5 DAP, respectively. Error bars
 259 associated with the histograms are ± 1 standard error of the mean. The vertical bars represent standard error of the
 260 difference (SED) for (1) day, (2) nutrient solution, (3) nutrient solution treatment, (4) cultivars; (1') day, (2') soil
 261 treatment, (3') cultivars. Below the vertical bars (SED) ANOVA for the main effects is presented as:
 262 *Significant at the 0.05 probability level; **Significant at the 0.01 probability level; ***Significant at the 0.001
 263 probability level; and ns = not significant. For figure A: means with the same letter are not significantly different
 264 between nutrient solution treatments within each nutrient solution type at each measurement time; for Scout 66
 265 (capital in NSD and capital with apostrophe in NSR, respectively) and for Sivka (small in NSD and small with
 266 apostrophe in NSR, respectively). For figure B: means with the same letter are not significantly different
 267 between soil treatments at each measurement time for Scout 66 (capital) and for Sivka (small).
 268

269 In nutrient solutions Al treatments reduced ($P < 0.001$) length of the longest seminal root
 270 of both cultivars (Scout 66 and Sivka), grown in both nutrient solutions (NSD and NSR) and
 271 at both measurement times (3 DAP and 5 DAP). In addition, there was a significant
 272 interaction of cultivar \times nutrient solution \times treatment ($P < 0.01$). No significant difference in
 273 length of the longest seminal root was obtained for Sivka grown in NSR0 ($P > 0.05$) (39.77
 274 mm) and NSD0 (34.68 mm), while cultivar Scout 66 produced longer seminal root in NSR0
 275 ($P < 0.001$) (51.25 mm) compared to NSD0 (31.78 mm). The opposite was obtained in Al
 276 treatment solutions where no significant difference ($P > 0.05$) was found between NSR1
