

Evaluating Calibrachoa (*Calibrachoa ×hybrida* Cerv.) Genotype Sensitivity to Iron Deficiency at High Substrate pH

Ryan W. Dickson

University of New Hampshire Cooperative Extension, University of New Hampshire Department of Biological Sciences, Durham, NH 03824

Paul R. Fisher¹

Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL 32611

Sonali R. Padhye

PanAmerican Seed, 622 Town Road, West Chicago, IL 60185

William R. Argo

The Blackmore Company, 10800 Blackmore Avenue, Belleville, MI 48111

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Abstract. Floriculture crop species that are inefficient at iron uptake are susceptible to developing iron deficiency symptoms in container production at high substrate pH. The objective of this study was to compare genotypes of iron-inefficient calibrachoa (*Calibrachoa ×hybrida* Cerv.) in terms of their susceptibility to showing iron deficiency symptoms when grown at high vs. low substrate pH. In a greenhouse factorial experiment, 24 genotypes of calibrachoa were grown in peat:perlite substrate at low pH (5.4) and high pH (7.1). Shoot dry weight, leaf SPAD chlorophyll index, flower index value, and shoot iron concentration were measured after 13 weeks at each substrate pH level. Of the 24 genotypes, analysis of variance (ANOVA) found that 19 genotypes had lower SPAD and 18 genotypes had reduced shoot dry weight at high substrate pH compared with SPAD and dry weight at low substrate pH. High substrate pH had less effect on flower index and shoot iron concentration than the pH effect on SPAD or shoot dry weight. No visual symptoms of iron deficiency were observed at low substrate pH. Genotypes were separated into three groups using *k*-means cluster analysis, based on the four measured variables (SPAD, dry weight, flower index, and iron concentration in shoot tissue). These four variables were each expressed as the percent reduction in measured responses at high vs. low substrate pH. Greater percent reduction values indicated increased sensitivity of genotypes to high substrate pH. The three clusters, which about represented high, medium, or low sensitivity to high substrate pH, averaged 59.7%, 42.8%, and 25.2% reduction in SPAD, 47.7%, 51.0%, and 39.5% reduction in shoot dry weight, and 32.2%, 9.2%, and 27.7% reduction in shoot iron, respectively. Flowering was not different between clusters when tested with ANOVA. The least pH-sensitive cluster included all four genotypes in the breeding series ‘Calipetite’. ‘Calipetite’ also had low shoot dry weight at low substrate pH, indicating low overall vigor. There were no differences between clusters in terms of their effect on substrate pH, which is one potential plant iron-efficiency mechanism in response to low iron availability. This experiment demonstrated an experimental and statistical approach for plant breeders to test sensitivity to substrate pH for iron-inefficient floriculture species.

Floriculture species differ in susceptibility to developing micronutrient disorders, particularly iron and manganese toxicity or deficiency, depending on the efficiency at which micronutrients are taken up by plant

roots and the solubility of micronutrients as a function of pH (Albano and Miller, 1998; Argo and Fisher, 2002). The solubility of inorganic Fe³⁺ decreases 1000-fold for each unit increase in pH (Lindsay, 1979). Decreased solubility results in low levels of water-extractable iron in soilless substrates when pH is above 6 (Peterson, 1981). Appearance of iron deficiency in iron-inefficient species such as calibrachoa (*Calibrachoa ×hybrida*) develops at high substrate pH levels (pH > 6.4) and often requires supplemental applications of chelated iron fertilizer (Fisher et al., 2003).

Cultivars of iron-efficient floriculture species have been shown to differ in their tendency to accumulate excess iron/manganese

at low substrate pH (Albano and Miller, 1998; Harbaugh, 1995). Marigold (*Tagetes erecta* L.) cultivars developed different degrees of “leaf bronzing” resulting from toxic iron levels in mature leaves after high micronutrient concentrations were applied to the substrate (Albano and Miller, 1998). Susceptible cultivars of pentas (*Pentas lanceolata* Benth.) developed lower leaf necrosis at substrate pH less than 5.5, which was correlated with high tissue iron levels (Harbaugh, 1995).

Cultivars of agronomic crop species grown at high pH and in calcareous soils are also known to differ in susceptibility to iron deficiency (Fröehlich and Fehr, 1981; Gao and Shi, 2007; Marschner, 1995; Norvell and Adams, 2006). Typical symptoms of iron deficiency include interveinal chlorosis of young shoots and reduced shoot growth during early stages and can progress to severe stunting and shoot tip death in later stages (Marschner, 1995; Römheld, 1987). Symptoms of iron deficiency are well documented for floriculture species, with photos of iron deficiency for a range of floriculture species including calibrachoa published by Argo and Fisher (2002), Gibson et al. (2007), and others.

