IRON AND NITRATE ASSIMILATION IN BLUEBERRIES (Vaccinium spp.)

By

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2003
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by

Umpika Poonnachit
This dissertation is dedicated in loving memory of my parents (Wicha and Boonterm) and my younger brother (Alongkorn)
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IRON AND NITRATE ASSIMILATION IN BLUEBERRIES (Vaccinium spp.)

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May 2003

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Major Department: Horticultural Science

The uptake and assimilation of iron (Fe), nitrate (NO$_3^-$) and ammonium (NH$_4^+$) were studied in the cultivated southern highbush blueberry (Vaccinium corymbosum L. interspecific hybrid ‘Misty’) and a wild species (V. arboreum Marsh.). Blueberries were grown hydroponically in either NO$_3^-$ or NH$_4^+$ at pH 5.5 or in NO$_3^-$ at various pHs and Fe concentrations. Iron, NO$_3^-$ and NH$_4^+$ uptake rate, root and leaf ferric chelate reductase (FCR) activity, and root nitrate reductase (NR) activity were quantified.

The results showed that blueberries can take up both nitrogen (N) forms but NH$_4^+$ uptake rates were greater than NO$_3^-$ uptake rates. V. arboreum had greater root NR activity than V. corymbosum except when the experiments were conducted during the winter (October to February in Gainesville, Florida). The greater root NR activity in V. arboreum was not always reflected in increased N uptake and/or growth. V. corymbosum had potentially greater root FCR activity than V. arboreum but this was not reflected in increased Fe uptake rates. In general, neither V. corymbosum nor V. arboreum had the
ability to increase root FCR activities under Fe deficient conditions compared with Fe sufficient conditions. Root FCR activity and Fe uptake rate increased as external Fe concentration increased.

Under Fe sufficient conditions, root FCR activity was greater at pH 6.5 compared with pH 5.5, but no pH effect on leaf FCR activity, root NR activity or NO$_3^-$ uptake rates was found in either species. There were no severe symptoms of leaf chlorosis in either species, although there was a reduction in leaf area at pH 6.5 compared with 5.5.

*V. arboreum* had greater leaf FCR and root NR activities compared with *V. corymbosum* under NO$_3^-$ conditions. The ability of *V. arboreum* to utilize Fe and NO$_3^-$ more efficiently explains why this species can thrive in high pH soils where NO$_3^-$ is the dominant N form.
CHAPTER 1
INTRODUCTION

Blueberries (*Vaccinium* sp.) are native to North America (Galletta and Ballington, 1996). They belong to the Ericaceae family, which are acid loving plants (Korcak, 1989). Blueberries grow well in acidic soils (Brown and Draper, 1980; Erb et al., 1993; Finn et al., 1993; Gough, 1996; Haynes and Swift, 1985,1986; Korcak et al., 1982; Townsend, 1971; Williamson and Lyrene, 1995), which are also well-aerated, high in organic matter, have readily-available iron (Fe), and contain nitrogen (N) primarily in the ammonium ($\text{NH}_4^+$) form. Soil pH can range from 3.5 to 5.5 (Williamson and Lyrene, 1995), with optimum pH about 4.8 (Gough, 1996).

The main production areas of blueberry are located in North America and Canada (Moore, 1993). Blueberry acreage in the United States has expanded rapidly in the northern states (Moore, 1993) and has spread to the southern states, which have earlier production (Williamson and Lyrene, 1995). The blueberry production area was projected to increase by 13%, from 73,585 hectares in 1992 to 82,819 hectares in 2000 (Moore, 1993). However, not all areas possess an optimum soil type for blueberry production. In the past, the approach to soil adaptation problems has focused on changing the soil conditions to fit plant demand (Marschner, 1995). High-input approaches, coupled with the heavy use of fertilizers, have been successful in enhancing crop production in areas that do not have extreme soil conditions. However, even with high inputs, some plants still have limited adaptation under adverse soil conditions. Thus, one area of focus in
Vaccinium breeding programs has been the selection of genotypes for growth on broader soil types with less dependence on acid, organic soils (Galletta and Ballington, 1996).

