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Rhizosphere Acidification is Not Part of the Strategy I Iron Deficiency Response of *Vaccinium arboreum* and the Southern Highbush Blueberry

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***Abstract.* *Vaccinium arboreum* (VA) is a wild blueberry species that exhibits wider soil pH tolerance and greater ability for iron and nitrate uptake than cultivated *Vaccinium* species, including southern highbush blueberry (SHB, *V. corymbosum* interspecific hybrids). The ability of VA and SHB to respond to iron deficiency by rhizosphere acidification was investigated. Rooted cuttings of the VA genotype FL09-502 and SHB 'Emerald' were transplanted to a hydroponic system filled with complete nutrient solution. After 14 days of acclimation at 45 μM iron, plants were transferred to unbuffered nutrient solutions containing 90 or 10 μM iron. 'Emerald' and FL09-502 plants grown in 10 μM iron exhibited less iron uptake and lower chlorophyll, total iron, and active iron contents than plants grown in 90 μM iron. Generally, there were no species-level differences in iron or nitrate uptake. Neither FL09-502 nor 'Emerald' acidified the rhizosphere in either the nutrient solution or in a gel-based assay, regardless of external iron concentration. A screen of 18 additional genotypes of VA and SHB confirmed that this response is absent in these taxa. Thus, rhizosphere acidification is not part of the iron deficiency response of SHB and VA. In addition, the ability to acidify the soil is not likely to be responsible for the wider soil pH tolerance of VA.**

Iron is an essential element for plant growth but its uptake by plants can be limited by biotic and abiotic factors (Kim and Guerinot, 2007). Dicots and nongraminaceous monocots respond to iron limitation through strategy I iron uptake. This strategy comprises the coordinated action of three complementary processes functioning at the plasma membrane (PM) of root epidermal cells: a) rhizosphere acidification, b) iron reduction, and c) transmembrane iron transport (Jeong and Conolly, 2009).

Rhizosphere acidification results from the action of PM-bound H^+-ATPases that extrude protons from the symplastic space into the rhizosphere (Kim and Guerinot, 2007). This facilitates iron uptake by increasing the solubility of iron-containing compounds in the soil (Lemanceau et al., 2009), providing an adequate microenvironment for iron reduction and generating the proton motive force for ion uptake (Dell'Orto et al., 2000). Yet despite these benefits, there is wide diversity in the extent and plasticity of rhizosphere acidification among plant species.

Herbaceous and woody species like cucumber (*Cucumis sativus*), cork oak (*Quercus suber*), and plum (*Prunus cerasifera*) are capable of acidifying their rhizosphere by developing or enhancing proton extrusion in response to iron deficiency (Dell'Orto et al., 2000; Gogorcena et al., 2001; Gonzalo et al., 2011). On the other hand, wild apple (*Malus baccata*) and peach-almond hybrids (*Prunus amygdalus* × *Prunus persica*) are not capable of this response (Gonzalo et al., 2011; Wu et al., 2012). Moreover, some species like grapevine (*Vitis vinifera*) exhibit intraspecific diversity of responses; 'Cabernet Sauvignon' is capable of acidifying its rhizosphere whereas 'Balta' is not (Jimenez et al., 2007; Ksouri et al., 2006).

Although iron reduction and uptake by *Vaccinum* sp. have been investigated previously (Darnell and Cruz-Huerta, 2011; Poonnachit and Darnell, 2004), to our knowledge rhizosphere acidification in *Vaccinium* has not been quantified. Southern highbush blueberry, like all cultivated blueberry, is adapted to acidic soils (Coville, 1910; Finn et al., 1993) and experiences iron deficiency when grown in higher pH soils (Gough, 1997). On the other hand, VA is a wild species that exhibits greater tolerance to high pH soils (Lyrene, 1997) and greater efficiency at iron assimilation than SHB (Darnell and Cruz-Huerta, 2011).

Additionally, VA and SHB also differ in their ability to take up nitrate from the soil. Several studies indicate that VA exhibits greater nitrate assimilation than SHB (Darnell and Cruz-Huerta, 2011; Darnell and Hiss, 2006; Poonnachit and Darnell, 2004). Since nitrate anions are transported across the PM in symport with H^+ (Pii et al., 2014; Santi et al., 2003), nitrate uptake in *Vaccinium* sp. could lead to pH increases in the rhizosphere, as it does in other woody plants (Jimenez et al., 2007; Sas et al., 2003). Hence, iron and nitrate uptake are closely related due to the antagonistic effect that these processes have on rhizosphere pH.

This study investigated the rhizosphere acidification capacity of two taxa in the genus *Vaccinium*—*V. corymbosum* interspecific hybrid (SHB) and VA. We hypothesized that a) SHB and VA would respond to iron deficiency by developing or enhancing rhizosphere acidification beyond levels necessary to offset the effect of nitrate uptake on pH, and b) VA has greater ability to acidify the rhizosphere than SHB and therefore is adapted to a wider range of soil conditions.

