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*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1644579> since 2017-07-20T10:40:26Z

*Published version:*

DOI:10.1007/s10681-017-1931-3

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(Article begins on next page)

**This is the author's final version of the contribution published as:**

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Euphytica, 213: 148, 2017, 1-15

DOI 10.1007/s10681-017-1931-3

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3 **Adaptation to iron deficiency and high pH in evergreen azaleas (*Rhododendron* spp.):**  
4 **potential resources for breeding**

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14 **Acknowledgments** The research was financially supported by the Italian ‘Ministero dell’Istruzione,  
15 dell’Università e della Ricerca’ (Project PRIN 2009, prot. 2009BW3KL4\_003). The mobility of SD  
16 was funded by ForESTFlowers Project (FP7-PEOPLE-2010-IRSES-269204). The authors wish to  
17 thank Kazuki Koiwai, Tomoki Takaku and Walter Gaino for providing technical assistance.

18

19 **Abstract**

20 The growth of evergreen azaleas (*Rhododendron* spp.) can be altered by iron (Fe)  
21 chlorosis when plants are cultivated in a neutral-alkaline substrate. In this study,  
22 morphological and physiological responses to alkalinity and Fe deficiency were evaluated in  
23 five diploid Japanese azaleas to assess their potential as resources for breeding. *R. obtusum*  
24 ‘Kirin’, *R. indicum* ‘Shinsen’, *R. × pulchrum* ‘Sen-e-oomurasaki’, *R. indicum* ‘Osakazuki’, and  
25 *R. ripense* were pot cultivated in a peat-based substrate for 10 weeks, in acid and alkaline  
26 growing media with both adequate and inadequate Fe nutrition. Plant performance was  
27 generally affected by high pH of the substrate, while Fe deficiency by itself influenced few of  
28 the evaluated parameters, possibly due to the complex adaptive response mechanisms of these  
29 slow growing ornamental shrubs. According to the biochemical and physiological variations  
30 recorded on a long period of cultivation, *R. indicum* ‘Osakazuki’ reported the best  
31 performance. This azalea could be a valuable resource for breeders.

32

33 **Key words:** abiotic stress / alkalinity / chlorosis / growing medium / pot cultivation

34

35 **Abbreviations:**

- 36 GI, growth index  
37 IO, *R. indicum* 'Osakazuki'  
38 KR, *R. obtusum* 'Kirin'  
39 RI, *R. ripense*  
40 SH, *R. indicum* 'Shinsen'  
41 SO, *R. × pulchrum* 'Sen-e-oomurasaki'

42 **Introduction**

43 In alkaline soils, the growth of many crops can be altered or compromised by macro and  
44 micro nutrients deficiencies. The pH is, in fact, a crucial feature amid substrate characteristics,  
45 since it affects solubility and therefore availability of mineral elements (Miller et al. 1984;  
46 Marschner 1995). High concentrations of calcium carbonate (CaCO<sub>3</sub>) and hydrogen carbonate  
47 (HCO<sub>3</sub><sup>-</sup>) in alkaline soils have proved to play a relevant role in limiting iron (Fe) and other  
48 micronutrients availability, such as manganese (Mn), zinc (Zn) and copper (Cu) (Loeppert et  
49 al. 1994). The Fe unavailability is mainly a problem of solubility and not of abundance, as Fe  
50 availability decreases dramatically with increasing pH due to its hydrolysatation,  
51 polymerization and precipitation with inorganic ions (Hell and Stephan 2003). Fe is involved  
52 in numerous biological redox systems and is necessary in respiration, chlorophyll biosynthesis  
53 and photosynthesis (Marschner 1995; Kim and Guerinot 2007; Kobayashi and Nishizawa  
54 2012). Therefore, a Fe deficiency can impair plant health causing the distinctive interveinal  
55 chlorosis in the youngest leaves, together with a decrease in root development and stunted  
56 growth (Marschner 1995; Schmidt 1999; Lucena 2000; Römheld 2000), and compromise the  
57 production of many crops (Tagliavini and Rombolà 2001; Hansen et al. 2006), including  
58 ornamentals (Albano and Miller 1998; Fisher et al. 2003; Smith et al. 2004a; Smith et al.  
59 2004b; Valdez-Aguilar and Reed 2006; Valdez-Aguilar and Reed 2007). However, within  
60 species, genotypes that grow well in soils with a pH outside of their native range have been  
61 recorded (Pestana et al. 2001; Symonds et al. 2001; Kaufman and Smouse 2001; Alcántara et  
62 al. 2012; Martínez-Cuenca et al. 2013; Stanton and Mickelbart 2014; Wulandari et al. 2014).

63 Evergreen azaleas (family Ericaceae, genus *Rhododendron*, subgenus *Tsutsusi*) suffer  
64 from Fe deficiency when cultivated outside their *optimum* pH range (4.5-6.0) (Kofranek and  
65 Lunt 1975; Wallace and Wallace 1986; Galle 1987; Chaanin and Preil 1994; Preil and  
66 Ebbinghaus 1994; Giel and Bojarczuk 2002; Giel and Bojarczuk 2011). However, the high  
67 genetic variability of wild evergreen azaleas generated a wide range of differences not only in

68 flower morphology and canopy characteristics (Heursel 1975; Galle 1987), but also in their  
69 adaptability to alkaline pH (Scariot and Kobayashi 2008).

70 The employment of genotypes highly tolerant to Fe deficiency is considered a good  
71 approach to overcome Fe unavailability, with the additional aim of avoiding the application of  
72 synthetic chelates during cultivation (Tagliavini and Rombolà 2001; Abadía et al. 2011; Jelali  
73 et al. 2011). In order to study this nutritional issue, Fe deficiency can be induced either  
74 directly, by removing Fe, or indirectly, by raising substrate pH (Alcántara et al. 2012). This  
75 approach has been successfully adopted in herbaceous crops and fruit trees (Alcántara et al.  
76 2012). In *Rhododendron*, Preil and Ebbinghaus (1994) selected *R.* 'Cunningham's White' as  
77 alkaline-tolerant cultivar. Moreover, *R. yakushimanum* cultivars grafted on alkaline tolerant  
78 rootstocks have been developed, currently known as Inkarho<sup>®</sup> rhododendrons (Nelson 1999).  
79 These cultivars have proved to be resistant to Fe chlorosis caused by elevated pH of the  
80 cultivation medium, up to 7.5 (Pfarr et al. 2015). Preliminary and explorative screenings of  
81 Japanese evergreen azaleas were performed in hydroponics (Scariot et al. 2013; Demasi et al.  
82 2015a). In these studies, two of the most appreciated cultivars showed opposite responses to  
83 high pH on the base of foliar chlorosis and mortality: *R. obtusum* 'Kirin' (KR) deeply suffered  
84 the alkalinity of the growing medium (pH 9), showing elevated foliar damages and high  
85 mortality rate (80%), while *R. × pulchrum* 'Sen-e-oomurasaki' (SO) showed low damages and  
86 mortality (20%). The influence of high pH on these two genotypes was also investigated  
87 through the evaluation of their root Ferric Chelate Reductase (FCR) activity (Demasi et al.  
88 2015b), a rapid screening for Fe deficiency tolerant genotypes before visual symptoms occur  
89 (Jolley et al. 1996). At pH 9, the FCR activity remained stable in KR, while in SO increased  
90 1.5-fold, indicating SO as a plant tolerant to Fe deficiency (Marschner et al. 1986). However,  
91 substantial scientific data on the variation for pH adaptability in the wide group of evergreen  
92 azaleas in realistic conditions are still missing. In this study, agronomical and physiological  
93 performances of five Japanese genotypes (*R. indicum* 'Osakazuki', IO; *R. obtusum* 'Kirin', KR;  
94 *R. ripense*, RI; *R. indicum* 'Shinsen', SH and *R. × pulchrum* 'Sen-e-oomurasaki', SO) were  
95 evaluated for the first time in pot cultivation.

96 Plants were tested in a 10 weeks variety trial, imposing two Fe nutrition regimes, both  
97 in acid and alkaline conditions. The surveys covered growth parameters (canopy diameter,  
98 plant height and leaf number), biomass production and leaf chlorosis, mineral elements  
99 concentration ( $\text{Ca}^{2+}$ ,  $\text{K}^{+}$  and  $\text{Fe}^{2+}$ ), abscisic acid (ABA) and chlorophyll content. Moreover,  
100 with the aim to evaluate the possibility of interspecific hybridization, the genome size and  
101 ploidy level of each genotype were assessed by using flow cytometric ploidy analysis.

## 102 **Materials and methods**

### 103 *Plant material*

104 Five evergreen azalea genotypes (*R. indicum* 'Osakazuki', IO; *R. obtusum* 'Kirin', KR; *R.*  
105 *ripense*, RI; *R. indicum* 'Shinsen', SH and *R. × pulchrum* 'Sen-e-oomurasaki', SO) were  
106 selected for their ornamental importance, widespread use in urban green areas (Greer 1984)  
107 and different response to Fe deficiency, as assessed previously (Scariot and Kobayashi 2008;  
108 Demasi et al. 2015a; Demasi et al. 2015b).

