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

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

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## Abstract

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

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

**Abbreviations**:

GI, growth index

IO, *R. indicum* 'Osakazuki'

KR, *R. obtusum* 'Kirin'

RI, *R. ripense*

SH, *R. indicum* 'Shinsen'

SO, *R.* × *pulchrum* 'Sen-e-oomurasaki'

## Introduction

In alkaline soils, the growth of many crops can be altered or compromised by macro and micro nutrients deficiencies. The pH is, in fact, a crucial feature amid substrate characteristics, since it affects solubility and therefore availability of mineral elements (Miller et al. 1984; Marschner 1995). High concentrations of calcium carbonate ($CaCO_3$) and hydrogen carbonate ($HCO_3^-$) in alkaline soils have proved to play a relevant role in limiting iron (Fe) and other micronutrients availability, such as manganese (Mn), zinc (Zn) and copper (Cu) (Loeppert et al. 1994). The Fe unavailability is mainly a problem of solubility and not of abundance, as Fe availability decreases dramatically with increasing pH due to its hydrolysation, polymerization and precipitation with inorganic ions (Hell and Stephan 2003). Fe is involved in numerous biological redox systems and is necessary in respiration, chlorophyll biosynthesis and photosynthesis (Marschner 1995; Kim and Guerinot 2007; Kobayashi and Nishizawa 2012). Therefore, a Fe deficiency can impair plant health causing the distinctive interveinal chlorosis in the youngest leaves, together with a decrease in root development and stunted growth (Marschner 1995; Schmidt 1999; Lucena 2000; Römheld 2000), and compromise the production of many crops (Tagliavini and Rombolà 2001; Hansen et al. 2006), including ornamentals (Albano and Miller 1998; Fisher et al. 2003; Smith et al. 2004a; Smith et al. 2004b; Valdez-Aguilar and Reed 2006; Valdez-Aguilar and Reed 2007). However, within species, genotypes that grow well in soils with a pH outside of their native range have been recorded (Pestana et al. 2001; Symonds et al. 2001; Kaufman and Smouse 2001; Alcántara et al. 2012; Martínez-Cuenca et al. 2013; Stanton and Mickelbart 2014; Wulandari et al. 2014).

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

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

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

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

## Materials and methods

### *Plant material*

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

### *Flow cytometry*

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

### *Cultivation conditions*

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

Agrochimica, Bolzano, Italy) and coconut fibre (Tref Ego Substrate BV, Moderdijk, The Netherlands) (Berruti and Scariot 2012). This substrate was supplemented with $CaCO_3$ to raise the pH and obtain the alkaline substrate used for treatments (3) and (4). Fe was added as iron sulphate ($FeSO_4$) at the beginning of the trial in treatments (2) and (4), while (1) and (3) did not received any Fe fertilisation. During the trial the pH gradually decreased in all treatments.

**Table 1** Calcium carbonate ($CaCO_3$) and iron (Fe) concentration of the four substrates and the pH course along the trial

| Treatment | $CaCO_3$ | Fe | pH | | |
|---|---|---|---|---|---|
| | | | Week 0 | Week 5 | Week 10 |
| (1) Acid-Fe | - | - | 4.8 | 4.2 | 3.9 |
| (2) Acid+Fe | - | 5 mg $L^{-1}$ | 4.7 | 4.7 | 4.3 |
| (3) Alkaline-Fe | 0.1 mol $dm^{-3}$ | - | 8.5 | 8.2 | 7.9 |
| (4) Alkaline+Fe | 0.1 mol $dm^{-3}$ | 5 mg $L^{-1}$ | 8.8 | 8.0 | 8.0 |