Strategies for evaluating agronomic crop species for sensitivity to iron deficiency include growing cultivars in noncalcareous and calcareous soils and measuring differences in shoot chlorosis, growth, and yield (Fröehlich and Fehr, 1981; Graham et al., 1992; Hintz et al., 1987; Niebur and Fehr, 1981). Fröehlich and Fehr (1981) used percent reduction in plant height and yield to compare soybean (*Glycine max* L.) cultivars grown in calcareous vs. noncalcareous soils. Gao and Shi (2007) used hierarchical cluster analysis to group peanut (*Arachis hypogaea* L.) cultivars by sensitivity to iron chlorosis based on leaf SPAD chlorophyll content, physiologically “active” leaf iron at flowering stage, and pod yield.

Genotypic differences in iron efficiency has not been studied in calibrachoa, which often shows iron deficiency symptoms at high substrate pH or low iron fertilizer level (Wik et al., 2006). The objective of this study was to compare 24 genotypes of calibrachoa for their sensitivity to showing iron deficiency symptoms (reduced shoot growth, chlorophyll content, tissue iron concentration, and flower number as well as chlorosis and necrosis on new shoots) when grown at high vs. low substrate pH. Twenty of the genotypes were commercial cultivars from four breeding companies, in addition to four experimental genotypes. Eleven genotypes were propagated from seed and the remainder from vegetative cuttings. We hypothesized that differences in sensitivity may be related to the tendency for a genotype to increase pH and thereby reduce iron solubility, and/or higher demand for iron (milligrams iron per plant, from either a high required iron concentration per unit dry weight, or high vigor in terms of dry weight gain).

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¹Corresponding author. E-mail: pfisher@ufl.edu.

In a greenhouse factorial experiment, seedling plugs and rooted liners of each genotype were transplanted into 11.4-cm-diameter containers and grown for 13 weeks in a soilless peat:perlite substrate at low (initial 5.4) and high (initial 7.1) substrate pH, with analysis of final substrate pH and substrate-electrical conductivity, leaf SPAD chlorophyll content, total shoot dry weight, tissue iron concentrations, and visual indexes of iron chlorosis symptoms and flower number.

Materials and Methods

Experimental design

The experiment was a 24 genotype by two substrate pH factorial using a randomized complete block design with eight blocks (one replicate per block). Genotypes were grown in plastic azalea containers at one plant per container, and each treatment replicate was an individual container (384 total containers). Blocks were divided evenly between two adjacent, identical greenhouses at one block per greenhouse bench. Greenhouse benches were oriented north to south.

Plant materials and propagation

On 30 Jan. 2014, 24 genotypes of iron-inefficient calibrachoa were transplanted from 128-count seedling trays into 11.4-cm (4.5-inch)-diameter azalea containers (500 cm³; Poppelman Plastics US LLC, Claremont, NC) at one plant per container. Twenty genotypes were from six commercial breeding series ('Aloha Kona', 'Cabaret', 'Calipetite', 'Crave', 'Kabloom', and 'Minifamous') in addition to four experimental genotypes. Eleven genotypes were propagated by seed and 13 by vegetative tip cuttings.

Vegetatively propagated genotypes included genotypes of 'Aloha Kona' ('Canary Yellow', 'Milk and Honey', 'True Blue', and 'Dark Red'), 'Cabaret' ('Deep Blue', 'Bright Red', 'Deep Yellow', and 'White 2015'), 'Calipetite' ('Red', 'Blue', 'Yellow', and 'White'), and 'Minifamous Pink 2014'. Seed-propagated genotypes included genotypes of 'Kabloom' ('Blue', 'Deep Pink', 'Denim', 'Red', 'White', and 'Yellow'), 'Crave Sunset', and the remaining experimental genotypes ('E113', 'E144', 'E153', and 'E15597').

Growing conditions and data collection

The substrate was (v/v) 80% Canadian Sphagnum peat (Sun Gro Horticulture, Agawam, MA) with long fibers and little dust (Von Post scale 1–2; Puustjarvi and Robertson, 1975) and 20% coarse perlite with preplant fertilizer (in g·m⁻³ 41.7N, 15.3P, 63.4K, 111.2Ca, 83.4S, 4.2Mg, 0.08B, 0.15Cu, 0.08Fe, 0.54Mn, 0.15Mo, and 0.46Zn) but with no initial liming agent. Nutrients were derived from ammonium nitrate, ammonium phosphate, calcium nitrate, boric acid, copper sulfate, iron ethylenediaminetetraacetic acid (EDTA), magnesium nitrate, manganese sulfate, potassium nitrate, sodium molybdate,