109

### 110 *Flow cytometry*

111 Genome sizes were measured using a Partec Cyflow Space (Partec, Münster, Germany)  
112 with a green solid state laser (100 mW, 532 nm). Sample preparation was performed  
113 according to Van Laere et al. (2009). Propidium iodide staining was performed using the PI  
114 Cystain kit (Partec). For every azalea sample, *Glycine max* L. 'Polanka' was used as an  
115 internal reference with a genome size of 2.50 pg  $2C^{-1}$  (Doležel and Greilhuber 2010).  
116 Genome sizes were calculated from the peak position ratios. Influence of plant cytosolic  
117 compounds on fluorochrome accessibility to nuclear DNA was tested as described by  
118 Greilhuber et al. (2007). The peak position of the internal reference standard was located on  
119 identical fluorescence channels in all measurements, either with or without sample. Genome  
120 sizes for each genotype were similar when measured on different flow cytometers and with  
121 different references. Therefore, average genome sizes with a low standard deviation could be  
122 calculated. Also Cx values were calculated using 1 pg = 978 Mbp, according to Doležel et al.  
123 (2003). The terminology on genome size was used as defined by Greilhuber et al. (2005).  
124 Obtained data were analysed using Flomax software.

125

### 126 *Cultivation conditions*

127 Three years old plants were cut-propagated and cuttings were cultivated in a peat-perlite  
128 substrate for one year before the beginning of the trial. In order to evaluate the effects of Fe  
129 deficiency both in acid and alkaline conditions, plants were subjected to four treatments  
130 (Table 1) according to a completely randomized design for 10 weeks (February-April 2013)  
131 in a glass greenhouse of the Department of Agricultural, Forest and Food Sciences of the  
132 University of Torino (Italy, 45°03'58.5"N; 7°35'29.1"E). The acid substrate of treatments (1)  
133 and (2) was composed by a mixture (1:1, by volume) of sphagnum peat (Silver Torf,

134 Agrochimica, Bolzano, Italy) and coconut fibre (Tref Ego Substrate BV, Moderdijk, The  
 135 Netherlands) (Berruti and Scariot 2012). This substrate was supplemented with CaCO<sub>3</sub> to  
 136 raise the pH and obtain the alkaline substrate used for treatments (3) and (4). Fe was added as  
 137 iron sulphate (FeSO<sub>4</sub>) at the beginning of the trial in treatments (2) and (4), while (1) and (3)  
 138 did not received any Fe fertilisation. During the trial the pH gradually decreased in all  
 139 treatments.

140

141 **Table 1** Calcium carbonate (CaCO<sub>3</sub>) and iron (Fe) concentration of the four substrates and the pH  
 142 course along the trial

Treatment	CaCO <sub>3</sub>	Fe	pH		
			Week 0	Week 5	Week 10
(1) Acid-Fe	-	-	4.8	4.2	3.9
(2) Acid+Fe	-	5 mg L <sup>-1</sup>	4.7	4.7	4.3
(3) Alkaline-Fe	0.1 mol dm <sup>-3</sup>	-	8.5	8.2	7.9
(4) Alkaline+Fe	0.1 mol dm <sup>-3</sup>	5 mg L <sup>-1</sup>	8.8	8.0	8.0

143

144 Substrates were allowed to equilibrate for seven days in a 0.6 L plastic pot before planting.  
 145 One pot containing one plant constituted a replication and 45 replicates per genotype were  
 146 subjected to the same treatment. Plants were fertilized weekly with 50 mL of a modified  
 147 Hoagland solution (Clark et al. 2003) consisted in: 1.79 mM L<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.79 mM L<sup>-1</sup>  
 148 Ca(NO<sub>3</sub>)<sub>2</sub>, 7 mM L<sup>-1</sup> CaCl<sub>2</sub>, 1 mM L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 1.66 mM L<sup>-1</sup> KCl, 0.42 mM L<sup>-1</sup> K<sub>2</sub>SO<sub>4</sub>, 2 mM  
 149 L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O. Micronutrients were supplied as a combination of Zn-EDTA (0.83 μM L<sup>-1</sup>  
 150 <sup>1</sup>), Cu-EDTA (0.33 μM L<sup>-1</sup>), Mn-EDTA (8.56 μM L<sup>-1</sup>), Na<sub>2</sub>B<sub>8</sub>O<sub>13</sub>·4H<sub>2</sub>O (10.71 μM L<sup>-1</sup>) and  
 151 Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O (0.08 μM L<sup>-1</sup>). Fertilisation resulted in a total amount of nutrients provided to  
 152 each plant as follows: 50 mg N, 15.5 mg P, 50 mg K, 50 mg Ca, 24 mg Mg, 60.5 mg S, 52 mg  
 153 Cl, 0.25 mg B, 0.01 mg Cu, 0.25 mg Mn, 0.005 mg Mo, and 0.025 mg Zn. Irrigation was  
 154 provided with deionized water (pH=4.8, EC=8 μS cm<sup>-1</sup>; soluble salts=6 mg L<sup>-1</sup>) at the bottom  
 155 of the pots when needed, in order to prevent the percolation of mineral elements. Plants were  
 156 kept in a glass greenhouse at an average temperature of 24°C during the day and 18°C during  
 157 the night, with a light/dark photoperiod of 16/8 h, under an additional Photosynthetically  
 158 Active Radiation (PAR) of 350 μmol m<sup>-2</sup> s<sup>-1</sup> at the canopy level, provided by high pressure  
 159 sodium lamps.

160

161 *Plant growth parameters*

162 The number of leaves and morphological characteristics were recorded weekly on six  
163 plants per treatment, measuring the number of leaves, the plant height and the canopy widths,  
164 which allowed to calculate the Growth Index (GI;  $\Pi * \{[(D'+D'')/2]\}^2 * H$ , where D' is the  
165 widest width, D'' is the perpendicular width and H is the height) according to Hidalgo and  
166 Harkess (2002). Plant height was measured from the substrate surface to the highest point of  
167 the canopy; canopy width was measured across the widest side, then the plant was turned 90°,  
168 and a second canopy width measurement was taken. The above ground part (leaves and  
169 stems) of three plants per treatment was collected at the beginning and at the end of the  
170 experiment (week 10) and oven-dried (70°C until no further weight loss was observed) to  
171 determine the dry biomass production.

172

### 173 *Foliar chlorosis and chlorophyll content*

174 Foliar chlorosis was recorded weekly on six plants per treatment through the evaluation  
175 of the total number of chlorotic leaves per plant. Mature, recently-expanded leaves were  
176 considered chlorotic when green veins were detectable among yellowing sections, as shown  
177 in Fig. 1. Besides, the total chlorophyll content was determined on new expanded leaves in  
178 three plants per treatment at the end of cultivation. According to the protocol described by  
179 Lichtenthaler (1987), 0.05 g of leaf tissue was ground and total chlorophylls were extracted  
180 with pure methanol ( $\geq 99\%$ ) under dark condition, at 4°C for 2 h. Afterwards, the absorbance  
181 of the extracting solution was determined at 665 and 652 nm by a spectrophotometer  
182 (Ultrospec 2100 Pro, GE Healthcare, USA).

183

### 184 *Iron, calcium and potassium determination*

185 Concentration of ferrous iron ( $\text{Fe}^{2+}$ ), calcium ( $\text{Ca}^{2+}$ ) and potassium ( $\text{K}^+$ ) in the leaves of  
186 the five azalea genotypes cultivated in acid and alkaline treatments supplemented with Fe was  
187 measured at the beginning and at the end of the experiment (week 10). New expanded leaves  
188 were harvested from three plants per treatment and oven-dried at 50°C for four days. After  
189 grinding, 4 mL of sulphuric acid ( $\text{H}_2\text{SO}_4$ ) and 4 mL of hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 30%) were  
190 added to 0.5 g of each sample and heated-digested in a Kjeldahl flask. Samples were later  
191 diluted to 100 mL with deionized water.  $\text{Ca}^{2+}$  and  $\text{K}^+$  content was determined through atomic  
192 absorption spectrophotometry (AA-7000, Shimadzu Corporation, Japan), while  $\text{Fe}^{2+}$  content  
193 was spectrophotometrically measured (UV-1700 PharmaSpec, Shimadzu Corporation, Japan)



194 after the application of the o-phenanthroline method (Saywell and Cunningham 1937; Gupta  
195 1968).

196

#### 197 *Abscisic acid determination*

198 The concentration of endogenous ABA in the leaves was quantified in three plants per  
199 treatment at the beginning of the experiment and at week 4, 6, 8 and 10. The plant hormone  
200 was quantified by High Performance Liquid Chromatography (HPLC), based on Solid Phase  
201 Extraction (SPE) purification method (modified from Bosco et al. 2013). New opened leaves  
202 were sampled and maintained at -80°C until analysis. Leaves were ground in liquid nitrogen  
203 and 0.5 g of each sample was suspended in 4 ml of the extraction solution (65% pure  
204 methanol, 25% ultrapure water, 10% aqueous hydrogen chloride 1 M) for 2 h at 4°C, in  
205 darkness condition and under magnetic stirring. Sample was then filtered and the eluate was  
206 added to a SPE cartridge (Supelclean SPE LC-NH<sub>2</sub>, Supelco Analytical, USA), previously  
207 washed with pure methanol (99%) and ultrapure water. ABA was eluted with 5% of  
208 phosphoric acid (H<sub>3</sub>PO<sub>4</sub>) in methanol. The procedure was carried out under artificial light  
209 with amber glassware, in order to prevent analyte degradation. The chromatographic analysis  
210 of the eluate was performed with HPLC 1200 Series (Agilent Technologies, Böblingen,  
211 Germany). The signal for ABA was monitored at 265 nm and hormone concentration was  
212 quantified according to a calibration curve, constructed from the matrix-matched calibration  
213 standards.

214

#### 215 *Statistical analysis*

216 Data were compared over time by repeated measures in General Linear Model (GLM),  
217 considering pH and Fe nutrition as separate factors. The Greenhouse-Geisser adjustment was  
218 used when the covariance matrix of data did not meet the assumption of sphericity. Data were  
219 then subjected to the homogeneity of variances and examined by one- (Fig. 2, 3, 4) or three-  
220 way ANOVA (Table 4). The arcsine transformation was performed on leaf chlorosis  
221 percentage before statistical analysis. Correlation between GI and foliar chlorosis was tested  
222 using Pearson correlation coefficients. All presented values are means of untransformed data  
223 using three replicates for biomass, total chlorophyll, ABA, and mineral elements and six  
224 replicates for growth index, leaf number and foliar chlorosis. Statistical analysis were  
225 performed with SPSS Statistics Software 21.0 (SPSS, Chicago, USA).