The limitation of blueberry growth under high pH soil conditions, which are also characterized by low Fe and N in the form of nitrate (NO$_3^-$), may be due to the Fe deficiency and/or the inability to assimilate NO$_3^-$. Limited Fe may be due to low Fe solubility in soils at high pH (Lindsay, 1984), or to a decrease in the activity of the enzyme, ferric chelate reductase (FCR). This plasma membrane localized enzyme catalyzes the reduction of Fe$^{3+}$ (ferric)-chelate to ferrous (Fe$^{2+}$), which is a prerequisite for Fe uptake in plants (Chaney et al., 1972). The activity of root FCR is pH dependent (Holden et al., 1991; Susin et al., 1996), with optimum pH ranging from 5.5 to 6.5, depending on species (Cohen et al., 1997; Holden et al., 1991; Susin et al., 1996). After Fe$^{2+}$ is taken up by plant roots, it is reoxidized and complexed with citrate to form Fe$^{3+}$-citrate which is translocating to the shoot (Brown and Ambler, 1976; Marschner, 1991; Mengel, 1994). Once in the leaves, Fe$^{3+}$-citrate must be reduced by a leaf FCR before the Fe$^{2+}$ can be taken up into the mesophyll cytoplasm (Mengel, 1994). Thus, the presence and/or activity of leaf FCR may play a key role in Fe deficiency (Mengel, 1994). The high leaf apoplastic pH found in plants grown under high pH, NO$_3^-$ containing soils can decrease leaf FCR activity and lead to Fe chlorosis (Kosegarten et al., 2001; Mengel, 1994). There is no information on root or leaf FCR activity in response to Fe deficiency in blueberries.

The inability to assimilate NO$_3^-$ may be due to the low activity of nitrate reductase (NR) (Korcak, 1989). Nitrate reductase is the enzyme responsible for the reduction of NO$_3^-$ to nitrite (NO$_2^-$) in the first step of NO$_3^-$ assimilation. Nitrate reductase activity in
blueberries has been detected in leaves (Dirr et al., 1972; Townsend, 1971; Wang and Korcak, 1995) and roots (Claussen and Lenz, 1999; Dirr et al., 1972; Merhaut, 1993; Townsend, 1970). However, NR activity in blueberry leaves is low (Dirr et al., 1972; Townsend, 1971) or non-detectable in some cultivars (Claussen and Lenz, 1999; Merhaut, 1993). Root NR activity is also low in blueberry. In southern highbush blueberry, for example, root NR activity averaged 30 nmol NO$_2^\text{-}$/g FW/h (Merhaut, 1993), which was relatively low compared with other fruit crops such as apple (150-280 nmol NO$_2^\text{-}$/g FW/h) (Hucklesby and Blanke, 1987; Lee and Titus, 1992) and citrus (370 nmol NO$_2^\text{-}$/g FW/h) (Hucklesby and Blanke, 1987). The low NR activity may be limiting the capacity of blueberries to utilize NO$_3^-$ (Merhaut, 1993) under high pH soils where NO$_3^-$ is the predominant N source, and lead to the reduction of growth compared with blueberries grown in soils containing NH$_4^+$. 

Information on the physiological responses of *Vaccinium* to simulated sub-optimal soil conditions for blueberries, e.g. high NO$_3^-$, limited Fe, and/or high pH, may be a valuable tool for use in plant selection in breeding programs or to improve the cultural practices for blueberries grown in high pH soils. The objectives of the present study were 1) to determine the effects of N form (NH$_4^+$ or NO$_3^-$) on N and Fe uptake, root NR activity and root FCR activity, 2) to determine the effect of pH (5.5 or 6.5) on N and Fe uptake, root NR activity and root and leaf FCR activity under NO$_3^-$ conditions, and 3) to determine the effect of the external Fe concentration on N and Fe uptake, root NR activity and root and leaf FCR activity under NO$_3^-$ conditions. Two blueberry species were used in this study, the cultivated southern highbush blueberry (*V. corymbosum* L. interspecific hybrid ‘Misty’) and a wild species, *V. arboreum* Marsh. *V. arboreum* grows
well under relatively high pH (~ pH 6.0) in which southern highbush blueberry tolerates poorly (Brooks and Lyrene, 1995; Lyrene, 1997). Thus, understanding the physiological differences between these two species that relate to the ability to adapt to high pH soils, can help in the developing of new blueberry cultivars with a wider range of soil adaptation.
CHAPTER 2
LITERATURE REVIEW