and zinc sulfate. Hydrated dolomitic limestone [Graymont Western Lime, Inc., Eden, WI, 97% Ca(OH)₂·MgO of which 92% passed through a 45- μ m mesh and had an acid neutralizing value of 140 calcium carbonate equivalents (CCEs)] was incorporated at rates of 1.1 and 2.0 kg·m⁻³ for initial substrate pH levels of 5.4 and 7.1, respectively. Substrate pH levels of 5.4 and 7.1 were considered low and high, respectively, according to the pH range (5.8 to 6.2) recommended for adequate nutrient availability for most bedding plant species by Peterson (1981). Plants at low pH (5.4) were expected to have adequate micronutrient availability for growth, whereas plants at high pH (7.1) were expected to develop micronutrient deficiency based on previous reports for calibrachoa (Argo and Fisher, 2002; Gibson et al., 2007). Each container was irrigated with 200 mL of nutrient solution at transplant. Macronutrients were supplied from a commercial water-soluble fertilizer at (in mg·L⁻¹) 150N, 16P, 124K, 35Ca, and 9Mg (GreenCare Fertilizers, Kakanee, IL) where 25% of total nitrogen was supplied as ammonium (NH₄⁺-N) and the remainder as nitrate (NO₃⁻-N). Micronutrients were supplied at (in mg·L⁻¹) 1.0Fe, 1.0B, 1.0Mn, 0.5Zn, 0.2Cu, and 0.04Mo derived from H₃BO₃, MnSO₄·H₂O, ZnSO₄·7H₂O, CuSO₄·5H₂O, (NH₄)₆MoO₇·H₂O, and Fe(II)EDTA (13% Fe; GreenCare Fertilizers). Fertilizer nutrients were derived from the same carriers previously mentioned, mixed in tap water which contained additional fertilizer nutrients (in mg·L⁻¹) at 0.3P, 4.3K, 32.2Ca, 22.1Mg, and 42.0S and alkalinity of 20.4 mg HCO₃⁻/L. Plastic saucers placed under each container collected leached solution and allowed for nutrient solution reabsorption following an irrigation event. After the initial irrigation at transplant, the remaining irrigation events consisted of 150 mL of nutrient solution applied to each container replicate.

On 21 Mar. 2014, all plants were pruned to a uniform height of 7.5 cm and width of 5.0 cm, and the pruned tissue was dried and saved for measuring total shoot dry weight at final data collection. Flowable Limestone (Limestone F, Clearys, NJ, containing 50% dolomitic limestone of which 89% passed through a 200 mesh and with an acid neutralizing value of 56.49% CCE) was prepared at a 1:75 ratio with tap water and 100 mL was applied once to each container at high substrate pH (pH 7.1) to ensure substrate pH remained above 6.5. Equivalent volumes of tap water were applied once to each container at low substrate pH (pH 5.4). The nutrient solution was modified for the remainder of the experiment by halving the original concentration of all micronutrients, to increase severity of micronutrient-deficiency symptoms.

Plants were harvested for final data collection on 2 May 2014, after a total of 13 weeks. The average daily temperature and daily light integral over the 13-week period were (mean \pm sd) 20.8 \pm 3.5 °C and

9.3 \pm 4.3 mol·m⁻²·d⁻¹ of photosynthetically active radiation, respectively (Watchdog A-Series and Mini Station Model 2475 data loggers; Spectrum Technologies, Inc., Aurora, IL).

Final substrate pH was measured (Orion Versa Star; ThermoFisher Scientific, Waltham, MA) on composite leachate samples using the pour-through method described by Whipker et al. (2003) for greenhouse container crops. Composite samples consisted of combining leachate from treatment replicates (\approx 50 mL leachate per replicate) in adjacent blocks for a total of four composite leachate samples per treatment.

Leaf chlorophyll content was measured nondestructively using a Minolta SPAD meter (Soil Plant Analysis Department; Ramsey, NJ). The SPAD meter indexes leaf chlorophyll content by measuring the ratio of light transmitted through leaves at 750- and 940-nm wavelengths. For this experiment, dark green leaves had SPAD index values greater than 35, whereas leaves that showed chlorosis had SPAD index values of 30 or less. SPAD data consisted of the average of three SPAD measurements taken from leaves on three separate shoot tips per replicate. Each SPAD measurement was taken on a fully expanded leaf within the top 5.1 cm of the shoot.

Severity of iron chlorosis was measured for each replicate using a six-point visual index. Chlorosis index values of 0, 1, 2, 3, 4, and 5 indicated shoots showed no symptoms of iron chlorosis, shoots were pale green, <50% of shoots showed marginal leaf chlorosis, <50% of shoots showed interveinal chlorosis, >50% of shoots showed interveinal chlorosis, and shoots were bleached and showed necrosis, respectively.

Flower number was measured for each replicate using a six-point visual index. Flower index values of 0, 1, 2, 3, 4, and 5 indicated that replicates had 0, 1 to 5, 6 to 10, 11 to 20, 21 to 40, and >40 flowers, respectively. Digital pictures were taken of representative plants with actual flower numbers corresponding to index values and were used as a visual guide to improve consistency in data collection.

Shoot dry weight was measured for each replicate by destructively sampling shoot mass. Shoots were cut at the base of the stem near the substrate, washed with 0.1 N HCl, rinsed with deionized water, and oven-dried at 70 °C for 48 h. Shoot dry weight was measured before pruning and was combined with shoot dry weight at the end of the experiment. Dried shoot mass was combined into composite samples the same as previously mentioned for leachate data. Composite samples were ground and analyzed for shoot iron concentration by inductively-coupled plasma atomic emission spectrophotometry (Karla, 1998) by Quality Analytical Laboratories (Panama City, FL).