Substrates were allowed to equilibrate for seven days in a 0.6 L plastic pot before planting. One pot containing one plant constituted a replication and 45 replicates per genotype were subjected to the same treatment. Plants were fertilized weekly with 50 mL of a modified Hoagland solution (Clark et al. 2003) consisted in: 1.79 mM $L^{-1}$ $(NH_4)_2SO_4$, 1.79 mM $L^{-1}$ $Ca(NO_3)_2$, 7 mM $L^{-1}$ $CaCl_2$, 1 mM $L^{-1}$ $KH_2PO_4$, 1.66 mM $L^{-1}$ KCl, 0.42 mM $L^{-1}$ $K_2SO_4$, 2 mM $L^{-1}$ $MgSO_4 \cdot 7H_2O$. Micronutrients were supplied as a combination of Zn-EDTA (0.83 µM $L^{-1}$), Cu-EDTA (0.33 µM $L^{-1}$), Mn-EDTA (8.56 µM $L^{-1}$), $Na_2B_8O_{13} \cdot 4H_2O$ (10.71 µM $L^{-1}$) and $Na_2MoO_4 \cdot 2H_2O$ (0.08 µM $L^{-1}$). Fertilisation resulted in a total amount of nutrients provided to 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 Cl, 0.25 mg B, 0.01 mg Cu, 0.25 mg Mn, 0.005 mg Mo, and 0.025 mg Zn. Irrigation was provided with deionized water (pH=4.8, EC=8 µS $cm^{-1}$; soluble salts=6 mg $L^{-1}$) at the bottom of the pots when needed, in order to prevent the percolation of mineral elements. Plants were kept in a glass greenhouse at an average temperature of 24°C during the day and 18°C during the night, with a light/dark photoperiod of 16/8 h, under an additional Photosynthetically Active Radiation (PAR) of 350 µmol $m^{-2}$ $s^{-1}$ at the canopy level, provided by high pressure sodium lamps.

*Plant growth parameters*

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

*Foliar chlorosis and chlorophyll content*

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

*Iron, calcium and potassium determination*

Concentration of ferrous iron ($Fe^{2+}$), calcium ($Ca^{2+}$) and potassium ($K^{+}$) in the leaves of the five azalea genotypes cultivated in acid and alkaline treatments supplemented with Fe was measured at the beginning and at the end of the experiment (week 10). New expanded leaves were harvested from three plants per treatment and oven-dried at 50°C for four days. After grinding, 4 mL of sulphuric acid ($H_2SO_4$) and 4 mL of hydrogen peroxide ($H_2O_2$, 30%) were added to 0.5 g of each sample and heated-digested in a Kjeldahl flask. Samples were later diluted to 100 mL with deionized water. $Ca^{2+}$ and $K^{+}$ content was determined through atomic absorption spectrophotometry (AA-7000, Shimadzu Corporation, Japan), while $Fe^{2+}$ content was spectrophotometrically measured (UV-1700 PharmaSpec, Shimadzu Corporation, Japan)

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

*Abscisic acid determination*

The concentration of endogenous ABA in the leaves was quantified in three plants per treatment at the beginning of the experiment and at week 4, 6, 8 and 10. The plant hormone was quantified by High Performance Liquid Chromatography (HPLC), based on Solid Phase Extraction (SPE) purification method (modified from Bosco et al. 2013). New opened leaves were sampled and maintained at -80°C until analysis. Leaves were ground in liquid nitrogen and 0.5 g of each sample was suspended in 4 ml of the extraction solution (65% pure methanol, 25% ultrapure water, 10% aqueous hydrogen chloride 1 M) for 2 h at 4°C, in darkness condition and under magnetic stirring. Sample was then filtered and the eluate was added to a SPE cartridge (Supelclean SPE LC-$NH_2$, Supelco Analytical, USA), previously washed with pure methanol (99%) and ultrapure water. ABA was eluted with 5% of phosphoric acid ($H_3PO_4$) in methanol. The procedure was carried out under artificial light with amber glassware, in order to prevent analyte degradation. The chromatographic analysis of the eluate was performed with HPLC 1200 Series (Agilent Technologies, Böblingen, Germany). The signal for ABA was monitored at 265 nm and hormone concentration was quantified according to a calibration curve, constructed from the matrix-matched calibration standards.