226

227 **Results**

228 *Genome size measurements*

229 Genome sizes and estimated ploidy level of each tested evergreen azaleas are  
 230 summarized in Table 2. The genome sizes varied from 0.94 pg/2C for SH to 1.07 pg/2C for  
 231 KR. The estimated ploidy of all the studied samples is diploid ( $2n = 2x = 26$ ).

232

233 **Table 2** Main plant characteristics, relative genome size measurements (pg) and estimated ploidy  
 234 level, determined by flow cytometry, for the five tested azaleas (*R. indicum* 'Osakazuki', IO; *R.*  
 235 *obtusum* 'Kirin', KR; *R. ripense*, RI; *R. indicum* 'Shinsen', SH; *R. × pulchrum* 'Sen-e-oomurasaki', SO).

Genotype	Plant characteristics	Relative 2C (pg) <sup>1</sup>	Estimated ploidy (x)
IO	Bushy growth; deep pink flowers with darker blotch	1.02±0.01	2
KR	Medium to tall dense shrub; strong pink, small hose in hose flowers	1.07±0.01	2
RI	Medium shrub; light purple large flowers	1.03±0.01	2
SH	Late blooming shrub; white flowers with deep pink and red speckles and stripes, many variations	0.94±0.04	2
SO	Double flower sports of <i>R. × pulchrum</i> 'Oomurasaki'	0.98±0.03	2

236 <sup>1</sup>Values represent mean 2C holoploid genome size ± standard error for three repetition

237

238 *Plant growth*

239 Plant growth was assessed by calculating the GI and evaluating the number of leaves  
 240 and biomass production. The repeated measures (Table 3) comparing GI over 10 weeks of  
 241 cultivation indicated significant overall effect for the pH of the substrate and the genotype.  
 242 Effects were also highlighted for the interaction of “pH × Fe × Genotype”.

243 The GI trend of the five azaleas tested in the trial is represented in Fig. 2. Plants grew to  
 244 a different extent from the beginning to the end of cultivation, with values that varied widely  
 245 from 1500 to 9500 cm<sup>3</sup>. On average, RI reported the highest increase along the trial (+4144  
 246 cm<sup>3</sup>), followed by KR (+2914 cm<sup>3</sup>), SO (+2830 cm<sup>3</sup>), SH (+1833 cm<sup>3</sup>) and IO (+1703 cm<sup>3</sup>).  
 247 Significant effects of the treatments started to appear in the first half of the trial in SO plants  
 248 (week 1 to 5), and in the second half (week 5 to 10) in KR, RI and SH. KR and RI plants had  
 249 higher GI under Acid+Fe treatment, while SH under Acid-Fe. Conversely, this treatment  
 250 constrained the growth of SO plants until week 5. No statistical differences were recorded in  
 251 IO between treatments. The three-way ANOVA performed on GI variation (GI week 10 - GI  
 252 week 0, Table 4) indicates that KR and RI plants generally grew significantly more than IO

253 and SH, while SO showed an intermediate growth along the trial. The characteristics of the  
 254 substrate partially influenced the development of the genotypes tested, since the alkaline pH  
 255 reduced plant growth by half, while Fe nutrition by itself did not cause effect.

256 The number of leaves increased in all genotypes along the experiment and was affected  
 257 by the treatments, except in RI (Fig. 2, boxes). Generally, in IO and SH, the leaf number was  
 258 limited by alkalinity (+/-Fe), while adding Fe alleviated symptoms in KR and to a lesser  
 259 extent in SO. Surprisingly, SH plants showed the highest number of leaves in Acid-Fe  
 260 substrate.

261

262 **Table 3** Level of significance of the repeated measures for the Growth Index (GI; cm<sup>3</sup>) and foliar  
 263 chlorosis (Chlorosis; %), comparing data over 10 weeks of cultivation

	GI	Chlorosis
Week	***§	***
Week × pH	***	***
Week × Fe	ns	***
Week × Genotype	***	***
Week × pH × Fe	ns	ns
Week × pH × Genotype	ns	***
Week × Fe × Genotype	ns	ns
Week × pH × Fe × Genotype	***	ns

264 § ns, \*\*\* indicates non-significant, or significant at  $P \leq 0.001$ , respectively

265

266

267 Biomass production of the above ground part (leaves and stems) increased during the  
 268 10-weeks experiment in all genotypes, regardless the treatment, with higher values in RI  
 269 plants. Alkaline pH of the substrate and inadequate Fe nutrition had a negative effect (Table  
 270 4).

271 **Table 4** Differences among genotypes and effect of pH and Fe nutrition on growth index variation (GI week 10-GI week 0; cm<sup>3</sup>), variation of biomass of the  
 272 above ground part (biomass week 10-biomass week 0; g of dry weight), total chlorophyll content (µg mg<sup>-1</sup>), foliar chlorosis (%), and ferrous iron (Fe<sup>2+</sup>),  
 273 calcium (Ca<sup>2+</sup>) and potassium (K<sup>+</sup>) content in the leaves of the five azaleas tested (*R. indicum* 'Osakazuki', IO; *R. obtusum* 'Kirin', KR; *R. ripense*, RI; *R.*  
 274 *indicum* 'Shinsen', SH; *R. × pulchrum* 'Sen-e-oomurasaki', SO) at the end of the trial

	GI variation (cm <sup>3</sup> )		Biomass variation (g)		Total chlorophylls (µg mg <sup>-1</sup> )		Chlorosis (%)		Fe <sup>2+</sup> (µg g <sup>-1</sup> )		Ca <sup>2+</sup> (mg g <sup>-1</sup> )		K <sup>+</sup> (mg g <sup>-1</sup> )
Genotype													
IO	1703	b	3.31	b	1.042	a	37.5	d	1.83	b	0.19	bc	4.87
KR	2914	a	3.23	b	0.642	b	90.6	ab	2.11	b	0.41	ab	5.10
RI	4144	a	4.49	a	1.054	a	72.9	c	1.87	b	0.12	c	5.03
SH	1833	b	2.26	b	0.851	ab	84.8	bc	2.21	b	0.63	a	4.95
SO	2830	ab	2.67	b	1.025	a	97.5	a	3.19	a	0.47	a	4.82
<i>P</i>	***§		**		***		***		***		***		ns
pH													
Acid	3384		3.7		1.079		72.2		2.44		0.45		4.95
Alkaline	1867		2.68		0.767		91.9		2.05		0.28		4.96
<i>P</i>	***		**		***		***		*		*		ns
Iron													
-Fe	2577		2.88		0.903		88		-		-		-
+Fe	2674		3.51		0.942		76		-		-		-
<i>P</i>	ns		*		ns		***		-		-		-
Genotype x pH	ns		ns		*		***		ns		ns		***
Genotype x Iron	ns		ns		ns		ns		-		-		-

275 § ns, \*, \*\* or \*\*\* indicates non-significant, or significant at  $P \leq 0.05$ , 0.01 or 0.001, respectively. Same letter denote no significant differences according to  
 276 REGW-F test ( $P < 0.05$ ).

277 *Leaf chlorosis and chlorophyll content*

278         The repeated measures comparing data over 10 weeks of cultivation indicated  
279 significant effect on leaf chlorosis for substrate pH, Fe nutrition, genotype, and the interaction  
280 of substrate pH and genotype (Table 3). Mean values of foliar chlorosis higher than 70% were  
281 recorded at the end of cultivation in every genotype, except for IO (Table 4). Alkaline pH and  
282 Fe deficiency were responsible for the higher damages detected. The appearance of foliar  
283 chlorosis and its trend along the trial is reported in Fig. 3, together with significant differences  
284 between treatments. IO showed slight chlorosis in alkaline substrates from week 7, which  
285 increased and was significant higher in weeks 8 to 10, while plants cultivated in acid  
286 substrates did not show considerable symptoms throughout the experiment. KR plants did not  
287 show considerable chlorosis until week 6 of cultivation, afterwards chlorotic leaves occurred  
288 rapidly over 15% in every treatment, with significant negative effects of both alkaline  
289 treatments at week 9, together with Acid+Fe treatment at week 10. RI showed slight chlorosis  
290 since week 2, with higher damages in Alkaline+Fe at week 4 and 5, with significantly higher  
291 values at week 8 and 9 in all treatments, except in Acid+Fe. SH showed leaf chlorosis in the  
292 second half of the trial, significantly higher in both alkaline treatments (weeks 5 and 6), and at  
293 week 9 also in Acid+Fe. SO showed strong chlorosis in Alkaline-Fe starting from week 7,  
294 while similar damages occurred later in the other treatments. In the last week of the trial KR,  
295 RI, SH and SO plants displayed chlorosis percentages higher than 50% in all treatments  
296 performed.

297         The leaf chlorophyll content resulted genotype dependent. Generally, the amount of  
298 total chlorophylls varied widely among genotypes and treatments, with the lowest chlorophyll  
299 content detected in KR at week 6 ( $0.408 \mu\text{g mg}^{-1}$ , Alkaline-Fe), and the highest recorded in RI  
300 at week 9 ( $2.366 \mu\text{g mg}^{-1}$ , Acid+Fe) (data not shown). At the end of the trial, differences  
301 between genotypes were null, except for KR plants, which showed the lower chlorophyll  
302 content (Table 4).