Materials and Methods

Plant material and growth conditions. About 1-year-old rooted cuttings of SHB 'Emerald' and the VA genotype FL09–502 were individually transplanted to 2-L bottles filled with continuously aerated nutrient solution. Plant size was uniform at the beginning of the experiment, with no differences in initial plant fresh weights (mean = 83.3 g). The nutrient solution contained: 1.0 mM KNO_3, 0.5 mM K_2PO_4, 1.0 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.045 mM H_3BO_3, 0.01 mM $MnSO_4$, 0.1 mM $ZnSO_4$ with 0.3 μM $CuSO_4$, and 0.2 μM Na_2MoO_4, and was changed weekly to maintain nutrient concentrations. Plants were acclimated to the hydroponic system in a nutrient solution buffered to pH 5.5 with 5.0 mM 2-(4-morpholino)-ethane sulfonic acid (MES). During acclimation, iron concentration in the nutrient solution was 45 μM for all plants. Iron was supplied in the form of Sequestrene 330 (10% Fe (III)-diethylenetriamine pentaacetic acid) (Becker Underwood, Inc., Ames, IA). After 14 d, one-half of the plants received 10 μM iron (lower iron) and the other half received 90 μM iron (higher iron) in their nutrient solutions. The initial pH of the nutrient solutions was adjusted to 5.5 for each weekly change, but solutions were prepared without MES to document pH changes in the rhizosphere starting on day 14 as well.

After 56 d of treatment (70 d in total), subjective interveinal chlorosis ratings of the plants were made on a scale of 1 to 5, where 5 was completely green, and decreasing rating levels showed increasing interveinal chlorosis with 1 being severely chlorotic. Following this, plants were destructively harvested, leaves, canes, and roots were separated and weighed, then dried at 72 °C to a constant weight before further analysis.

Rhizosphere pH. The pH of the nutrient solution in each bottle was measured three times per week using an Accumet AP110 portable pH meter (Thermo Fisher Scientific, Inc., Waltham, MA).

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At the end of the experiment (day 70), a representative root sample (1.4% of root fresh weight on average) was removed from each plant. A representative root sample included a root of third order or higher and all its attached lower order roots. Root order was determined as per Valenzuela-Estrada et al. (2008). Root samples were rinsed in 1.0 mM ethylenediaminetetraacetic acid for 5 min to remove any extracellular iron. Samples were then rinsed in ultrapure water (pH 4.6) three times and blotted dry before embedding in reactive gels. Gels contained 2.2 mM bromocresol purple (pH 6.0) and 6.25 $g \cdot L^{-1}$ low-melting point agarose (adapted from Marchner et al., 1982). Pea (*Pisum sativum*) seeds were germinated in tissue paper that had been moistened with tap water starting on day 63. On day 70, pea roots were used as positive controls for the reactive gels (White and Robson, 1989). Embedded *Vaccinium* and pea roots were incubated in the dark at room temperature and photographed on a light table 0, 6, and 24 h after gel set.

Iron and nitrate uptake. Iron and nitrate uptake were measured as depletion from the nutrient solution during all but one of the weeks during the treatment period. Iron concentration in the nutrient solution samples was determined via inductively coupled plasma-atomic emission spectrometry (U.S. Environmental Protection Agency, 1994). Nitrate concentration in the nutrient solution was determined as per Darnell and Cruz-Huerta (2011). Briefly, 10 µL of the nutrient solution were diluted in 1.5 mL of ultrapure water and acidified with 15 µL 12 N HCl. The absorbance of this dilution at 210 nm was measured (Biotek Synergy HT; Biotek Instruments, Inc., Winooski, VT) and used to determine nitrate concentration with a standard curve.

Tissue chlorophyll and mineral content. Chlorophyll content was determined from the youngest fully mature leaf of two branches in each plant at the start (day 14) and end (day 70) of the treatment period. Leaf squares (14.5 mg on average) were cut out of each leaf avoiding the leaf edge and midvein. Two leaf squares per plant were pooled and macerated in liquid nitrogen under low light conditions before adding 1.0 mL 80% acetone. Samples were vortexed and centrifuged before reading the absorbance of the liquid phase of the extraction at 645 and 663 nm (Shimadzu ultraviolet-160, Kyoto, Japan). Chlorophyll content per gram of fresh weight was calculated as per Arnon (1949).