*Statistical analysis*

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

## Results

### *Genome size measurements*

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

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

| Genotype | Plant characteristics | Relative 2C (pg)[1] | 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 *R.* × *pulchrum* 'Oomurasaki' | 0.98±0.03 | 2 |

[1]Values represent mean 2C holoploid genome size ± standard error for three repetition

### *Plant growth*

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

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

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

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

**Table 3** Level of significance of the repeated measures for the Growth Index (GI; $cm^3$) and foliar 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 |

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

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

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

| | GI variation ($cm^3$) | | Biomass variation (g) | | Total chlorophylls (µg $mg^{-1}$) | | Chlorosis (%) | | $Fe^{2+}$ (µg $g^{-1}$) | | $Ca^{2+}$ (mg $g^{-1}$) | | $K^+$ (mg $g^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 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 |
| *P* | ***§ | | ** | | *** | | *** | | *** | | *** | | 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 |
| *P* | *** | | ** | | *** | | *** | | * | | * | | ns |
| Iron | | | | | | | | | | | | | |
| -Fe | 2577 | | 2.88 | | 0.903 | | 88 | | - | | - | | - |
| +Fe | 2674 | | 3.51 | | 0.942 | | 76 | | - | | - | | - |
| *P* | ns | | * | | ns | | *** | | - | | - | | - |
| Genotype x pH | ns | | ns | | * | | *** | | ns | | ns | | *** |
| Genotype x Iron | ns | | ns | | ns | | ns | | - | | - | | - |

§ 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 REGW-F test ($P < 0.05$).

*Leaf chlorosis and chlorophyll content*

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

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

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

306 **Table 5** Correlation coefficients (*r*, n=10) between GI ($cm^3$) 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 |

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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, $Fe^{2+}$ content was
314 stable (except for KR, in which decreased in Alkaline+Fe substrate), while $Ca^{2+}$ content
315 decreased and $K^+$ increased (Table 6). Looking at the genotypes, $Fe^{2+}$ concentration was
316 higher in SO leaves, and was negatively affected by high pH only in SH. $Ca^{2+}$ 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 $K^+$
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

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

| Genotype | Treatment | $Fe^{2+}$ (μg $g^{-1}$) | | | $Ca^{2+}$ (mg $g^{-1}$) | | | $K^{+}$ (mg $g^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Week 0 | Week 10 | *P* | Week 0 | Week 10 | *P* | Week 0 | Week 10 | *P* |
| IO | Acid+Fe | 2.244 | 1.830 | ns[§] | 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 | |

[§] ns, * or ** indicates non-significant, or significant at $P \leq 0.05$ or 0.01, respectively

*Abscisic acid content*

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

## Discussion

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

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

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

A high correlation level was found between GI and chlorosis in many genotypes, together with elevated damages in almost all treatments/genotypes at the end of cultivation. Even though foliar chlorosis was related to Fe deficiency, these data could suggest both that 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 $CaCO_3$
381 in the substrate did not correspond to an increase in foliar $Ca^{2+}$ concentration. Similarly to
382 Chaanin and Preil (1994) and Giel and Bojarczuk (2002), higher levels of $Ca^{2+}$ 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 $Ca^{2+}$
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 $CO_2$ fixation. For this reason, a higher $K^+$ requirement is usually considered a
398 positive response of plants under environmental stress conditions (Cakmak 2005). In our
399 experiment no differences in $K^+$ 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 $Fe^{3+}$ reduction by mesophyll cells, with a decrease of Fe