303         Along the 10-weeks experiment, foliar chlorosis was positively correlated with GI  
304 (Table 5).

305

306 **Table 5** Correlation coefficients ( $r$ ,  $n=10$ ) between GI (cm<sup>3</sup>) and foliar chlorosis (%) and related  $P$ -  
 307 values in the five azaleas tested (*R. indicum* 'Osakazuki', IO; *R. obtusum* 'Kirin', KR; *R. ripense*, RI; *R.*  
 308 *indicum* 'Shinsen', SH; *R. × pulchrum* 'Sen-e-oomurasaki', SO)

Genotype	Treatment	$r$	$P$
IO	Acid-Fe	0.29	0.396
	Acid+Fe	0.30	0.369
	Alkaline-Fe	0.42	0.204
	Alkaline+Fe	0.46	0.157
KR	Acid-Fe	0.77	0.005
	Acid+Fe	0.80	0.003
	Alkaline-Fe	0.77	0.005
	Alkaline+Fe	0.83	0.002
RI	Acid-Fe	0.65	0.032
	Acid+Fe	0.61	0.045
	Alkaline-Fe	0.40	0.218
	Alkaline+Fe	0.18	0.603
SH	Acid-Fe	0.80	0.003
	Acid+Fe	0.71	0.015
	Alkaline-Fe	0.94	0.000
	Alkaline+Fe	0.80	0.003
SO	Acid-Fe	0.92	0.000
	Acid+Fe	0.90	0.000
	Alkaline-Fe	0.79	0.004
	Alkaline+Fe	0.56	0.071

309

310

311 *Foliar mineral elements concentration*

312 The effect of substrate pH on mineral element concentration in the leaves of azalea  
 313 plants under adequate Fe nutrition is reported in Table 4. Along the trial, Fe<sup>2+</sup> content was  
 314 stable (except for KR, in which decreased in Alkaline+Fe substrate), while Ca<sup>2+</sup> content  
 315 decreased and K<sup>+</sup> increased (Table 6). Looking at the genotypes, Fe<sup>2+</sup> concentration was  
 316 higher in SO leaves, and was negatively affected by high pH only in SH. Ca<sup>2+</sup> content varied  
 317 according to the genotype, being higher in SO and SH plants (Table 4), and was influenced by  
 318 alkalinity in KR and RI (Table 6). Finally, no differences were highlighted in leaf K<sup>+</sup>  
 319 concentration between genotypes (Table 4), despite alkalinity induced higher concentration in  
 320 IO and RI, and a lower content in SH (Table 6).

321

322 **Table 6** Foliar mineral elements concentration in the leaves of the five azaleas tested (*R. indicum*  
 323 'Osakazuki', IO; *R. obtusum* 'Kirin', KR; *R. ripense*, RI; *R. indicum* 'Shinsen', SH; *R. × pulchrum* 'Sen-  
 324 e-oomurasaki', SO), at the beginning (Week 0) and at the end of the trial (Week 10)

Genotype	Treatment	Fe <sup>2+</sup> (µg g <sup>-1</sup> )			Ca <sup>2+</sup> (mg g <sup>-1</sup> )			K <sup>+</sup> (mg g <sup>-1</sup> )		
		Week 0	Week 10	P	Week 0	Week 10	P	Week 0	Week 10	P
IO	Acid+Fe	2.244	1.830	ns <sup>§</sup>	1.163	0.221	*	4.051	4.563	ns
	Alkaline+Fe	2.244	1.830	ns	1.163	0.156	*	4.051	5.170	*
	P	-	ns		-	ns		-	*	
KR	Acid+Fe	2.557	2.444	ns	1.272	0.534	**	4.234	4.869	*
	Alkaline+Fe	2.557	1.780	*	1.272	0.284	**	4.234	5.330	**
	P	-	ns		-	*		-	ns	
RI	Acid+Fe	2.118	1.880	ns	0.230	0.141	**	4.685	4.875	ns
	Alkaline+Fe	2.118	1.868	ns	0.230	0.101	**	4.685	5.192	*
	P	-	ns		-	**		-	*	
SH	Acid+Fe	2.181	2.645	ns	1.691	0.884	*	3.296	5.350	**
	Alkaline+Fe	2.181	1.792	ns	1.691	0.382	**	3.296	4.557	*
	P	-	*		-	ns		-	**	
SO	Acid+Fe	2.407	3.385	ns	1.302	0.453	**	4.093	5.104	*
	Alkaline+Fe	2.407	2.996	ns	1.302	0.492	*	4.093	4.546	*
	P	-	ns		-	ns		-	ns	

325 <sup>§</sup> ns, \* or \*\* indicates non-significant, or significant at P ≤ 0.05 or 0.01, respectively

326

### 327 *Abscisic acid content*

328 ABA production in the tested azaleas differed considerably among genotypes (Fig. 4).  
 329 SO and KR plants produced very low amount (up to 0.047 µg g<sup>-1</sup>), compared to the others (up  
 330 to 0.41 µg g<sup>-1</sup>). In IO, ABA peaked at week 4 and week 10 in Acid-Fe and Alkaline-Fe. In  
 331 KR, the hormone concentration remained stable during the trial in acid substrates, while in  
 332 alkaline conditions significantly peaked at week 4. In RI the hormone peaked at week 4 and  
 333 10 in absence of Fe nutrition. In SH, ABA peaked at week 10 under Alkaline-Fe treatment. At  
 334 last, SO plants had almost null ABA content.

335

336

### 337 **Discussion**

338 Plants that suffer from Fe deficiency usually show a typical interveinal chlorosis on new  
 339 developed leaves and stunted growth (Marschner 1995; Schmidt 1999; Lucena 2000). For this  
 340 reason, in our experiment we evaluated GI, leaves number and biomass. However, evergreen  
 341 azaleas are slow-growing plants which can have extremely different development depending  
 342 on the genotype (Galle 1987; Scariot et al. 2013; Demasi et al. 2015a). In this study, plants

343 started to be influenced by treatments from week 5. The alkaline substrate was more effective  
344 than Fe deficiency in reducing the extent of growth of the azaleas tested. Looking at GI trend,  
345 in fact, high pH limited plant growth if compared to acid substrate. Similarly to the plant  
346 development, also leaves number varied widely according to genotype and was negatively  
347 influenced by high pH, particularly in Fe deficiency, in all genotypes, except for RI.  
348 Interestingly, Fe nutrition helped to promote foliar production in KR and SO plants when  
349 cultivated under high pH. Overall, the five azaleas tested displayed different growth rates,  
350 affected either by alkalinity and Fe deficiency, in particular after seven weeks of cultivation,  
351 from which significant differences between treatments were recorded. GI of IO was  
352 comparable under all treatments performed and plants grew considerably less than the other  
353 genotypes. This characteristic suggests a possibly lower nutrient requirements, resulting in  
354 lesser damages when plants are cultivated under Fe deficiency or high pH conditions.  
355 Conversely to the present trial, KR and SO plants did not show variation in the above ground  
356 growing traits in the previous screening performed by Demasi et al. (2015a), probably due to  
357 the shorter length of the experiment (21 days).

358 Plant responses to the Acid+Fe treatment can give information about the nutrient  
359 requirements of the studied genotypes, until now unknown. Based on the number of weeks  
360 without chlorotic leaves in Acid+Fe (SH: week 7, KR: week 8, SO: week 9, RI: week 10, and  
361 IO: no chlorosis), it can be assumed that SH, KR and SO require more frequent Fe  
362 fertilizations than RI and IO. The absence of chlorosis in IO also in Acid-Fe treatment  
363 suggests a lower necessity of Fe comparing to the other genotypes. Considering the substrate  
364 pH, alkalinity worsen chlorosis development in all azaleas, in particular in SO plants under Fe  
365 deficiency. This results counteract what previously observed in Demasi et al. (2015a; 2015b),  
366 where SO plants showed extremely low chlorosis and mortality rate and high FCR activity in  
367 high pH hydroponic conditions. Conversely, in KR plants results were comparable to  
368 previous observation in hydroponics (Demasi et al. 2015a), that highlighted a strong  
369 sensitivity to alkaline substrate either with or without Fe nutrition. The composition of  
370 growing medium is probably the reason for this opposite behaviour, being the solid substrate  
371 more buffered and complex comparing to a nutrient solution.

372 A high correlation level was found between GI and chlorosis in many genotypes,  
373 together with elevated damages in almost all treatments/genotypes at the end of cultivation.  
374 Even though foliar chlorosis was related to Fe deficiency, these data could suggest both that  
375 Fe became limiting at the end of the trial but also that a nitrogen deficiency could occur. Even



376 if the fertilisation program was assessed based on realistic protocols, nitrogen deficiency  
377 could be involved in the chlorosis detected at the end of cultivation.