 277 (21.40 mm) and NSD1 (16.37 mm) grown Scout 66, while significantly ($P < 0.05$) longer
 278 seminal roots were obtained for NSR1 (23.24 mm) compared to NSD1 (17.55 mm) grown
 279 Sivka (Figure 3A). In soil, length of the longest seminal root was affected by measurement
 280 time ($P < 0.01$), by cultivar ($P < 0.01$) with average length of 55.97 mm for Scout 66

281 compared to 74.08 mm for Sivka, and by treatment ($P < 0.01$) with average length 55.35 mm
282 in acid soil compared to 74.71 mm in limed soil (Figure 3B).

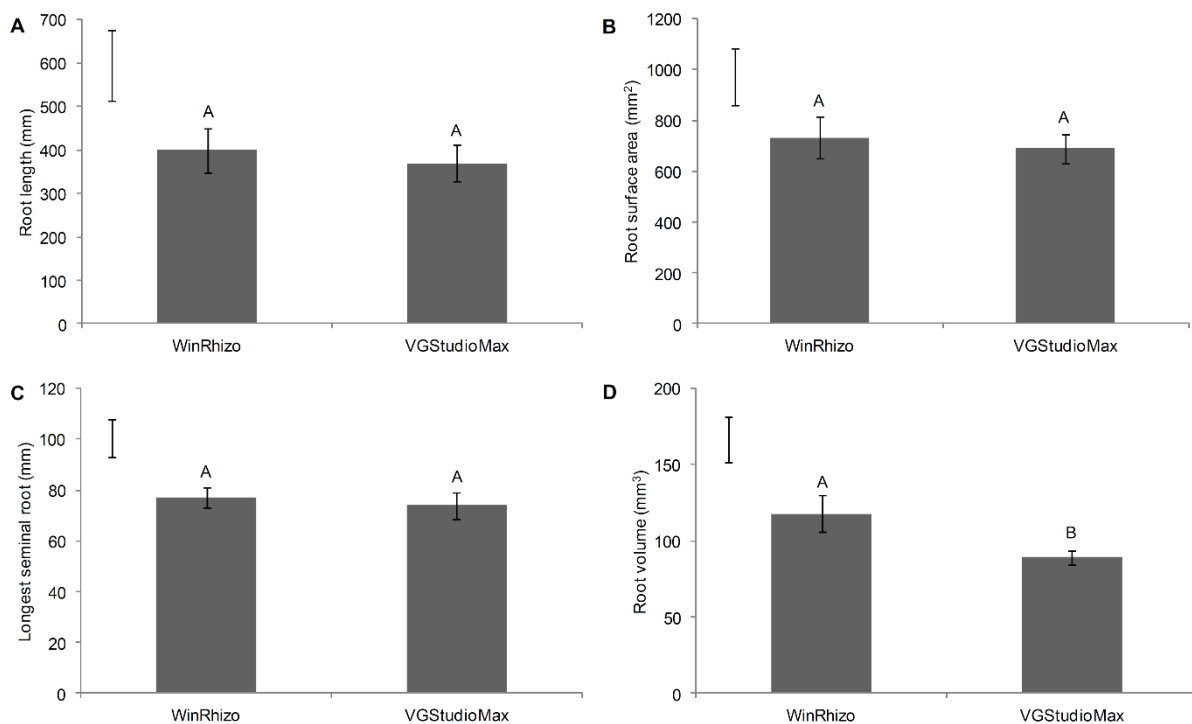
283 In nutrient solutions, root surface area was affected by nutrient solution \times treatment ($P <$
284 0.01) and cultivar \times treatment ($P < 0.001$) interaction. Al treatments reduced root surface area
285 in both nutrient solutions, as well as for both cultivars. However, this reduction was more
286 pronounced in NSR (344.51 mm² in NSR0 vs. 165.35 mm² in NSR1) compared to NSD
287 (258.65 mm² in NSD0 vs. 142.57 mm² in NSD1) and for cultivar Scout 66 (332.99 mm² in
288 control solutions vs. 142.1 mm² in Al-treatment solutions) compared to Sivka (270.17 mm²
289 and in control solutions vs. 165.82 mm² in Al-treatment solutions) (Figure 4A). When grown
290 in soil, the largest mean root surface area was obtained for Sivka (634.41 mm²) compared to
291 Scout 66 (475.14 mm²) ($P < 0.05$), and the interaction of treatment \times measurement time was
292 significant ($P < 0.05$). No significant differences ($P > 0.05$) in root surface area were found
293 between plants grown in acid (422.1 mm²) and limed (417.27 mm²) soil at 3 DAP, while at 5
294 DAP plants grown in limed soil produced root systems with bigger ($P < 0.01$) surface area
295 (788.59 mm²) compared to those grown in acid soil (591.16 mm²) (Figure 4B)