For mineral content determination, dried tissue was ground using a Mini-Mill (Thomas Scientific, Swedesboro, NJ) until it passed through a size of 20 mesh. Total iron content was determined by digesting 0.20 g of tissue in 4.0 mL concentrated HNO_3 and 0.5 mL concentrated HCl for 12 min at 103 °C in a rotating microwave. Once samples had cooled to 50 °C, 15.5 mL of deionized water was added for a final dilution of 1:100. Dilutions were filtered and iron content was measured using inductively coupled plasma atomic emission spectrometry (U.S. Environmental Protection Agency, 2007). Active iron content of the dried tissue was determined as per Darnell and Cruz-Huerta (2011). Active iron is the fraction of total iron extracted via digestion in dilute HCl and is considered a better indicator of the plant's iron status than total iron. About 0.05 g of tissue was incubated with 1.0 mL of 0.1 M HCl for 24 h under continuous shaking at 11,000 rpm. Following incubation, 400 µL of the liquid phase were mixed with 250 µL of 500 mM ascorbic acid, 250 µL of 1.0 mM bathopenanthroline (BP), and 400 µL of 2.5 M sodium

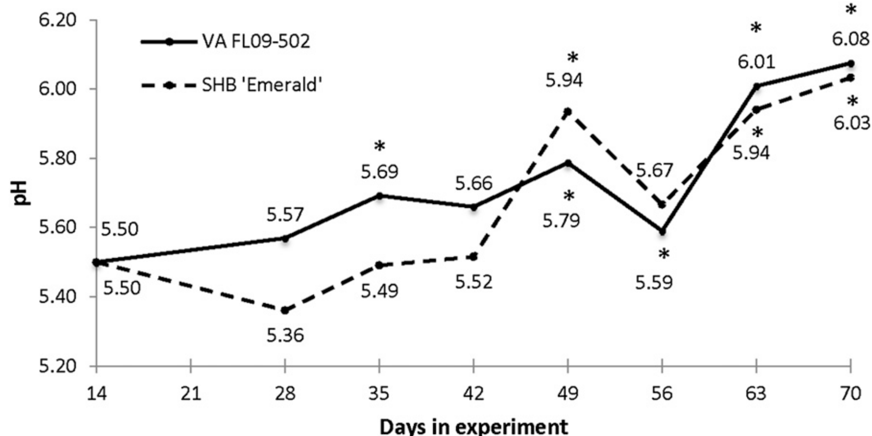

Fig. 1. Nutrient solution pH of hydroponically grown *Vaccinium arboreum* (VA) FL09-502 (solid line) and southern highbush blueberry (SHB) 'Emerald' (dashed line) for 70 d. Starting on day 14, plants were supplied with fresh, unbuffered nutrient solution (initial pH 5.5) with 10 or 90 µM iron on a weekly basis. Values shown are means of n = 6 replications per genotype pooled across iron treatments. Asterisks indicate significant differences from the initial solution pH as determined by posthoc *t* tests (Ho: $\mu_{genotype} = 5.5$; $P \leq 0.05$).

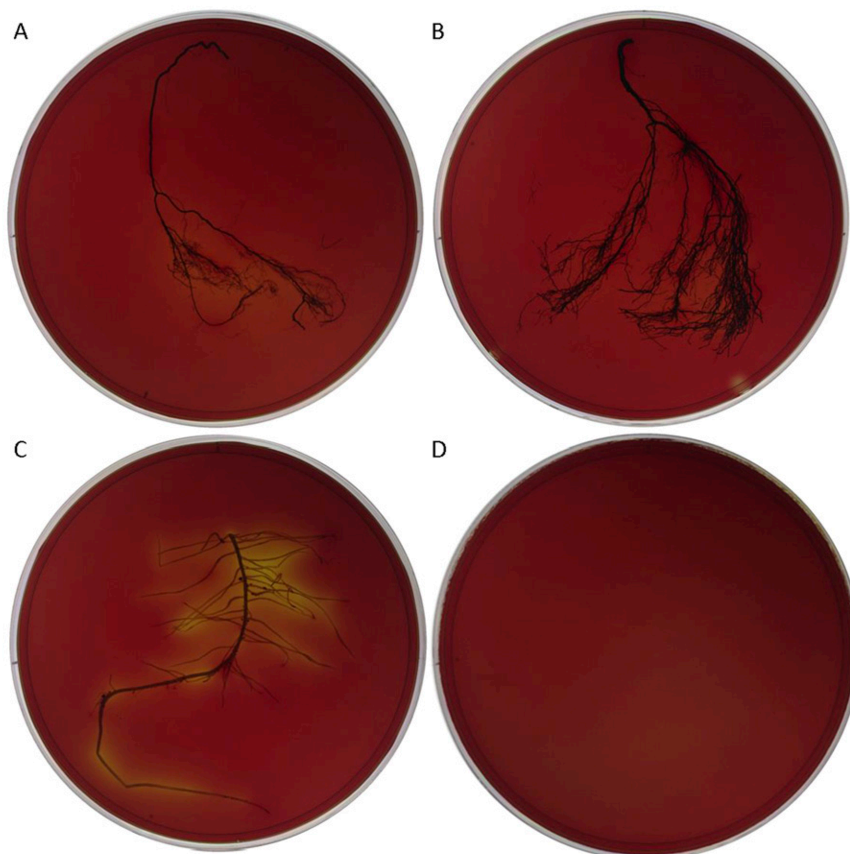

Fig. 2. (**A**) Southern highbush blueberry 'Emerald', (**B**) *Vaccinium arboreum* FL09-502, and (**C**) *Pisum sativum* roots embedded in agarose gels containing pH indicator bromocresol purple (pH 6.0). The indicator changes color from purple to red to yellow as the acidity of the medium increases from pH 7.0 to 5.0. (**D**) Blank gel without roots. No rhizosphere acidification by *Vaccinium* sp. roots was detected. *P. sativum* exhibited rhizosphere acidification in its roots. Photographs taken 24 h after gel set.