378         The presence of calcium carbonate and hydrogen carbonate in soil can interfere with the  
379 availability of nutrients and their uptake by plants (Giel and Bojarczuk 2002; Smith et al.  
380 2004b; Giel and Bojarczuk 2011; Alcántara et al. 2012). In our study, the addition of CaCO<sub>3</sub>  
381 in the substrate did not correspond to an increase in foliar Ca<sup>2+</sup> concentration. Similarly to  
382 Chaanin and Preil (1994) and Giel and Bojarczuk (2002), higher levels of Ca<sup>2+</sup> in the  
383 cultivation medium did not correspond to higher leaf chlorosis, confirming that the ion itself  
384 is not toxic and not involved in azalea sensitivity to high pH (Tod 1959), while different Ca<sup>2+</sup>  
385 contents could be ascribable to different transpiration rates (Clark et al. 2003). Mineral  
386 concentration in foliar tissues is a complex trait to be investigated since it is the result of  
387 nutrients accumulation over an extended growing period (Smith et al. 2004b). In fact, even  
388 though an increasing proton concentration in acidic soils has been found to reduce cation  
389 uptake in crop plants (Islam et al. 1980; Marschner 1995), the influence of substrate  
390 characteristics on nutritional status cannot be predicted solely on nutrient solubility (Smith et  
391 al. 2004b). It is fundamental to consider that growing media can have characteristics deeply  
392 different from that of natural soils, especially in terms of physical properties and nutrients  
393 availability (Lemaire 1994; Riviere and Caron 1999; Raviv et al. 2001). High percentages of  
394 nitrogen, potassium and phosphorous, for example, can be lost by leaching and mineralisation  
395 of fertilisers can vary widely depending to the growing medium type (Bunt 1973). Potassium  
396 is essential in several physiological processes and is highly required for maintenance of  
397 photosynthetic CO<sub>2</sub> fixation. For this reason, a higher K<sup>+</sup> requirement is usually considered a  
398 positive response of plants under environmental stress conditions (Cakmak 2005). In our  
399 experiment no differences in K<sup>+</sup> content were highlighted in azalea plants, conversely to what  
400 observed in tolerant rhododendrons by Chaanin and Preil (1994) and in sensitive *Prunus*  
401 *persica* and *Helianthus annuus* (Alcántara et al., 1988, 2000).

402         The increase of chlorosis under Fe deficiency is usually related to a decrease of  
403 chlorophyll content (Smith et al. 2004b; Jelali et al. 2011), since Fe controls the δ-  
404 aminolevulinic acid (ALA), a precursor of chlorophyll synthesis (Marschner 1995). Despite  
405 about 90% of cellular Fe is located in the chloroplasts (Thomine and Lanquar 2011;  
406 Kobayashi and Nishizawa 2012), Fe is present within the plant in different environments and  
407 in specific chemical specie(s), which are still to be clarified (Abadía et al. 2011).  
408 Nevertheless, it is well known that high pH of leaf apoplast in plants cultivated under alkaline  
409 conditions may be able to depress Fe<sup>3+</sup> reduction by mesophyll cells, with a decrease of Fe

410 transport across the plasmamembrane: the metal remains in metabolically inactive forms that  
411 prevent the chlorophyll formation (Marschner 1995; Tagliavini and Rombolà 2001). Fe  
412 inactive pools such as ferritin, vacuolar Fe and the Fe sequestered in old organs are suggested  
413 to be important sources during Fe shortage (Thomine and Lanquar 2011; Abadía et al. 2011;  
414 Kobayashi and Nishizawa 2012; Lei et al. 2014). Moreover, the uptake of Fe is highly  
415 regulated (Kim and Guerinot 2007), thus plants that are unable to take up sufficient Fe and do  
416 not induce adaptive responses under Fe deficiency are usually considered Fe-inefficient (Briat  
417 and Lobreáux 1997; Tagliavini and Rombolà 2001). Fe-inefficient plants may also display  
418 lower Fe translocation rates from roots to leaves, as observed in deciduous azaleas (Clark et  
419 al. 2003). In our research, foliar Fe<sup>2+</sup> concentration was significantly lower under alkaline  
420 conditions, similarly to the observation made in both tolerant and sensitive citrus rootstocks  
421 (*Murraya exotica* and *Poncirus trifoliata*, Wulandari et al. 2013), and in *Rhododendron* plants  
422 (Chaanin and Preil 1994). However further studies about root responses and Fe translocation  
423 are needed to better understand Fe deficiency issue in the studied evergreen azaleas.

424 Fe deficiency responses can be stimulated and regulated by several signalling molecules  
425 such as nitric oxide, auxin, carbon dioxide, ethylene and ABA (Kobayashi and Nishizawa  
426 2012). Although ABA involvement in response to Fe deficiency is not completely clear (Lei  
427 et al. 2014), abiotic stresses involving the root system, such as drought, are proven to increase  
428 the ABA concentration in leaves (Wilkinson and Davies 2002). Moreover, ABA controls  
429 many stress-induced genes that encode enzymes for the biosynthesis of compatible osmolytes  
430 and Late Embryogenesis Abundant (LEA)-like proteins, which collectively increase plant  
431 stress tolerance to adverse environmental conditions (Vogler and Kuhlemeier 2003; Xiong  
432 and Zhu 2003; Peleg and Blumwald 2011). ABA also regulates stomatal movement and  
433 cellular growth in response to other hormones, developmental and environmental signals, and  
434 water and nutrient levels (Wilkinson and Davies 2002; Cutler et al. 2010; Peleg and  
435 Blumwald 2011). ABA was seen to alleviate Fe deficiency by promoting reutilization and  
436 transport of Fe from root to shoot, inducing sub-apical root hairs formation and also  
437 regulating ferritin expression (Lei et al. 2014). ABA production depends on different factors,  
438 for instance plant species, developmental stage and plant organ (Xiong and Zhu 2003; Bosco  
439 et al. 2013; Bosco et al. 2014). The magnitude of the increase can also considerably differ  
440 according to the abiotic stress type (Thomashow 1999). In our study, minute quantities of  
441 ABA were detected in azalea leaves, if compared to other ornamental plants (Arve et al. 2013;  
442 Bosco et al. 2013) but differences were highlighted according to substrate pH and Fe  
443 nutrition. Both KR and SO had extremely low levels of ABA in the leaves during the

444 experiment and, generally, Acid+Fe treatment induced the lowest hormone production. The  
445 highest foliar chlorosis percentages did not correspond to the highest ABA content in the leaf  
446 tissue, making the hormone evaluation not a suitable indicator of plant response to Fe  
447 deficiency in a long term experiment. Nonetheless further studies should be performed to  
448 understand ABA origin and translocation inside azalea plants.

449 Lastly, ploidy level is an important consideration for plant breeders, because it can  
450 influence cross fertility, plant vigour, gene expression and speciation (Wendel 2000). In the  
451 present study, all the genotypes resulted diploid. This is in accordance with previous data  
452 observed in a wide range of *Rhododendron* species and cultivars belonging to subgenus  
453 *Tsutsusi* (De Schepper et al. 2001; Jones et al. 2007). Regarding the relative genome sizes, the  
454 studied genotypes presented lower content than the diploid evergreen azaleas analysed by  
455 Jones et al. (2007). Authors indicated a range for 2C genome from 1.2 to 1.3 pg instead of an  
456 average of 1.0 pg. Differences could be explained by the different origin of plants, since Jones  
457 et al. (2007) examined genotypes originated from USA material (North Carolina State  
458 University), while here we analysed five evergreen azaleas of Japanese origin.

459

## 460 **Conclusion**

461 Elucidating the genotype differential tolerance to adverse soil pH is highly requested  
462 among floriculture breeders and growers. Our investigation on physiological and biochemical  
463 responses to alkalinity and Fe deficiency in five evergreen azaleas highlighted the complexity  
464 of the issue. The plant responses, in fact, involve multiple cross-talk signalling pathways,  
465 which varied within closely related species and hybrids. The divergent results obtained in this  
466 study with the previous hydroponic trials suggest that the survey of early stress markers (e.g.  
467 FCR activity) to predict Fe deficiency tolerance can be inappropriate in slow growing plants,  
468 and that pot cultivation and long term experiments are necessary to understand azalea  
469 adaptation to Fe deficiency stress. Overall, the present study highlighted that alkalinity of the  
470 substrate hampered evergreen azalea ornamental performance more than Fe deficiency.  
471 Among the five genotype studied, IO reported the best performance in terms of high GI and  
472 low foliar chlorosis, while SO in terms of low ABA production, high leaves number,  
473 chlorophylls and Ca<sup>2+</sup> and Fe<sup>2+</sup> content. Moreover, the reported genomic information proves  
474 the possibility of interspecific hybridization between the studied evergreen azaleas. Taken  
475 together, these findings provide insights for breeders involved in azalea breeding  
476 programmes.

477 **Compliance with ethical standards**

478 **Conflict of interest** The authors declare that have no conflict of interest.

479

480 **References**

481 Abadía J, Vázquez S, Rellán-Álvarez R, et al (2011) Towards a knowledge-based correction  
482 of iron chlorosis. *Plant Physiol Biochem* 49:471–482. doi: 10.1016/j.plaphy.2011.01.026

483 Albano JP, Miller WB (1998) Marigold cultivars vary in susceptibility to iron toxicity.  
484 *HortScience* 33:1180–1182.

485 Alcántara E, Montilla I, Ramírez P, et al (2012) Evaluation of quince clones for tolerance to  
486 iron chlorosis on calcareous soil under field conditions. *Sci Hortic (Amsterdam)* 138:50–  
487 54. doi: 10.1016/j.scienta.2012.02.004

488 Alcántara E, Romera FJ, Cañete M, De la Guardia MD (2000) Effects of bicarbonate and iron  
489 supply on Fe(III) reducing capacity of roots and leaf chlorosis of the susceptible peach  
490 rootstock “Nemaguard.” *J Plant Nutr* 23:1607–1617. doi: 10.1080/01904160009382127

491 Alcántara E, Romera FJ, de la Guardia MD (1988) Genotypic differences in bicarbonate-  
492 induced iron chlorosis in sunflower. *J Plant Nutr* 11:65–75. doi:  
493 10.1080/01904168809363785

494 Arve LE, Terfa MT, Gislørød HR, et al (2013) High relative air humidity and continuous light  
495 reduce stomata functionality by affecting the ABA regulation in rose leaves. *Plant Cell*  
496 *Environ* 36:382–392. doi: 10.1111/j.1365-3040.2012.02580.x

497 Berruti A, Scariot V (2012) Coconut fiber: A peat-like substrate for acidophilic plant  
498 cultivation. In: *Acta Horticulturae*. pp 629–636

499 Bosco R, Caser M, Ghione GG, et al (2014) Dynamics of abscisic acid and indole-3-acetic  
500 acid during the early-middle stage of seed development in *Rosa hybrida*. *Plant Growth*  
501 *Regul* 75:265–270. doi: 10.1007/s10725-014-9950-8

502 Bosco R, Caser M, Vanara F, Scariot V (2013) Development of a rapid LC-DAD/FLD  
503 method for the simultaneous determination of auxins and abscisic acid in plant extracts. *J*  
504 *Agric Food Chem* 61:10940–10947. doi: 10.1021/jf4034305

505 Briat JF, Lobréaux S (1997) Iron transport and storage in plants. *Trends Plant Sci.* 2:187–193.

506 Bunt AC (1973) Some physical and chemical characteristics of loamless pot-plant substrates  
507 and their relation to plant growth. In: *I Symposium on Artificial Media in Horticulture*  
508 37. pp 1954–1965

509 Cakmak I (2005) The role of potassium in alleviating detrimental effects of abiotic stresses in  
510 plants. *J Plant Nutr Soil Sci* 168:521–530.