296 In nutrient solutions, root volume was affected by interactions of cultivar \times treatment ($P <$
297 0.001) and cultivar \times nutrient solution \times measurement time ($P < 0.05$). Al treatments reduced
298 root volume of cv. Scout 66 at both measurements and in both nutrient solutions. On the other
299 hand, significant reduction of root volume of cv. Sivka was found only at 5 DAP in NSR ($P <$
300 0.01) (35.9 mm³ in NSR1 compared to 51.6 mm³ in NSR0) (Figure 5A). In soil, the
301 interaction of cultivar \times treatment ($P < 0.05$) for root volume was significant. Root volume of
302 cultivar Sivka was greater ($P < 0.05$) in acid (74.22 mm³) compared to limed soil (56.11
303 mm³), while no significant differences ($P > 0.05$) were found for Scout 66 grown in acid
304 (58.14 mm³) and limed soil (62.63 mm³) (Figure 5B).

305

306 **Comparison of the scanning techniques: X-ray μ CT versus WinRhizo**

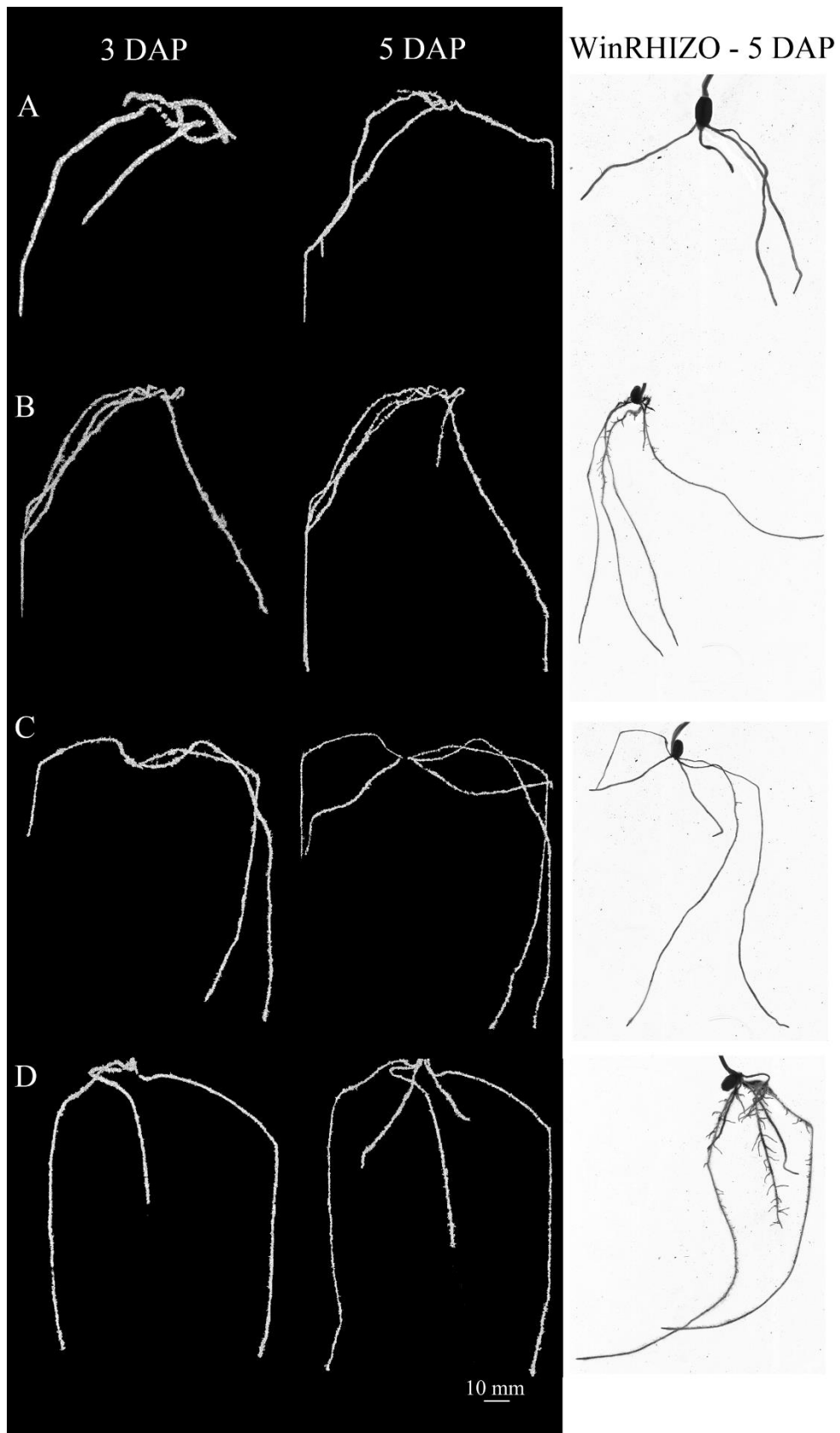
307 A comparison of the root traits (root length, length of the longest seminal root, root
308 surface area, and root volume) measured by VGStudioMax after x-ray μ CT scanning and by
309 WinRhizo (after washing soil from roots) at 5 DAP are shown in Figure 6. Although all
310 measured root traits were slightly larger when measured by WinRhizo compared to
311 VGStudioMax, there were no significant difference in root length ($P > 0.05$), root surface area
312 ($P > 0.05$), and the length of the longest seminal root ($P > 0.05$) when these two techniques
313 were compared. However, a significantly larger ($P < 0.05$) root volume was obtained by
314 WinRhizo (117.96 mm^3) compared to VGStudioMax (89.44 mm^3) which can be attributed to
315 the former capturing more of the finer roots (Figure 7).



316

317 Figure 6. Comparison of the root traits of wheat cultivars Scout 66 and Sivka obtained by different scanning
318 techniques, μ CT (VGStudioMax) and WinRhizo at 5 DAP: mean root length (A), root surface area (B), length of
319 the longest seminal root (C), and root volume (D). Error bars associated with the histograms are ± 1 standard
320 error of the mean. The vertical bars represent minimum significant difference (Tukey's HSD test, $p=0.05$) for
321 comparing the mean values between scanning techniques; means with the same letter are not significantly
322 different.

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327 Figure 7. Root system images of wheat cultivars Scout 66 (A and C) and Sivka (B and D) at 3 DAP and 5 DAP,
 328 grown in acid (A and B) and in limed soil (C and D), scanned by μ CT (left) and WinRhizo (right).
 329