Table 1. Leaf chlorophyll concentration in two *Vaccinium* genotypes grown hydroponically in nutrient solutions containing 10 or 90 μM Fe.

	Leaf chlorophyll (mg·g^{-1} fresh wt)		
	Day 14[z]	Day 70	Difference
Genotype[z]			
Emerald	1.40	1.48	0.08NS
FL09–502	1.00	0.88	–0.12NS
Difference	0.40*	0.60NS	
Fe concentration (μM)			
90	1.19	1.52	–0.36*
10	1.20	0.84	0.33NS
Difference	–0.01NS	0.68*	
Genotype × Fe concentration	NS	NS	

[z]'Emerald' southern highbush blueberry and *Vaccinium arboreum* (FL09–502).
NS, *Nonsignificant and significant at $P \leq 0.05$.

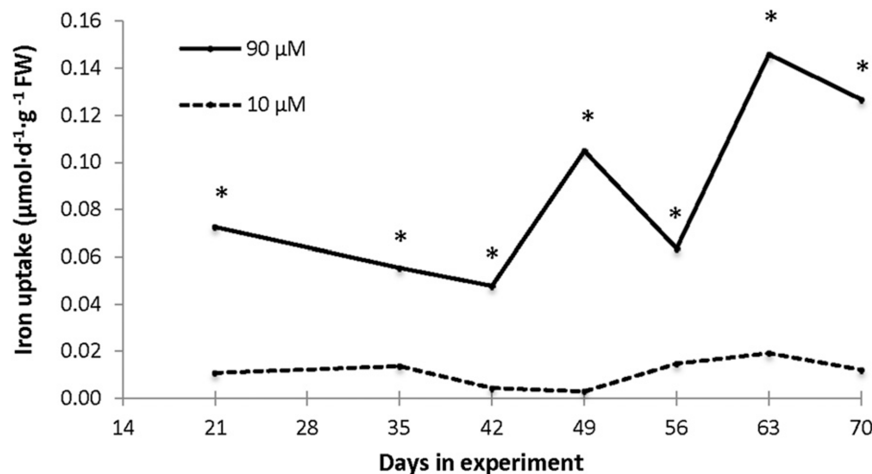

Fig. 3. Iron uptake of hydroponically grown *Vaccinium arboreum* FL09-502 and southern highbush blueberry 'Emerald' in either 90 μM (solid line) or 10 μM iron (dashed line). Values shown are means of n = 6 replications per iron treatment pooled across genotypes. Asterisks indicate significant differences at $P \leq 0.05$. FW = fresh weight.

Table 2. Total and active iron concentrations in leaves, canes, and roots of two *Vaccinium* genotypes grown hydroponically for 70 d in nutrient solutions containing 10 or 90 μM Fe.

	Total iron (μg·g^{-1} dry wt)			Active iron (μg·g^{-1} dry wt)		
	Leaves	Canes	Roots	Leaves	Canes	Roots
Genotype[z]						
Emerald	89.27	78.50	500.50	20.43	26.03	152.19
FL09–502	87.50	105.25	666.36	29.29	30.40	150.92
Difference	1.77NS	–26.75NS	–136.86NS	–8.86NS	–4.37NS	1.27NS
Fe concentration (μM)						
90	98.64	90.58	759.45	31.02	29.01	159.99
10	78.92	93.17	415.17	18.70	27.30	143.12
Difference	19.72*	–2.59NS	344.28*	12.32*	1.71NS	16.87*
Genotype × Fe concentration	NS	NS	NS	NS	NS	NS

[z]'Emerald' southern highbush blueberry and *V. arboreum* (FL09–502).
NS, *Nonsignificant and significant at $P \leq 0.05$.

acetate (pH 4.5). After adding 500 mL of 1-octanol, the Fe^{2+}–BP complex was allowed to migrate to the octanol fraction for 30 min at room temperature. Finally, the absorbance of the octanol fraction was read at 537 nm. A standard curve was used to quantify the active iron content in the samples.

The total nitrogen content of the sample was determined by combustion of ≈0.15 g of tissue under high-purity oxygen at 850 °C and subsequent quantitation by thermal conductivity detection (Association of Analytical Communities, 2012) in a LECO FP628 instrument (LECO Corporation, St. Joseph, MI). To extract nitrate, 0.50 g of tissue was mixed with 20-mL 2.0 N KCl and incubated for 30 min under continuous shaking. Extracts were then filtered and the nitrate concentration of the samples was determined using the automated cadmium reducing method (American Public Health Association, 2000) in a Lachat QuikChem 8000 Series Flow Injection Analysis System (Hatch Company, Loveland, CO).