Genotype sensitivity to substrate pH was calculated as the percent reduction in shoot dry weight, leaf SPAD, flower index value, and shoot iron concentration for plants grown at high substrate pH compared with these

measurement parameters when plants were grown at low substrate pH using Eq. [1]. Percent reduction data consisted of eight replicates for shoot dry weight, leaf SPAD, and flower index value and four replicates for shoot iron concentration.

Percent reduction

$$= (1 - (\text{value at high pH} \div \text{value at low pH})) \times 100$$

[1]

Statistical and cluster analysis. PROC GLIMMIX ANOVA in SAS 9.4 (SAS Institute, Cary, NC) was used to evaluate genotype and substrate pH effects on absolute values at high and low pH for leaf SPAD, shoot dry weight, flower index value, iron chlorosis index, and shoot iron concentration. PROC GLIMMIX was also used to analyze genotype main effects on percent reduction (pH sensitivity) of leaf SPAD, shoot dry weight, flower index value, and shoot iron concentration. Genotype main effects on substrate pH were evaluated at high and low pH levels. PROC FASTCLUS *k*-means cluster analysis was used in combination with PROC FREQ to separate genotypes into clusters of high, medium, and low sensitivity to substrate pH. Cluster centroid values were based on genotype least-square means for percent reduction in leaf SPAD, shoot dry weight, flower index value, and shoot iron concentration. ANOVA with PROC GLM was used to evaluate cluster differences in the genotype means for the four clustering measured variables. Tukey's honestly significant difference at

$\alpha = 0.05$ significance level was used for mean separation in all analyses.

Results and Discussion

Calibrachoa genotype (24 genotypes tested) interacted with the two substrate pH levels in terms of shoot dry weight ($P < 0.0001$), leaf SPAD ($P < 0.0001$), flower index value ($P = 0.0024$), and shoot iron concentration ($P < 0.0001$) (least-square means shown in Fig. 1). Analysis of the visual iron chlorosis index also showed an interaction between genotype and substrate pH level, whereby no obvious leaf discoloration was observed at low substrate pH but genotypes differed in iron chlorosis index at high pH. Because iron chlorosis index values at high substrate pH were negatively correlated with leaf SPAD measured at high substrate pH ($R^2 = 0.847$), only SPAD values are reported.

We interpreted a low shoot dry weight at low substrate pH to indicate a low inherent shoot vigor for a particular genotype. Shoot dry weight ranged from 3.42 g ('Calipetite Yellow') to 23.56 g ('Kabloom Deep Pink') at low substrate pH, when micronutrient solubility would not be expected to be limiting to growth. In contrast, we interpreted the shoot dry weight of a genotype grown at high substrate pH, especially in comparison with growth at low pH, to represent shoot vigor under high pH stress. Shoot dry weight was lower for plants grown at high substrate pH compared with growth at low pH for 18 genotypes (Fig. 1A). Shoot dry weight of 'Crave Sunset', 'Cabaret White 2015', and

all 'Calipetite' genotypes did not differ between plants grown at high vs. low pH levels. Genotypes of the 'Calipetite' breeding series overall had the lowest shoot dry weight at both high and low pH.

Leaf SPAD index values were lower at high substrate pH for 19 genotypes (least-square means in Fig. 1B). SPAD at low substrate pH ranged from 37.9 ('Aloha Kona Canary Yellow') to 51.2 ('E153'). SPAD at high substrate pH ranged from 15.5 ('Cabaret Red') to 42.3 ('Calipetite Blue'). SPAD values did not differ between substrate pH levels for the genotypes 'Cabaret Deep Blue', 'Calipetite Red', 'Calipetite Blue', 'Calipetite Yellow', and 'Minifamous Pink 2014'. Genotypes of the 'Calipetite' breeding series overall had the highest SPAD values and darkest green foliage at high substrate pH. 'Calipetite White' was developing symptoms of chlorosis at the end of the experiment, but emerging young leaves were too narrow to measure with the SPAD meter.

Flower index value was lower for seven genotypes at high substrate pH compared with low pH (Fig. 1C). Flower index at low substrate pH ranged from 3.6 ('E15597') to 4.8 ('Aloha Kona Canary Yellow', 'Aloha Kona Dark Red', and 'E113'). At high substrate pH, flower index ranged from 2.8 ('E15597') to 4.1 ('Aloha Kona Canary Yellow' and 'Cabaret Deep Yellow'). Flowering in calibrachoa can be reduced at high substrate pH and when plants are iron deficient (Fisher et al., 2003).