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

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

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

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

## Conclusion

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

**Compliance with ethical standards**

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

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## Figure legends

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

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

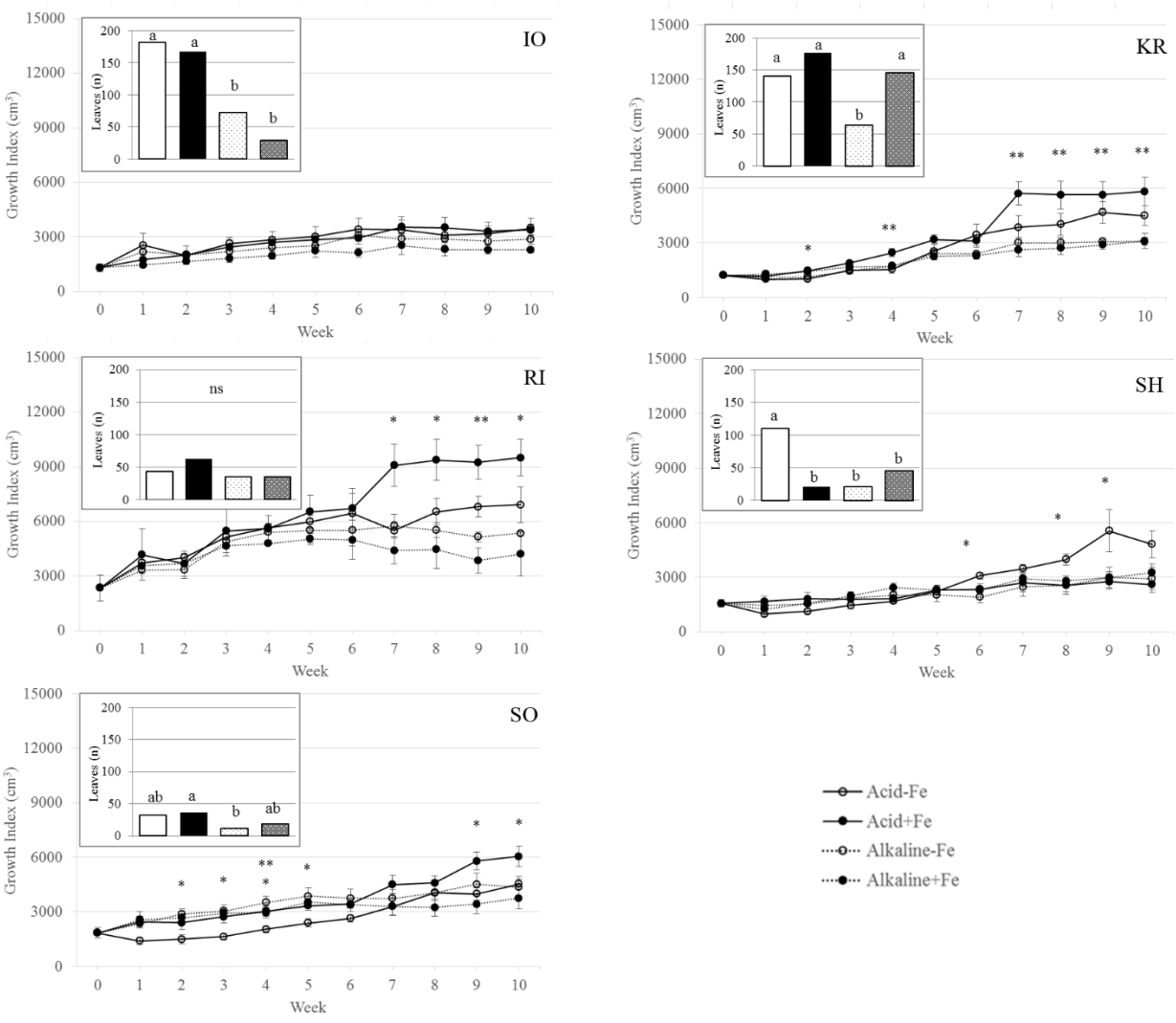


**Fig. 3** Means of chlorotic leaves (%) per plant showed by the five azaleas tested (*R. indicum* 'Osakazuki', IO; *R. obtusum* 'Kirin', KR; *R. ripense*, RI; *R. indicum* 'Shinsen', SH; *R.* × *pulchrum* 'Sen-e-oomurasaki', SO) during 10 weeks of cultivation in four different treatments (Acid-Fe, Acid+Fe,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