Intra-specific variation. In a subsequent experiment, a wider pool of SHB and VA genotypes was screened for rhizosphere acidification.Rooted cuttings of SHB 'Abundance', 'Chickadee', 'Farthing', 'Jewel', 'Kestrel', 'Meadowlark', 'Primadonna', 'Snowchaser', and 'Springhigh', eight VA plants (four seedlings and four rooted cuttings) grown from open-pollinated seed, and one micropropagated VA genotype (North American Plants, Inc., Lafayette, OR) were used for this experiment. Rooted cuttings of 'Emerald' and FL09–502 were included as control plants. On average, plants were 13.7 g (fresh weight) at the start of the experiment. Plants were transplanted to 2-L bottles and acclimated to the hydroponic growth system as described above. Iron concentration was maintained at 45 μM for the duration of the experiment. Starting on day 14, buffer MES was withheld from the nutrient solution and pH was measured three times each week. On day 28, representative root samples from each plant were embedded in reactive gels containing pH indicator bromocresol purple. This gel-based method has been successfully used by others to investigate the within-species variability in rhizosphere acidification (Santi and Schmidt, 2009).

Statistical analysis. Treatments were arranged in a 2 × 2 factorial (genotype × iron concentration) in a randomized complete block design with six replications, using a single plant per replication. The pH data collected at the midpoint between solution changes (4th d of every week) were used to fit a general linear mixed model with repeated measurements. Time (measured in weeks) was the quantitative variable, and genotype, iron treatment, and their interaction were the qualitative variables. The interaction term was sliced to test the effect of iron within a genotype. Post-hoc *t* tests were used to compare the nutrient solution pH change each week with the initial pH of 5.5. All data were analyzed using the SAS software (version 9.3; SAS Institute, Cary, NC).

Results

Rhizosphere acidification. The nutrient solution pH of 'Emerald' and FL09-502 measured midway between solution changes increased gradually during the treatment period in both species (Fig. 1) and in both iron treatments (data not shown). When data were fit to a general linear mixed model, only time was a significant predictor of nutrient solution pH change ($P < 0.001$). Neither genotype ($P = 0.11$), iron concentration ($P = 0.33$), nor the sliced interaction of species × iron ($P = 0.06$ for 'Emerald', $P = 0.37$ for FL09-502, respectively) significantly affected the pH change of the nutrient solution. On average, nutrient solution pH increased by ≈0.07 units each week.

Roots of 'Emerald' and FL09-502 embedded in pH-reactive gels for 24 h did not exhibit rhizosphere acidification, regardless of iron treatment (Fig. 2A and B). Pea roots, which were used as a positive control, acidified the gels (Fig. 2C) within 30 min. Gels with no roots did not change color (Fig. 2D).

Plant growth. At the beginning of the treatment period (day 14), chlorophyll concentration in 'Emerald' leaves was significantly greater than in FL09-502 leaves (Table 1). However, there were no significant differences between genotypes at day 70. At the beginning of the treatment period, leaves of plants grown in 10 and 90 μM Fe in the nutrient solution exhibited similar chlorophyll concentrations. By the end of the treatment period, leaves of plants grown under lower iron concentrations contained significantly less chlorophyll than those of plants grown under higher iron concentrations. The interaction between genotype and iron treatment was not significant ($P = 0.66$ at day 14, $P = 0.63$ at day 70). These findings agree with the subjective interveinal chlorosis assessment made on day 70, where plants grown in 90 μM iron averaged a chlorosis score of 4.0 out of 5 while plants grown in 10 μM iron averaged 3.5 out of 5.

After 70 d of growth, neither genotype nor iron treatment affected leaf (mean = 3.6 g), root (mean = 8.8 g), or whole plant (mean = 23.5 g) dry weights. However, cane dry weight in FL09-502 was significantly greater than that in 'Emerald' (15.5 vs. 11.5 g, respectively, $P = 0.04$), but cane dry weights were not different between iron treatments.

Iron nutrition. There were no significant differences in iron uptake between FL09-502 and 'Emerald' during the 56 d treatment period (data not shown). For both genotypes, plants grown under higher iron concentrations exhibited significantly greater iron uptake than plants grown under lower iron concentrations (Fig. 3). The interaction of genotypes × iron concentration was not significant.

The total iron and active iron concentrations did not differ significantly between the genotypes in any organ at day 70 (Table 2). At this time, leaves and roots of plants grown under 90 μM iron exhibited significantly greater total iron and active iron concentrations than those of plants grown under 10 μM iron. There was no effect of iron concentration on total or active iron concentration in canes and there was no significant interaction between genotype and iron treatment.