Shoot iron ranged from (in $\mu\text{g Fe/g}$ of dry weight) 49.6 ('E153') to 88.0 ('Calipetite Red') at low substrate pH. Shoot iron levels

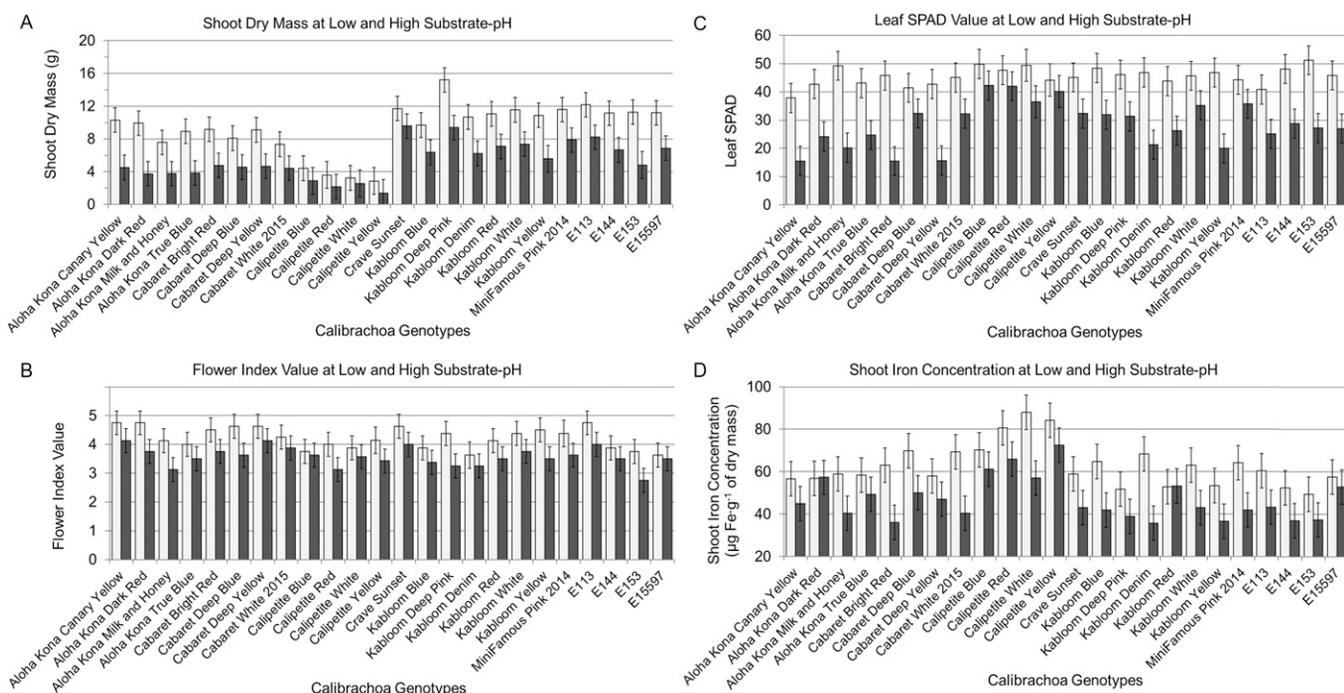


Fig. 1. (A) Shoot dry weight, (B) leaf SPAD chlorophyll content, (C) flower index value, and (D) shoot iron concentration in calibrachoa genotypes grown for 13 weeks at low (pH 5.4, open bars) and high substrate pH (pH 7.1, dark bars). Data are least-square means for shoot dry weight ($n = 8$), leaf SPAD ($n = 8$), flower index value ($n = 8$), and shoot iron concentration ($n = 4$). Error bars indicate $\pm 95\%$ confidence intervals using Tukey's honestly significant difference at the $\alpha = 0.05$ significance level.

at low substrate pH were low compared with the reported iron sufficiency range [61 to 150 µg Fe/g for calibrachoa from Gibson et al. (2007), or 61 to 150 µg Fe/g of dry weight, Vetanovetz (1996) for general greenhouse crops]. However, poor correlations between total leaf iron and chlorophyll content have been found in other research (Marschner, 1995; Mengel, 1994) because only a proportion of iron in shoot tissue is physiologically active. In this study, shoot iron was also poorly correlated with leaf

SPAD chlorophyll content (data not shown) and plants at low substrate pH had dark green foliage. Shoot iron concentration was lower at high substrate pH compared with plants grown at low pH for 11 genotypes (Fig. 1D). At high substrate pH, shoot iron concentration ranged from 35.7 (‘Kabloom Denim’) to 72.5 (‘Calipetite Yellow’) (µg Fe/g of dry weight). Shoot iron concentration was negatively correlated with shoot dry weight at both low substrate pH ($R^2 = 0.605$) and at high pH ($R^2 = 0.413$). In other words, smaller

plants tended to have higher iron concentration. This negative correlation between plant size and iron concentration may have been a nutrient dilution effect in rapidly expanding shoot tissue with more vigorous plants (Marschner, 1995).