511 Chaanin A, Preil W (1994) Influence of bicarbonate on iron deficiency chlorosis in  
512 Rhododendron. *Acta Horti* 364:71–78.

513 Clark MB, Mills HA, Robacker CD, Latimer JG (2003) Influence of nitrate:ammonium ratios  
514 on growth and elemental concentration in two azalea cultivars. *J Plant Nutr* 26:2503–  
515 2520. doi: 10.1081/PLN-120025475

516 Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2010) Abscisic acid: emergence of a  
517 core signaling network. *Annu Rev Plant Biol* 61:651–79. doi: 10.1146/annurev-arplant-  
518 042809-112122

519 De Schepper S, Leus L, Mertens M, et al (2001) Flow cytometric analysis of ploidy in  
520 Rhododendron (subgenus Tsutsusi). *HortScience* 36:125–127.

521 Demasi S, Caser M, Kobayashi N, et al (2015a) Hydroponic screening for iron deficiency  
522 tolerance in evergreen azaleas. *Not Bot Horti Agrobot Cluj-Napoca* 43:210–213. doi:  
523 10.15835/nbha4319929

524 Demasi S, Handa T, Scariot V (2015b) Ferric chelate reductase activity under iron deficiency  
525 stress in azalea. *Int J Horti Floric* 3:157–160.

526 Doležel J, Bartos J, Voglmayr H, Greilhuber J (2003) Nuclear DNA content and genome size  
527 of trout and human. *Cytometry A* 51:127–8; author reply 129. doi: 10.1002/cyto.a.10013

528 Doležel J, Greilhuber J (2010) Nuclear genome size: are we getting closer? *Cytom Part A*  
529 77:635–642.

530 Fisher PR, Wik RM, Smith BR, et al (2003) Correcting iron deficiency in calibrachoa grown  
531 in a container medium at high pH. *Hort Technol* 13:308–313.

532 Galle FC (1987) *Azaleas* (Revised and enlarged edition). Timber Press, Portland

533 Giel P, Bojarczuk K (2002) The effect of high concentration of selected calcium salts on  
534 development of microcuttings of rhododendron R. “Catawbiense Grandiflorum” in in  
535 vitro cultures. *Dendrobiology* 48:23–29.

536 Giel P, Bojarczuk K (2011) Effects of high concentrations of calcium salts in the substrate  
537 and its pH on the growth of selected rhododendron cultivars. *Acta Soc Bot Pol* 80:105–  
538 114.

539 Greer H (1984) *The Satsuki Azaleas*.

540 Greilhuber J, Doležel J, Lysák MA, Bennett MD (2005) The origin, evolution and proposed  
541 stabilization of the terms “genome size” and “C-value” to describe nuclear DNA  
542 contents. *Ann Bot*. doi: 10.1093/aob/mci019

543 Greilhuber J, Temsch EM, Loureiro J (2007) Nuclear DNA content measurement. *Flow*  
544 *Cytom with plant cells Anal genes, Chromosom genomes* 67–101.

545 Gupta UC (1968) Studies on the O-phenanthroline method for determining iron in plant  
546 materials. *Plant Soil* 28:298–305. doi: 10.1007/BF01880247

547 Hansen NC, Hopkins BG, Ellsworth JW, Jolley VD (2006) Iron nutrition in field crops. In:  
548 Barton LL, Abadia J (eds) *Iron Nutrition in Plants and Rhizospheric Microorganisms*.  
549 Springer Netherlands, Dordrecht, pp 23–59

550 Hell R, Stephan UW (2003) Iron uptake, trafficking and homeostasis in plants. *Planta*  
551 216:541–551. doi: 10.1007/s00425-002-0920-4

552 Heursel J (1975) Inheritance of the flavonols azaleatin and quercetin in *Rhododendron simsii*  
553 Planch. and *Rh. obtusum* Planch. *Z Pflanzenzüchtg* 74:62–70.

554 Hidalgo PR, Harkess RL (2002) Earthworm castings as a substrate amendment for  
555 chrysanthemum production. *HortScience* 37:1035–1039.

556 Islam AKMS, Edwards DG, Asher CJ (1980) pH optima for crop growth - Results of a  
557 flowing solution culture experiment with six species. *Plant Soil* 54:339–357. doi:  
558 10.1007/BF02181830

559 Jelali N, Salah I Ben, M'sehli W, et al (2011) Comparison of three pea cultivars (*Pisum*  
560 *sativum*) regarding their responses to direct and bicarbonate-induced iron deficiency. *Sci*  
561 *Hortic (Amsterdam)* 129:548–553. doi: 10.1016/j.scienta.2011.06.010

562 Jolley VD, Cook KA, Hansen NC, Stevens WB (1996) Plant physiological responses for  
563 genotypic evaluation of iron efficiency in strategy I and strategy II plants - A review. *J.*  
564 *Plant Nutr.* 19:1241–1255.

565 Kaufman SR, Smouse PE (2001) Comparing indigenous and introduced populations of  
566 *Melaleuca quinquenervia* (Cav.) Blake: response of seedlings to water and pH levels.  
567 *Oecologia* 127:487–494. doi: 10.1007/s004420000621

568 Kim SA, Guerinot ML (2007) Mining iron: Iron uptake and transport in plants. *FEBS Lett*  
569 581:2273–2280. doi: 10.1016/j.febslet.2007.04.043

570 Kobayashi T, Nishizawa NK (2012) Iron uptake, translocation, and regulation in higher  
571 plants. *Annu Rev Plant Biol* 63:131–152. doi: 10.1146/annurev-arplant-042811-105522

572 Kofranek AM, Lunt OR (1975) Mineral nutrition. In: Kofranek AM, Larson RA (eds)  
573 *Growing Azaleas Commercially*. Division of Agricultural Sciences, University of  
574 California, pp 36–46

575 Lei GJ, Zhu XF, Wang ZW, et al (2014) Abscisic acid alleviates iron deficiency by promoting  
576 root iron reutilization and transport from root to shoot in *Arabidopsis*. *Plant, Cell*  
577 *Environ* 37:852–863. doi: 10.1111/pce.12203

578 Lemaire F (1994) Physical, chemical and biological properties of growing medium.

579 Hydroponics Transpl Prod 396 273–284.

580 Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic  
581 biomembranes. Elsevier

582 Loeppert RH, Wei LC, Ocumpaugh WR (1994) Soil factors influencing the mobilization of  
583 iron in calcareous soils. In: Manthey JA, Crowley DA, Luster DG (eds) Biochemistry of  
584 metal micronutrients in the rhizosphere. Lewis Publisher, Boca Raton, pp 343–360

585 Lucena JJ (2000) Effects of bicarbonate, nitrate and other environmental factors on iron  
586 deficiency chlorosis. A review. J Plant Nutr 23:1591–1606. doi:  
587 10.1080/01904160009382126

588 Marschner H (1995) Mineral Nutrition of Higher Plants, 2nd edn. Academic Press,  
589 Cambridge

590 Marschner H, Römheld V, Kissel M (1986) Different strategies in higher plants in  
591 mobilization and uptake of iron. J Plant Nutr 9:695–713. doi:  
592 10.1080/01904168609363475

593 Martínez-Cuenca M-R, Forner-Giner MÁ, Iglesias DJ, et al (2013) Strategy I responses to Fe-  
594 deficiency of two Citrus rootstocks differing in their tolerance to iron chlorosis. Sci  
595 Hortic (Amsterdam) 153:56–63. doi: 10.1016/j.scienta.2013.01.009

596 Miller GW, Pushnik JC, Welkie GW (1984) Iron chlorosis, a world wide problem, the relation  
597 of chlorophyll biosynthesis to iron. J Plant Nutr 7:1–22. doi:  
598 10.1080/01904168409363172

599 Nelson EC (1999) So many really fine plants—an epitome of Japanese plants in Western  
600 European gardens. Curtis’s Bot Mag 16:52–68.