Nitrate nutrition. There was a high degree of plasticity in the nitrate uptake of the two *Vaccinium* genotypes assayed. 'Emerald' exhibited similar (21, 49, 63, and 70 d) or significantly greater (35, 42, and 56 d) nitrate uptake than FL09-502 (Fig. 4A). The effect of the iron treatment was equally inconsistent. Plants grown in 90 μM iron exhibited significantly greater nitrate uptake than plants grown in 10 μM iron only at days 35 and 56 after treatment (Fig. 4B). The interaction of genotype × iron treatment was not significant.

Leaves, canes, and roots of FL09-502 had significantly greater total nitrogen concentration than those of 'Emerald' (Table 3). Total nitrogen concentration was unaffected by iron treatment or the interaction of genotype × iron (data not shown). Nitrate concentration in leaves of FL09-502 was significantly lower than that in leaves of 'Emerald'. Plants grown under 90 μM iron exhibited greater leaf nitrate concentration than the leaves of plants grown under 10 μM iron. There were no significant differences in the nitrate concentration of canes and roots. There was a significant interaction between genotype and iron on root nitrate concentration. Roots of 'Emerald' grown under 90 μM iron exhibited significantly greater nitrate concentration than roots of 'Emerald' grown under 10 μM iron, but they were not significantly different from roots of FL09-502 at either iron concentration (data not shown). All other interactions were not significant.

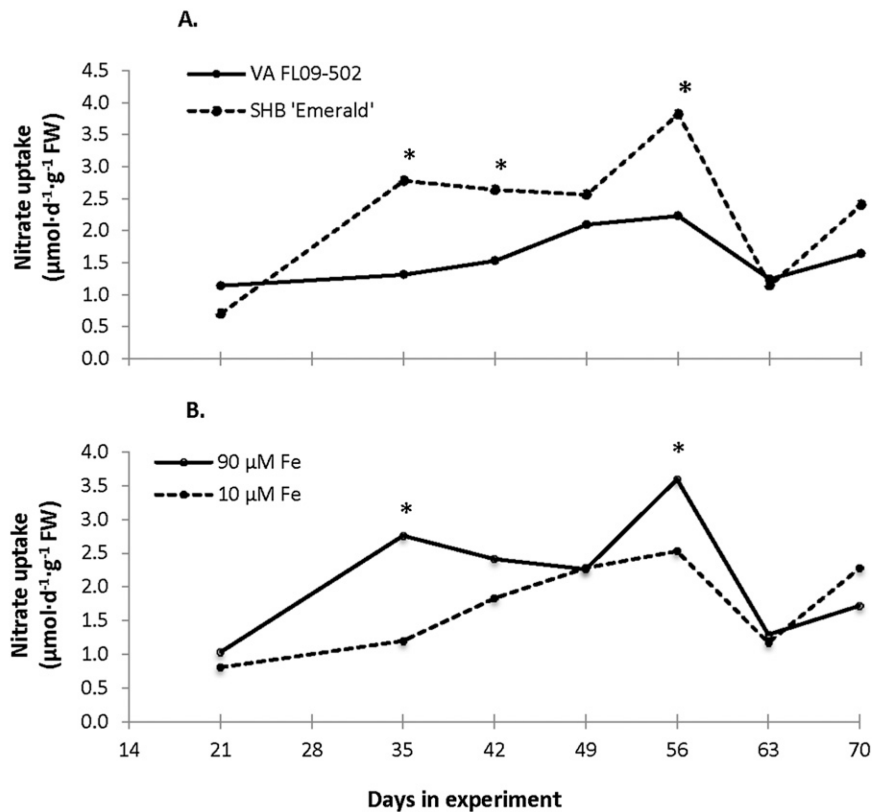

Fig. 4. Nitrate uptake of hydroponically grown *Vaccinium arboreum* (VA) FL09-502 and southern highbush blueberry (SHB) 'Emerald' in either 90 μM or 10 μM iron. The interaction of genotype × iron combination was not significant. Hence, data were pooled by genotype (**A**) and iron treatment (**B**). Asterisks indicate significant differences at $P \leq 0.05$; n = 6. FW = fresh weight.

Table 3. Total nitrogen and nitrate-nitrogen concentrations in leaves, canes, and roots of two *Vaccinium* genotypes grown hydroponically for 70 d in nutrient solutions containing 10 or 90 μM Fe.

	Total nitrogen (mg·g⁻¹ dry wt)			Nitrate nitrogen (μg·g⁻¹ dry wt)		
	Leaves	Canes	Roots	Leaves	Canes	Roots
Genotype[z]						
Emerald	15.37	5.04	8.28	8.45	7.00	20.92
FL09–502	21.69	7.81	12.31	3.45	4.83	22.17
Difference	–6.32*	–2.77*	–4.03*	5.00*	2.17NS	–1.25NS
Fe concentration (μM)						
90	18.88	6.48	10.50	6.45	6.33	23.17
10	18.48	6.38	10.08	5.45	5.50	19.92
Difference	0.40NS	0.10NS	0.42NS	1.00*	0.83NS	3.25NS
Genotype × Fe concentration	NS	NS	NS	NS	NS	*

[z]'Emerald' southern highbush blueberry and *V. arboreum* (FL09–502).
NS, *Nonsignificant and significant at $P \leq 0.05$.