330 **Discussion**

331 Although all measured root traits were larger when measured by WinRhizo compared to
332 VGStudioMax, comparison of the results of root traits obtained by these two scanning
333 techniques showed that they did not differ significantly, except for root volume. Relatively
334 poor correlation between root volumes measured destructively by WinRhizo and non-
335 destructively, after x-ray μ CT scan, was already described by Tracy et al. (2012). Tracy et al.
336 (2012) have attributed these discrepancies to the better contrast between roots and their
337 surroundings which can be obtained using WinRhizo and on the other hand to the image
338 resolution limitation which were gained by x-ray μ CT. This could also be the truth for our
339 results (Figure 7). Additional technical disadvantage of the x-ray μ CT scanning technique is
340 the limited soil volume that can be used for growing plants which disables this technique to
341 study older plants with more complex root architecture. Namely, all roots of the plants used in
342 this study reached bottom and/or side walls of the columns by the 5 DAP (Figure 7).
343 However, results of this study showed that x-ray μ CT scanning technique provide reliable and
344 good quality 3-D scans of roots in the soil, and despite its current limitations, new
345 developments of this technique, such as automated root segmentation, and bigger, faster and
346 more precise x-ray CT scanners with greater resolution would give the opportunity to study
347 older more complex root systems (for the review see Mooney et al. 2012).

348 Aluminum toxicity reduced all examined root traits in the experiment with nutrient
349 solutions while in soil based experiments it caused reduction of root length, length of the
350 longest seminal root and root surface area. Al induced reduction of root size is most likely the
351 primary cause of commonly described symptoms of Al toxicity, such as impairment of
352 nutrient and water acquisition. Al toxicity, both in acid soil and in Al treatment nutrient
353 solutions, caused a more pronounced reduction of all examined root traits for Al sensitive cv.
354 Scout 66 compared to Al tolerant cv. Sivka (Figure 1, 2, 3, 4, and 5). Differences in root traits

355 determined between cv. Scout 66 and cv. Sivka are in accordance to their tolerance to
356 aluminium. It is well known that there is significant genetic variability in Al tolerance among
357 wheat cultivars and cv. Scout 66 was used as a model of an Al sensitive cultivar in previous
358 studies related to Al toxicity (e.g. Rengel and Jurkić 1992; Ryan et al. 1992), on the other
359 hand cv. Sivka was evaluated as moderately tolerant cultivar in a screening for Al tolerance
360 among Yugoslavian wheat cultivars (Rengel and Jurkić 1992).

361 The first and most easily recognized symptom of Al toxicity is the inhibition of root
362 growth (Delhaize and Ryan 1995). Barceló and Poschenrieder (2002) stated that sensitive
363 plants exhibit statistically significant inhibition of root elongation after approximately 30 min
364 to 2 h exposure. Our results show that Al toxicity caused slower reduction of root growth in
365 acid soil compared to those that were obtained in experiments with nutrient solutions. For
366 example, reduction of root length and root surface area for plants grown in Al treatment
367 solutions was evident at 3 DAP while reduction of root length for acid soil grown cv. Sivka
368 and reduction of root surface area for both acid soil grown cultivars was evident only at 5
369 DAP. These delayed response to Al toxicity observed for acid soil grown plants could be
370 explained as a lag phase. Barceló and Poschenrieder (2002) described the lag phase as the
371 time or concentration required for Al to interfere with key processes in root growth. It was
372 estimated (Delhaize et al. 1993a) that significant Al inhibition of root growth in wheat occurs
373 at root tip Al concentrations around 1000 $\mu\text{g Al g}^{-1}$. Therefore, these results indicate that acid
374 soil grown plants, especially cv. Sivka, can tolerate a longer period of exposure to toxic Al
375 concentrations.

376 Although there are some reports about the Al induced inhibition of lateral roots in
377 sensitive genotypes of rice (*Oryza sativa* L.) (Famoso et al. 2011), soybean (*Glycine max* L.).
378 (Vilagarcia et al. 2001; Silva et al. 2001) maize (*Zea mays* L.) (Clark et al. 2013), there is lack
379 of data about the effect of Al toxicity on other root traits, especially under real acid soil

380 conditions. Villagarcia et al. (2001) developed a sand based screening technique which
381 simulated growth in acid soil. In their experiments, they made comparison between
382 hydroponic and sand based experiments by measurements of different root traits of soybean.
383 These authors reported Al toxicity (eighteen days of exposure to $450 \mu\text{M Al L}^{-1}$) in sand
384 based experiments did not greatly affect the tap root length, while it caused significant
385 reduction of root surface area (by 58%) compared to control, probably due to reduction in
386 length of basal roots and branches. In our experiments Al toxicity induced reduction of early
387 stage root volume for both cultivars grown in Al treatment nutrient solutions (Figure 5A),
388 while soil acidity did not affect root volume of cv. Scout 66 and that of cv. Sivka was greater
389 when grown in acid compared to limed soil (Figure 5B). Aluminium-injured roots are often
390 described as stubby and brittle, with thickened lateral roots (Foy et al. 1978). Possible
391 explanations of equal root volume (limed and acid soil grown cv. Scout 66) or increased root
392 volume in acid soil grown cv. Sivka could be the Al induced increase in viscous and elastic
393 extensibility of cell wall of the root apices (Ma et al. 2004) or Al induced reduction of cell
394 length accompanied by radial cell expansion which was found on Al treated rice roots
395 (Alvarez et al. 2012).