601 Peleg Z, Blumwald E (2011) Hormone balance and abiotic stress tolerance in crop plants.  
602 Curr Opin Plant Biol 14:290–295. doi: 10.1016/j.pbi.2011.02.001

603 Pestana M, David M, de Varennes A, et al (2001) Responses of “Newhall” orange trees to  
604 iron deficiency in hydroponics: effects on leaf chlorophyll, photosynthetic efficiency,  
605 and root ferric chelate reductase activity. J Plant Nutr 24:1609–1620. doi: 10.1081/PLN-  
606 100106024

607 Pfarr E, Hokanson S, McNamara S (2015) Evaluating high soil ph tolerance of rhododendron  
608 cultivars. University of Minnesota Digital Conservancy

609 Preil W, Ebbinghaus R (1994) Breeding lime-tolerant *Rhododendron* rootstocks. Acta Hortic  
610 364:61–70.

611 Raviv M, Wallach R, Blom TJ (2001) The effect of physical properties of soilless media on  
612 plant performance-A review. In: International Symposium on Growing Media and

613 Hydroponics 644. pp 251–259

614 Riviere L-M, Caron J (1999) Research on substrates: state of the art and need for the coming  
615 10 years. In: International Symposium on Growing Media and Hydroponics 548. pp 29–  
616 42

617 Römheld V (2000) The chlorosis paradox: Fe inactivation as a secondary event in chlorotic  
618 leaves of grapevine. J Plant Nutr 23:1629–1643. doi: 10.1080/01904160009382129

619 Saywell LG, Cunningham BB (1937) Determination of iron: Colorimetric o-phenanthroline  
620 method. Ind. Eng. Chem. 9:67–69.

621 Scariot V, Caser M, Kobayashi N (2013) Evergreen azaleas tolerant to neutral and basic soils:  
622 Breeding potential of wild genetic resources. Acta Hort 990:287–292.

623 Scariot V, Kobayashi N (2008) Evaluation of variability in Japanese wild azaleas and  
624 application of lime-tolerant genetic resources for breeding. B. Abstr. First Symp. Hortic.  
625 Eur. 268–269.

626 Schmidt W (1999) Mechanisms and regulation of reduction-based iron uptake in plants. New  
627 Phytol 141:1–26. doi: 10.1046/j.1469-8137.1999.00331.x

628 Smith BR, Fisher PR, Argo WR (2004a) Water-soluble fertilizer concentration and pH of a  
629 peat-based substrate affect growth , nutrient uptake , and chlorosis of container-grown  
630 seed geraniums. J Plant Nutr 27:497–524. doi: 10.1081/PLN-120028875

631 Smith BR, Fisher PR, Argo WR (2004b) Nutrient uptake in container-grown impatiens and  
632 petunia in response to root substrate pH and applied micronutrient concentration.  
633 HortScience 39:1426–1431.

634 Stanton KM, Mickelbart M V. (2014) Growth and foliar nutrition of *Spiraea alba* Du Roi and  
635 *Spiraea tomentosa* L. in response to root zone pH. Sci Hortic (Amsterdam) 165:23–28.  
636 doi: 10.1016/j.scienta.2013.10.027

637 Symonds WL, Campbell LC, Clemens J (2001) Response of ornamental Eucalyptus from  
638 acidic and alkaline habitats to potting medium pH. Sci Hortic (Amsterdam) 88:121–131.  
639 doi: 10.1016/S0304-4238(00)00202-8

640 Tagliavini M, Rombolà AD (2001) Iron deficiency and chlorosis in orchard and vineyard  
641 ecosystems. Eur J Agron 15:71–92. doi: 10.1016/S1161-0301(01)00125-3

642 Thomashow MF (1999) Plant cold acclimation: Freezing tolerance genes and regulatory  
643 mechanisms. Annu Rev Plant Biol 50:571–599.

644 Thomine S, Lanquar V (2011) Iron transport and signaling in plants. In: Transporters and  
645 Pumps in Plant Signaling. Springer, pp 99–131

646 Tod H (1959) Rhododendrons and lime. RHS Rhododendron Camellia Yearb. 13:19–24.



647 Valdez-Aguilar LA, Reed DW (2006) Comparison of growth and alkalinity-induced  
648 responses in two cultivars of hibiscus (*Hibiscus rosa-sinensis* L.). HortScience 41:1704–  
649 1708.

650 Valdez-Aguilar LA, Reed DW (2007) Response of selected greenhouse ornamental plants to  
651 alkalinity in irrigation water. J Plant Nutr 30:441–452. doi:  
652 10.1080/01904160601171983

653 Van Laere K, Leus L, Van Huylenbroeck J, Van Bockstaele E (2009) Interspecific  
654 hybridisation and genome size analysis in *Buddleja*. Euphytica 166:445–456. doi:  
655 10.1007/s10681-008-9844-9

656 Vogler H, Kuhlemeier C (2003) Simple hormones but complex signalling. Curr Opin Plant  
657 Biol 6:51–56. doi: 10.1016/S1369-5266(02)00013-4

658 Wallace A, Wallace GA (1986) Ornamental plants most likely to be killed by iron deficiency  
659 and some control measures. J Plant Nutr 9:1009–1014.

660 Wendel JF (2000) Genome evolution in polyploids. Plant Mol Biol 42:225–249. doi:  
661 10.1023/A:1006392424384

662 Wilkinson S, Davies WJ (2002) ABA-based chemical signalling: The co-ordination of  
663 responses to stress in plants. Plant, Cell Environ 25:195–210. doi: 10.1046/j.0016-  
664 8025.2001.00824.x

665 Wulandari C, Muraki S, Hisamura A, et al (2014) Effect of iron deficiency on root ferric  
666 chelate reductase, proton extrusion, biomass production and mineral absorption of citrus  
667 root stock Orange Jasmine (*Murraya exotica* L.). J Plant Nutr 37:50–64. doi:  
668 10.1080/01904167.2013.837178

669 Xiong L, Zhu JK (2003) Regulation of abscisic acid biosynthesis. PLANT Physiol 133:29–36.  
670 doi: 10.1104/pp.103.025395