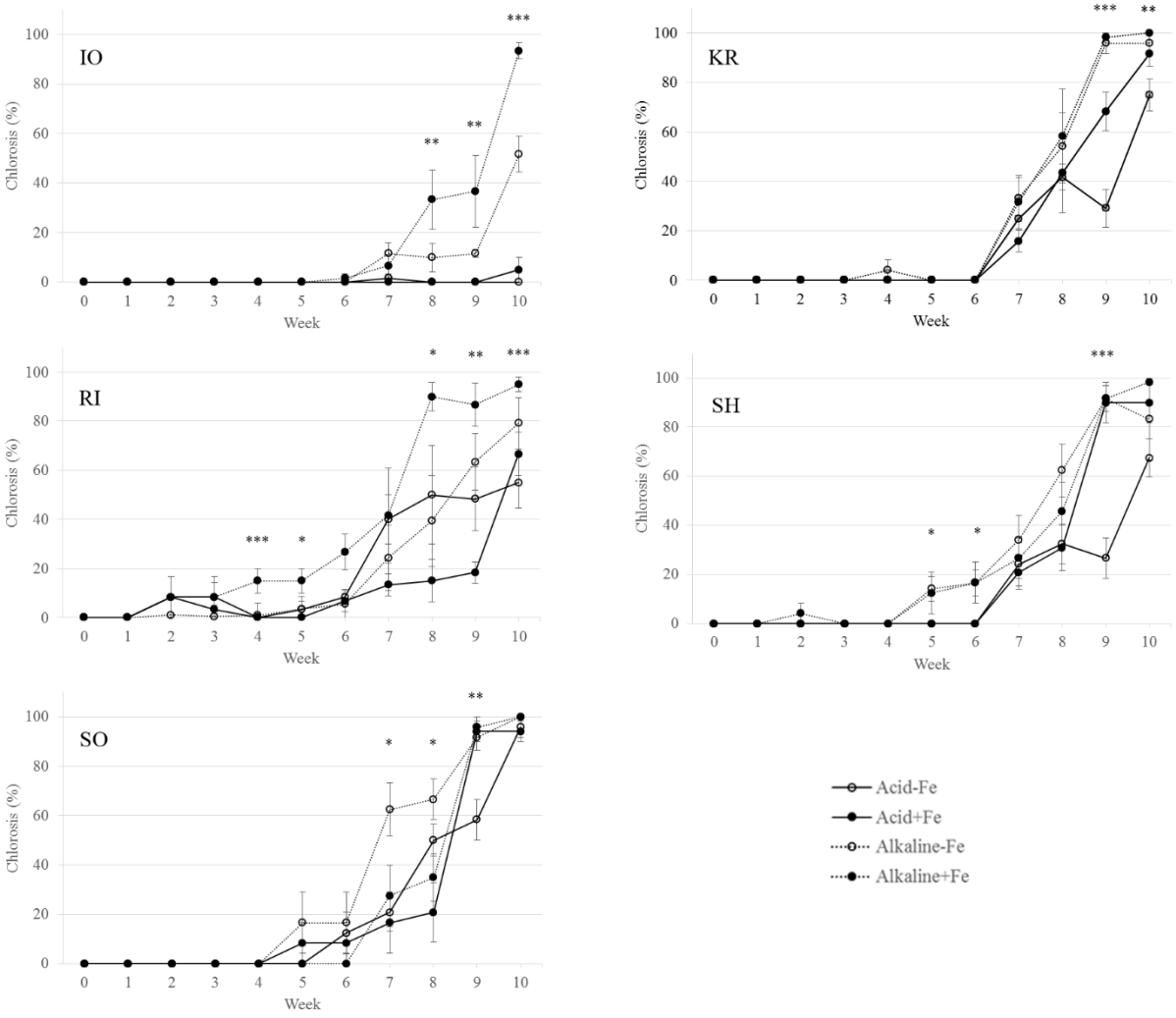
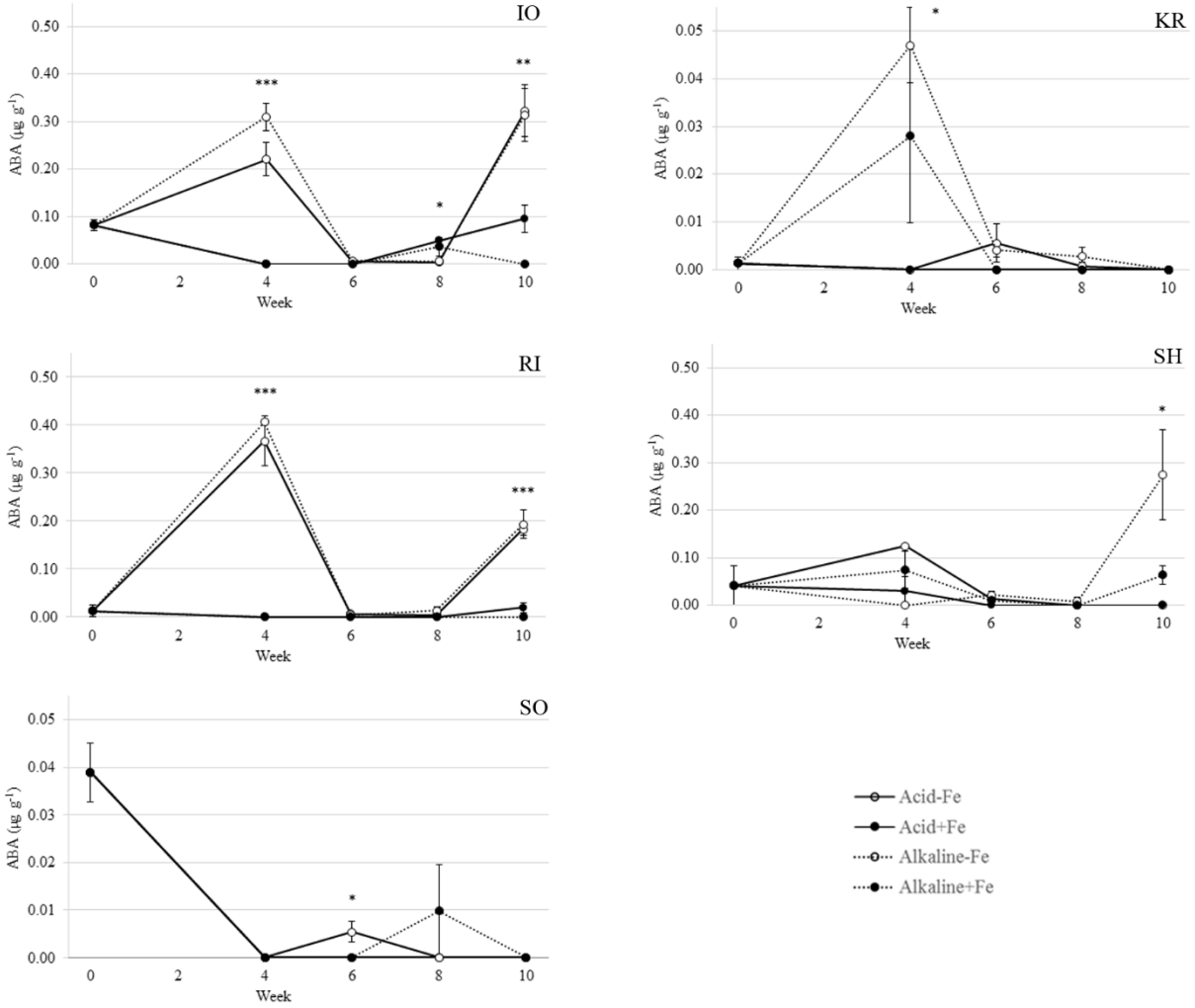


**Fig. 4** Abscisic acid content (μg $g^{-1}$) in the leaves of the five azaleas tested (*R. indicum* 'Osakazuki', IO; *R. obtusum* 'Kirin', KR; *R. ripense*, RI; *R. indicum* 'Shinsen', SH; *R.* × *pulchrum* 'Sen-e-oomurasaki', SO) during 10 weeks of cultivation in four different treatments (Acid-Fe,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



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