Genotypes differed in sensitivity to substrate pH, as quantified by the percent reduction in shoot dry weight ($P = 0.0003$), leaf SPAD ($P < 0.0001$), flower index value ($P = 0.0458$), and shoot iron ($P = 0.0006$) at high substrate pH compared with low substrate pH

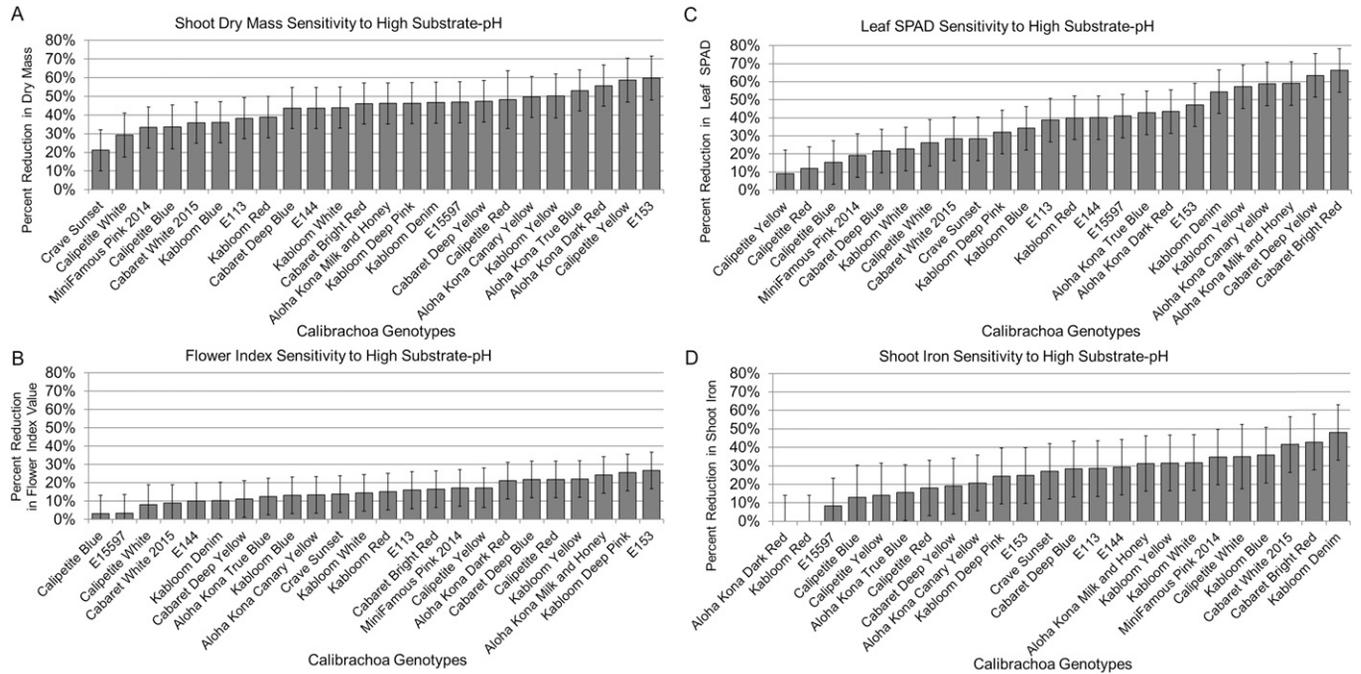


Fig. 2. Genotype sensitivity to substrate pH quantified as the percent reduction in (A) shoot dry weight, (B) leaf SPAD, (C) flower index value, and (D) shoot iron concentration at high (pH 7.1) compared with low (pH 5.4) substrate pH. Genotypes are ordered left to right from lowest to highest sensitivity. Data are least-square means for percent reduction in shoot dry weight ($n = 8$), leaf SPAD ($n = 8$), flower index value ($n = 8$), and shoot iron ($n = 4$). Error bars indicate $\pm 95\%$ confidence intervals using Tukey’s honestly significant difference at the $\alpha = 0.05$ significance level.

Table 1. Genotypes clustered into groups of about high, medium, and low sensitivity to substrate pH by luster analysis. Clusters were based on genotype least-square means for percent reduction in shoot dry weight, leaf SPAD, flower index value, and shoot iron concentration. Percent reduction at high substrate pH data are cluster least-square means for percent reduction in leaf SPAD, shoot dry weight, flower index value, and shoot iron concentration. Mean separation used Tukey’s honestly significant difference at the $\alpha = 0.05$ significance level.

Percent reduction at high substrate pH	Genotypes clustered by sensitivity to high substrate pH		
	High sensitivity (cluster 1)	Medium sensitivity (cluster 2)	Low sensitivity (cluster 3)
Leaf SPAD chlorophyll content	59.6% a	42.8% b	25.2% c***
Shoot dry weight	47.7% ab	51.0% a	39.5% b*
Shoot iron concentration	32.2% a	9.2% b	27.8% a**
Flower index value	16.2% a	15.8% a	14.7% a ^{ns}
	High sensitivity (cluster 1)	Medium sensitivity (cluster 2)	Low sensitivity (cluster 3)
	Aloha Kona Canary Yellow	Aloha Kona Dark Red	Cabaret White 2015
	Aloha Kona Milk and Honey	Aloha Kona True Blue	Cabaret Deep Blue
	Cabaret Bright Red	E153	Calipetite Red
	Cabaret Deep Yellow	E15597	Calipetite White
	Kabloom Yellow	Kabloom Red	Calipetite Yellow
	Kabloom Denim		Calipetite Blue
			Crave Sunset
			E113
			E144
			Kabloom Deep Pink
			Kabloom White
			Kabloom Blue
			Minifamous Pink 2014