671

672 **Figure legends**

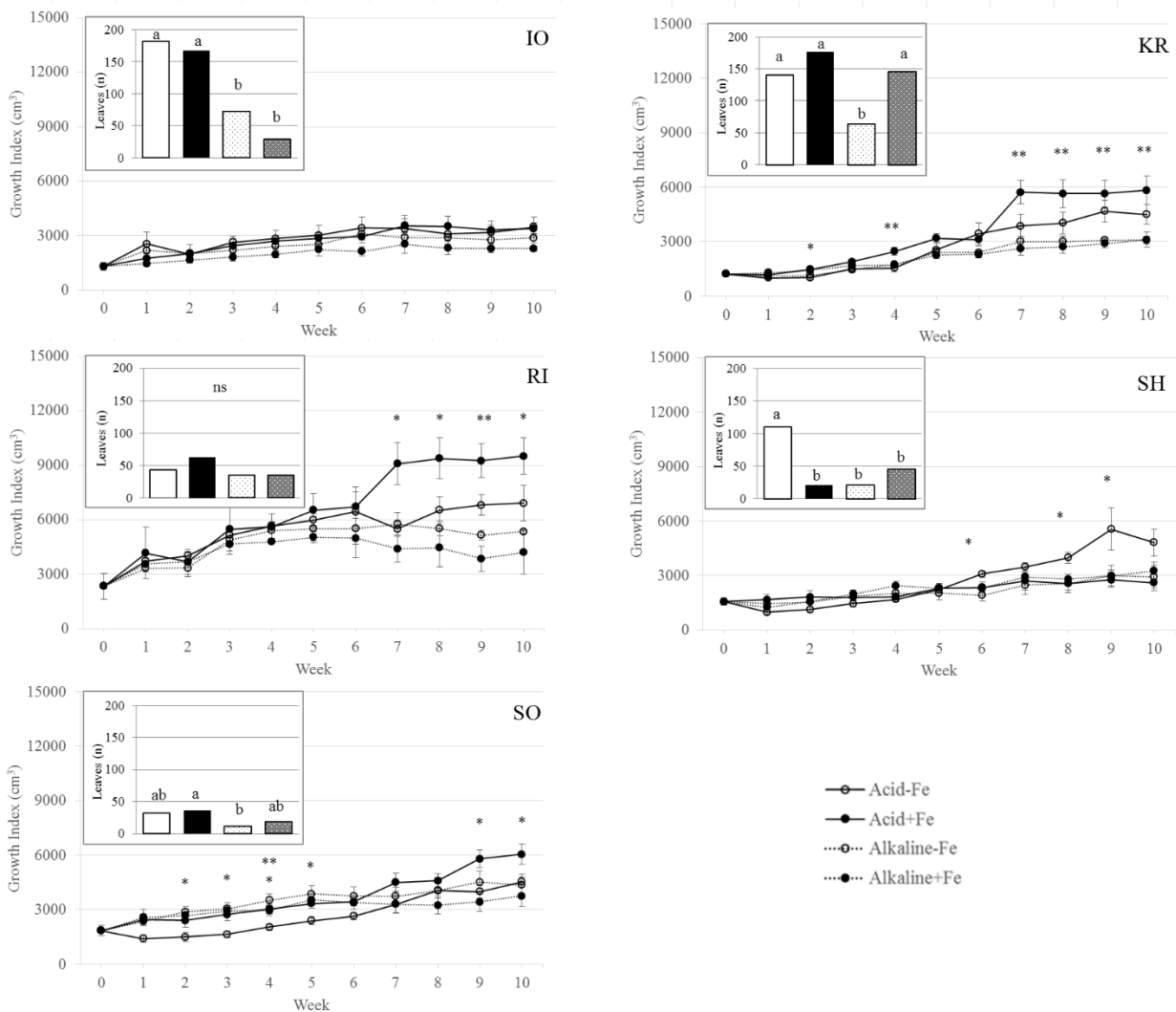
673

674 **Fig. 1** Interveinal chlorosis in new expanded leaf of azalea



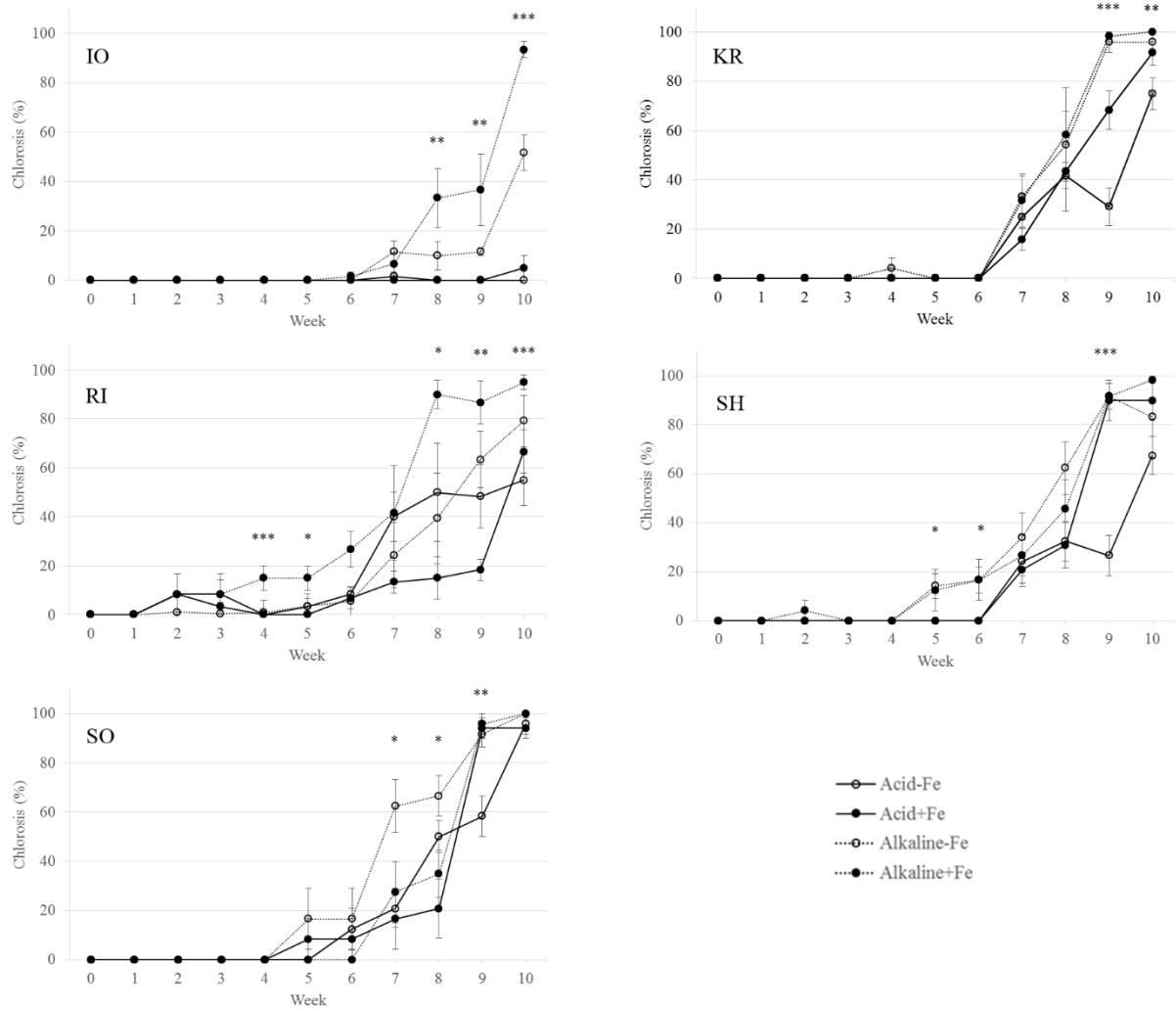
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676 **Fig. 2** Growth index (cm<sup>3</sup>) trend of the five azaleas tested (*R. indicum* 'Osakazuki', IO; *R. obtusum*  
 677 'Kirin', KR; *R. ripense*, RI; *R. indicum* 'Shinsen', SH; *R. × pulchrum* 'Sen-e-oomurasaki', SO) during  
 678 10 weeks of cultivation in four different treatments (Acid-Fe, Acid+Fe, Alkaline-Fe and Alkaline+Fe).  
 679 \*, \*\* or \*\*\* indicates significant differences at  $P \leq 0.05$ , 0.01 or 0.001, respectively. Boxes: leaf  
 680 number variation along the trial (number of leaves week 10 – number of leaves week 0) in the five  
 681 azaleas. Same letter denote no significant differences according to REGW-F test ( $P < 0.05$ )



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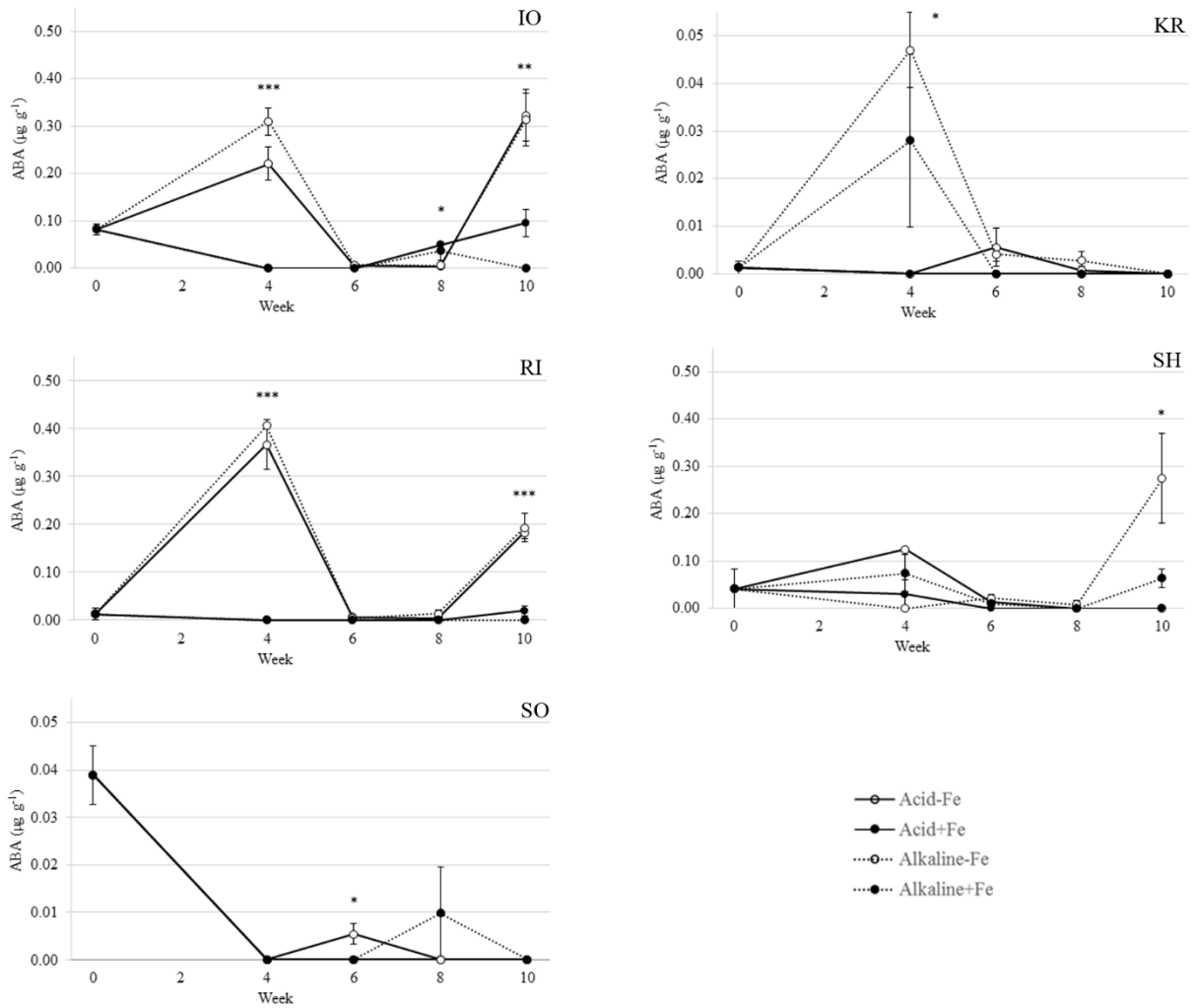
683 **Fig. 3** Means of chlorotic leaves (%) per plant showed by the five azaleas tested (*R. indicum*  
 684 'Osakazuki', IO; *R. obtusum* 'Kirin', KR; *R. ripense*, RI; *R. indicum* 'Shinsen', SH; *R. × pulchrum* 'Sen-  
 685 e-oomurasaki', SO) during 10 weeks of cultivation in four different treatments (Acid-Fe, Acid+Fe,  
 686 Alkaline-Fe and Alkaline+Fe). \*, \*\* or \*\*\* indicates significant differences at  $P \leq 0.05$ , 0.01 or 0.001,  
 687 respectively



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689

690 **Fig. 4** Abscisic acid content ( $\mu\text{g g}^{-1}$ ) in the leaves of the five azaleas tested (*R. indicum* 'Osakazuki',  
 691 IO; *R. obtusum* 'Kirin', KR; *R. ripense*, RI; *R. indicum* 'Shinsen', SH; *R. × pulchrum* 'Sen-e-  
 692 oomurasaki', SO) during 10 weeks of cultivation in four different treatments (Acid-Fe, Acid+Fe,  
 693 Alkaline-Fe and Alkaline+Fe). \*, \*\* or \*\*\* indicates significant differences at  $P \leq 0.05$ , 0.01 or 0.001,  
 694 respectively. Scales of y-axis are different: up to  $0.05 \mu\text{g g}^{-1}$  in KR and SO and up to  $0.5 \mu\text{g g}^{-1}$  in SH,  
 695 IO and RI



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