396 Plants grown in acid soil produced larger root system (root length, length of the longest
397 seminal root, root surface area and root volume) compared to plants grown in Al treatment
398 solutions. These results could be explained by higher activities of toxic Al in Al treatment
399 solutions (Table 2), as well as possible mitigating effect of soil compounds like plant nutrients
400 and organic matter on Al toxicity. Despite the high Al saturation percentage of soil used in
401 this experiment (Table 1), Delhaize and Ryan (1995) found that exchangeable Al in soil is a
402 poor indicator of Al toxicity. In sand based experiments, Villagarcia et al. (2001) reported that
403 an approximate 100-fold increase in Al concentration was required to inhibit root growth to a
404 comparable degree to hydroponic based experiments. However, high concentrations of toxic

405 Al are not the only reason for decreased root size in nutrient solutions. This statement is
406 supported by the fact that acid soil grown plants produced a larger root system compared to
407 plants grown in the control nutrient solutions. Reduced root growth of plants grown in
408 nutrient solutions could be explained by stress caused by transfer of young seedlings to
409 hydroponics (Tamas et al. 2006). Another possible explanation could be the more efficient
410 detoxification of Al in soil due to slower diffusion rates of organic acids (malate) away from
411 root surface and Al toward root surface. Kinraide et al. (2005) proposed biphasic diffusion
412 hypothesis of Al detoxification, which suggests that majority of Al detoxification occurs just
413 beneath the root epidermis. Our observed increase in root volume in acid soil grown plants
414 possibly caused by radial expansion of epidermal and cortex cells may represent the evidence
415 for such detoxification.

416 Despite equal concentrations of free Al in both NSD1 and NSR1 solutions (Table 2), Al
417 toxicity caused more pronounced reduction of root growth in NSD compared to NSR.
418 Possible explanation may lay in the different concentration of nutrients in these two nutrient
419 solutions, especially those of calcium and magnesium, and differences in ionic strength of the
420 solutions (Table 2). With the increasing ionic strength of the nutrient solution increases the
421 competition between Al^{3+} and other cations for negatively charged sites within the root cell
422 wall and plasma membrane. Due to complex chemistry of Al and its multiple interactions with
423 different nutrients in solution, in previous studies of Al toxicity researchers used simple
424 nutrient solutions with low ionic strength and wide range of Al concentrations (from 5 to 200
425 $\mu\text{M L}^{-1}$) (Wang et al. 2006), often avoiding usage of different plant nutrients, such as sulphur
426 and phosphorus (Samac and Tesfaye 2003). However, it has been well documented that
427 different concentrations nutrients such as nitrate, phosphate, sulphate and iron can lead to
428 alterations in root growth and architecture (for review see López-Bucio et al. 2003).

429 Results of this study indicate that beside the well-known reduction of root length Al
430 toxicity also has a profound effect on other root traits, e.g. in nutrient solutions Al toxicity
431 reduced the number of seminal roots, the length of the longest seminal root, the root surface
432 area, root volume. In addition, differences obtained in Al induced effects on root architecture
433 between different nutrient solutions (NSD and NSR) and even more profound differences
434 found between two growing systems (soil and nutrient solutions) emphasize the need for
435 further investigation of wheat root architecture under specific conditions of Al toxicity. In
436 previous experiments Al toxicity was studied under simplified conditions. X-ray μ CT
437 provides the opportunity to non-destructively study 3-D root system development in their
438 natural environment of soil. With the further development of this technique, it will be possible
439 to examine larger number of samples and to monitor root development over a more prolonged
440 period across the growth cycle of a plant and to include different environmental factors or
441 plant microbial interactions that could have significant effect on Al toxicity. For example, it
442 would be useful to investigate Al induced root architecture changes across specific soil pH
443 ranges (pH 4.0 – 6.0) in which Al toxicity occurs in arable soils. Furthermore, considering
444 that in many arable soils Al toxicity occurs in acid subsoil layer, further research should focus
445 on larger number of genotypes and on root architectures of mature more established plants.

446

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