^{ns}, *, **, ***Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

(Fig. 2). A greater percent reduction indicated greater sensitivity. Percent reduction ranged from 21.1% ('Crave Sunset') to 59.9% ('E153') for shoot dry weight (Fig. 2A), from 9.1% ('Calipetite Yellow') to 66.3% ('Cabaret Bright Red') for SPAD (Fig. 2B), from 3.1% ('Calipetite Blue') to 26.8% ('E153') for flower index value (Fig. 2C), and from -1.0% ('Aloha Kona Dark Red') to 48% ('Kabloom Denim') for shoot iron concentration (Fig. 2D). Negative values indicated increased shoot iron at high substrate pH compared with low pH.

Varietal effects on substrate pH were not correlated with genotype sensitivity to high substrate pH, and therefore substrate pH data were not shown. Substrate pH in low pH treatments remained within the recommended range (pH 5.4 to 6.2) for calibrachoa (Argo and Fisher, 2002). Only 'Aloha Kona Dark Red' affected substrate pH in high pH treatments and had a final substrate pH of 6.6, whereas all other genotypes had final substrate pH not different from initial 7.1.

Genotypes were separated into three clusters using *k*-means cluster analysis that corresponded to about high, medium, and low sensitivity to substrate pH based on percent reduction in leaf SPAD, shoot dry weight, flower index value, and shoot iron concentration (Table 1). Grouping plant species into three pH-management groups is a commonly used strategy for nutrient management (Argo and Fisher, 2002). Three clusters were, therefore, chosen as a practical and manageable number for grouping and evaluating genotypes by differences in sensitivity. The influence of each variable in terms of separating genotypes into clusters was shown by their R^2 values, which were 0.801, 0.452, 0.296, and 0.011 for percent reduction in SPAD, shoot iron, shoot dry weight, and flower index, respectively. ANOVA showed that genotype clusters differed in percent reduction in leaf SPAD ($P < 0.0001$), shoot iron ($P = 0.0018$), and shoot dry weight ($P = 0.0250$), but not percent reduction in flower index ($P = 0.8938$). High, medium, and low sensitivity cluster least-square means were 59.7%, 42.8%, and 25.2% for SPAD, 47.7%, 51.0%, and 39.5% for shoot dry weight, and 32.2%, 9.2%, and 27.7% for shoot iron (Table 1).

One of several iron-efficiency mechanisms is a drop in substrate pH by plant roots to increase iron solubility and uptake. However, there was no difference between clusters in terms of their least-square mean substrate pH at the high lime level (i.e., the high substrate pH treatment, data not shown). Other iron-efficiency mechanisms were not measured in this experiment, such as physiologically "active" iron levels in shoot tissue, root iron reductase activity, and root efflux of organic compounds which solubilize iron (Albano and Miller, 1996; Bienfait, 1988; Gao and Shi, 2007; Landsberg, 1986; Marschner et al., 1986; Mengel, 1994; Römheld and Marschner, 1983). However, lack of overall plant vigor did appear to reduce sensitivity to pH under the trial

conditions, as illustrated by the 'Calipetite' genotypes in the least pH-sensitive group. 'Calipetite' genotypes had low shoot dry weight at low substrate pH, indicating low overall vigor. Low vigor would reduce total iron requirement per plant, which may have delayed the onset of stunting and chlorosis symptoms at high substrate pH.

The clusters can be visually illustrated in two dimensions by plotting genotypes based on percent reduction in shoot dry weight vs. leaf SPAD (Fig. 3). Manuel and Alcántara (2002) plotted cultivars of pear (*Pyrus communis* L.), olive (*Olea europaea* L.), and quince (*Cydonia oblonga* Mill.) by relative reduction in shoot growth vs. reduction in SPAD to evaluate cultivars for sensitivity to iron deficiency when grown in iron-limited nutrient solution. In Fig. 3A, vertical and

horizontal dashed lines indicate median values for percent reduction in shoot dry weight (46.2%) and leaf SPAD (39.3%), respectively, and separate genotypes into 50th percentile groups. Figure 3B shows genotypes clustered by high (solid line), medium (dashed line), and low (dotted line) sensitivity. The irregular shape indicated for 'Kabloom Red', 'E113', and 'E144' occurs because two additional dimensions (shoot iron concentration and flower index) were also used in clustering but are not shown in Fig. 3B.