Intra-specific variation. Nutrient solution pH increased gradually across all VA and SHB genotypes tested in the screening study (Fig. 5). The within-species variance was between 0.01 and 0.03 pH units for both species. Representative roots samples of each genotype harvested on day 28 did not change the color of the pH-reactive gels up to 24 h after gel set (data not shown). Results for pea roots and blanks were similar to those described previously.

Discussion

Although FL09-502 and 'Emerald' plants grown at both iron concentrations were of similar size and dry weight, plants grown in 10 μM iron developed interveinal chlorosis and exhibited reduced chlorophyll, total iron,

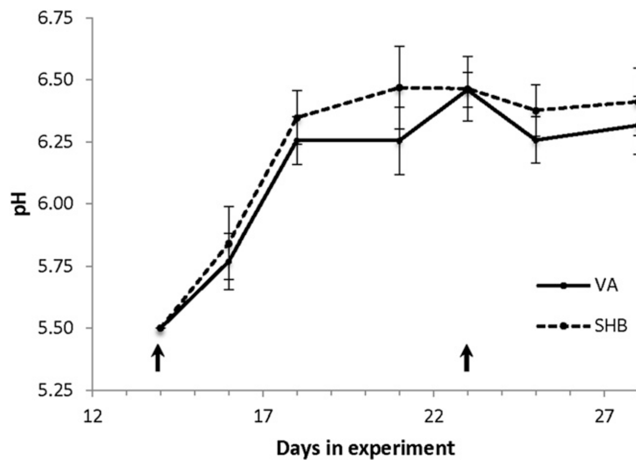

Fig. 5. Nutrient solution pH of a wide selection of hydroponically grown *Vaccinium arboreum* (VA) (solid line) and southern highbush blueberry (SHB) (dashed line) genotypes for 28 d. Starting on day 14, plants were supplied with fresh, unbuffered nutrient solution (initial pH 5.5) on a weekly basis (arrows). Values shown are means of n = 10 genotypes per species. Vertical bars represent the standard deviation at each data point per species.

and active iron compared with plants grown in 90 μM iron. Since these parameters are widely used to diagnose iron deficiency in woody plants (Darnell and Cruz-Huerta, 2011; Gogorcena et al., 2000; Gonzalo et al., 2011; Ksouri et al., 2006), it was expected that if rhizosphere acidification was a strategy employed by these two genotypes, it would be observed in the 10 μM iron treatment. However, neither genotype acidified their rhizosphere in response to iron deficiency. In fact, the nutrient solution pH gradually increased at both higher and lower iron concentrations. Similar absence of acidification has been reported in genotypes in the *Malus*, *Prunus*, and *Vitis* genera (Gogorcena et al., 2000; Gonzalo et al., 2011; Jimenez et al., 2007; Ksouri et al., 2006; Wu et al., 2012).

Given that nitrogen was supplied in the nitrate form, there is a possibility that the observed rhizosphere alkalinization could be a product of NO_3^-/H^+ symport (McClure et al., 1990). VA genotype FL09-502 exhibited lower rates of nitrate uptake than VA genotypes used in previous work (Darnell and Cruz-Huerta, 2011; Poonnachit and Darnell, 2004). Conversely, 'Emerald' took up nitrate at higher rates than observed in other SHB studied previously (Darnell and Cruz-Huerta, 2011; Poonnachit and Darnell, 2004). In this study, both genotypes depleted nitrate from the nutrient solution and raised the nutrient solution pH to a similar extent. Nitrate uptake by grapevine and strawberry (*Fragaria* ×*ananassa*) also led to alkalinization of soil and nutrient solution, respectively (Jimenez et al., 2007; Sas et al., 2003). Hence, nitrate-free reactive gels were used to determine the effects of iron deficiency vs. nitrate uptake on rhizosphere pH. The unchanged color of the reactive gels in this experiment confirmed that neither 'Emerald' nor FL09-502 extruded protons in response to iron deficiency. Similar results were reported for roots of plum and the *Prunus* rootstock 'GF677' (Gogorcena et al., 2000; Gonzalo et al., 2011). However, roots of cork oak, strawberry, wild apple, and peach-almond hybrids exhibited rhizosphere acidification in similar gel-based assays (Gogorcena et al., 2001; Gonzalo et al., 2011; Sas et al., 2003; Wu et al., 2012).

In this experiment, VA genotype FL09-502 did not take up or contain more iron than 'Emerald', which contrasts with previous work where iron uptake and assimilation increased in VA genotypes compared with SHB (Darnell and Cruz-Huerta, 2011). Given that rhizosphere pH was above 5.5 for most of the treatment period in this experiment, it is possible that the ability of FL09-502 to assimilate more iron than 'Emerald' was impeded by the unfavorable environment for iron reduction or transmembrane transport (Dell'Orto et al., 2000) provided by the high pH. Previous reports comparing the iron uptake capabilities of VA and SHB used solutions buffered at pH 5.5 (Darnell and Cruz-Huerta, 2011; Poonnachit and Darnell, 2004).

VA genotype FL09-502 took up significantly less nitrate than 'Emerald' and its leaves exhibited significantly lower nitrate concentration than leaves of 'Emerald'. This could be caused by FL09-502 reducing its leaf nitrate pools to compensate for the limited uptake of nitrate in this experiment. However, nitrate reductase activity in *Vaccinium* leaves is low or entirely absent (Claussen and Lenz, 1999; Darnell and Hiss, 2006). Alternatively, the low nitrate concentration in the leaves of FL09-502 could be the product of the translocation of already reduced nitrogen from roots to leaves. Previous studies found that VA genotypes exhibited greater root-level nitrate reduction capability than SHB cultivars (Darnell and Cruz-Huerta, 2011; Poonnachit and Darnell, 2004). However, neither of these scenarios explains the significantly greater total nitrogen concentration in plant organs of FL09-502 compared with organs of 'Emerald'. It is possible that FL09-502 had significantly higher total nitrogen concentration in its organs at the start of the experiment, and that the limited nitrate uptake of this genotype did not affect this concentration. Initial nitrogen concentrations were not measured in this experiment.

The nitrate uptake of 'Emerald' relative to FL09-502 is also intriguing. In previous work, VA seedlings exhibited greater nitrate uptake than SHB (Darnell and Cruz-Huerta, 2011; Poonnachit and Darnell, 2004). Since this experiment was carried out with softwood rooted cuttings of VA, it is possible that the discrepancies between our results and those in previous work are due to physiological differences between seedling vs. vegetatively propagated VA. Previous research has identified root-level morphological differences between seedlings and rooted cuttings of lowbush blueberry (*Vaccinium angustifolium*) (Jamierson and Nickerson, 2003; Morrison et al., 2000). It is possible that root morphological differences may also occur in seedling vs. rooted cuttings of VA. Further work is necessary to determine if this occurs and if so, what effect it would have on nitrogen nutrition.

Considering the reported intraspecific diversity in rhizosphere acidification in other species (Jimenez et al., 2007; Ksouri et al., 2006), a diverse pool of SHB and VA genotypes was screened for this response. When grown in 45 μM iron, all genotypes tested failed to acidify the nutrient solution in which they were grown. While there is a possibility that this iron concentration was not low enough to cause iron deficiency, we chose this midrange concentration based on the lack of response to iron concentration observed in the experiment with only FL09–502 and 'Emerald'. The low within-species variances suggest that SHB and VA do not exhibit intraspecific diversity in their rhizosphere acidification. The pH change in the nutrient solution during this screen was more pronounced than in the previous experiment, despite the smaller size of the plants used, which suggests that size or developmental differences might affect the rhizosphere acidification capacity. Nevertheless, we found no evidence suggesting that any of the SHB or VA genotypes were capable of acidifying their nutrient solution in the reactive gel assay where the sampling method used—based on root order—accounted for size and developmental differences.

The failure of both VA and SHB to acidify the nutrient solution and the gels suggests that the wider soil adaptation of VA compared with SHB (Hancock et al., 2008; Lyrene, 1997; Lyrene and Brooks, 1995) is not related to the former species' capacity to create an acidic microenvironment around its roots. It is tempting to propose that these and other *Vaccinium* sp. might have lost the ability to acidify their rhizosphere because this trait does not confer a selective advantage in the generally acidic soils where they are found in

the wild and in cultivation (Coville, 1910; Finn et al., 1993; Lyrene, 1997; Lyrene and Brooks, 1995). In acidic soils, relaxed selection (Lahti et al., 2009) on rhizosphere acidification could have led to the reduction or disappearance of this trait in *Vaccinium* sp. Alternatively, H^+-ATPase mediated rhizosphere acidification has been proposed to be an ‘‘emergency’’ reaction instead of a constitutive part of the iron uptake mechanism (Santi and Schmidt, 2009). *Vaccinium* sp. did not display this reaction under the experimental conditions despite the development of iron deficiency symptoms. Further investigation of the rhizosphere pH changes of the members of this genus will be required to discern between these two scenarios.

In conclusion, our results indicate that when grown with nitrate as the sole source of nitrogen, SHB and VA do not respond to iron deficiency by developing or enhancing rhizosphere acidification. SHB ‘Emerald’ and VA FL09-502 alkalinized their rhizosphere both in higher and lower iron concentrations, which is consistent with nitrate uptake and suggests that rhizosphere acidification is not part of their strategy I iron deficiency response. A screen of additional SHB and VA genotypes confirmed that rhizosphere acidification is not observed throughout these genotypes when nitrate is used as the nitrogen source. In absence of this process, the main driver of rhizosphere pH for these species is likely to be nitrate uptake.

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