Conclusions

Reduced shoot growth and increased chlorosis occurred in calibrachoa grown at high substrate pH, which are common symptoms

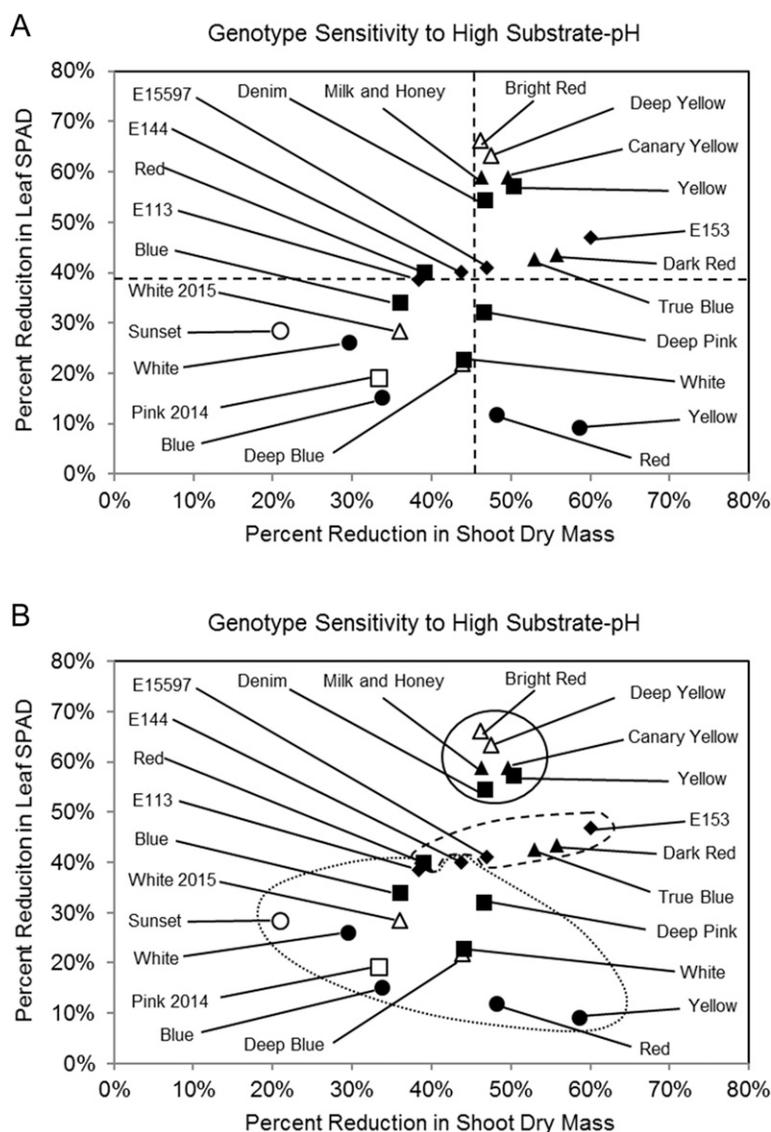


Fig. 3. Genotypes plotted by percent reduction in shoot dry weight (x axis) vs. percent reduction in leaf SPAD (y axis). Data represent least-square means of eight replicates. Horizontal and vertical dashed lines in Fig. 3A indicate median values for percent reduction in leaf SPAD (39.3%) and shoot dry weight (46.2%), respectively. Figure 3B groups genotypes with about high (solid line), medium (dashed line), and low (dotted line) sensitivity to substrate pH from *k*-means cluster analysis. Breeding series is indicated by the data point style (▲ = 'Aloha Kona', △ = 'Cabaret', ● = 'Calipetite', ○ = 'Crave', ■ = 'Kabloom', □ = 'Minifamous', ◆ = experimental genotypes).

of iron deficiency in floriculture species. Genotype sensitivity to substrate pH was quantified by calculating the percent reduction in shoot vigor, leaf SPAD, flower index value, and shoot iron concentration at high vs. low substrate pH. Genotypes were then grouped into high, medium, and low sensitivity to substrate pH using a *k*-means cluster analysis approach.

Genotypes with high, medium, and low sensitivity did not differ in the mean substrate pH at the high substrate pH treatment, which is one potential plant response influencing iron solubility and uptake. 'Calipetite' genotypes were least vigorous at low substrate pH, but were also the least sensitive to high substrate pH. Further investigation of varietal differences, including the parentage of calibrachoa genotypes and breeding lines, could provide greater guidance for calibrachoa breeding.

This experiment demonstrated an experimental and statistical approach for plant breeders to test genotype sensitivity to substrate pH for iron-inefficient floriculture species. This approach may also be used for screening genotypes when breeding genetic series with higher pH tolerance. Plants grown at low substrate pH where micronutrient solubility is not limiting can be compared with growth at high substrate pH. Measurement variables could include shoot dry weight, SPAD, flower number, and shoot iron concentration, in addition to other horticulturally important parameters. In this experiment, shoot dry weight and leaf SPAD were more sensitive than flower index or shoot iron concentration. Growth at low substrate pH was useful to compare inherent vigor of genotypes and provided a baseline to compare against growth under high pH stress. Percent reduction in growth at high vs. low substrate pH was calculated to indicate relative sensitivity to substrate pH. Cluster analysis using *k*-means provides breeders with a statistical approach to grouping genotypes where each genotype is assigned to only one cluster and clusters can be evaluated using ANOVA. Figure 3

demonstrates a simple and visual method for breeders to evaluate genotypes by shoot vigor and leaf color.

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