Genetic Diversity in *Vaccinium*

Blueberries belong to the Ericaceae Family, in the genus *Vaccinium*. This genus includes approximately 400 species (Galletta and Ballington, 1996). However, there are only 7 categories of cultivated *Vaccinium*, namely lingonberry (*V. vitis-idaea* L. in section Vitis-idaea), cranberry (*V. oxycoccus* L. in section Oxycoccus) and five groups in section Cyanococcus: lowbush (*V. angustifolium* Ait.), rabbiteye (*V. ashei* Reade), half-high (hybrid of *V. angustifolium* x *V. corymbosum*), highbush (*V. corymbosum* L.), and southern highbush blueberry (*V. corymbosum* L. interspecific hybrid). In Florida, only rabbiteye and southern highbush blueberry are grown commercially due to their low-chilling requirement. The ability of southern highbush blueberry to grow successfully under the mild weather conditions of Florida is inherited from the very low-chilling species *V. darrowi*, which is one of its parental species (Galletta and Ballington, 1996; Lyrene, 1997). The cultivated blueberries are well-known as acid-loving plants (Korcak, 1988, 1989). Production areas are limited to acidic (pH 4.5 to 5.5), well-drained sandy soils with high organic matter (>3%) and nitrogen (N) primarily in the ammonium (NH$_4^+$) form (Gough, 1996; Korcak, 1989; Williamson and Lyrene, 1995). However, increased demand for fresh blueberries and pick-your-own operations has led to the expansion of production acreage to areas with sub-optimal soil conditions (Korcak, 1989), such as high pH, low iron (Fe) availability and N primarily in the nitrate (NO$_3^-$) form. Under these soil conditions, blueberry growth and productivity are restricted,
possibly due to limitation in NO$_3^-$ (Korcak, 1989) and/or Fe uptake and assimilation (Gough, 1996). There have been attempts to breed blueberry genotypes that can be grown under widespread soil (primarily upland high pH soils) and/or weather conditions through several breeding programs (Galletta and Ballington, 1996; Lyrene, 1993, 1997; Patel and Goerge, 1996).

There are several *Vaccinium* species that belong to sections other than Cyanococcus but can cross with Cyanococcus species (Galletta and Ballington, 1996; Lyrene, 1997). One of them is sparkleberry or farkleberry (*V. arboreum* Marsh.), which belongs to the section Batodendron (Galletta and Ballington, 1996; Lyrene, 1993). This species is native to the semiarid area of south Texas and can also be found in other southeastern states of the U.S. (Brooks and Lyrene, 1995). It tolerates a wider range of soil types than any species in the section Cyanococcus. It is native to and thrives in the areas where soil pH is higher than 6.0 (Lyrene, 1993), which highbush and southern highbush blueberry tolerate poorly (Brooks and Lyrene, 1995; Lyrene, 1997). This suggests that *V. arboreum* may be more efficient than cultivated *Vaccinium* in acquisition/assimilation of Fe and NO$_3^-$ from these higher pH soils. *V. arboreum* also does well in sandy soils that have low water holding capacity and low organic matter (Brooks and Lyrene, 1995; Lyrene, 1993). *V. arboreum* has a coarse root system with a tap root, which differs from the shallow, fibrous root systems of other *Vaccinium* species (Lyrene, 1997). The undesirable traits of *V. arboreum* are small, dark, and scarcely palatable berries, large seeds, abundant sclerids, low juice content and occasionally astringent flesh (Lyrene, 1997). However, its ability to grow in high pH soils makes it a useful species for breeding wider soil adaptation into cultivated *Vaccinium*. 
Plant Iron Deficiency Responses

Leaf chlorosis, associated with low Fe availability, is usually the main problem associated with fruit crops such as blueberry (Brown and Draper, 1980), peach (Egilla et al., 1994; Morales et al., 1998; Shi et al., 1993; Tagliavini et al., 1995a), and pear (Morales et al., 1998; Tagliavini et al., 1995b), grown in high pH soils. Chlorosis is often enhanced by poor soil aeration caused by soil compaction or high water content, and low soil temperatures (Marschner, 1995). Leaf chlorosis begins in young leaves (Mengel and Kirkby, 2001) and is characterized by interveinal yellowing, while the midrib remains green (Egilla et al., 1994; Mengel and Kirkby, 2001). The youngest leaves may often be completely white (Mengel and Kirkby, 2001). The chlorosis results from the inhibition of chlorophyll biosynthesis under Fe-deficient conditions (Egilla et al., 1994; Abadia, 1992), which decreases the photosynthetic rate and growth of plants (Abadia, 1992). Leaf chlorosis can be induced by the combination of low Fe, high pH, high bicarbonate and high phosphorus (Shi et al., 1993).

The oxidized (ferric) form of Fe ($\text{Fe}^{3+}$) plays an important role in Fe deficiency problems under mineral soil conditions. Generally, the reduced (ferrous) form of Fe ($\text{Fe}^{2+}$) is the form that is absorbed by most plants, while $\text{Fe}^{3+}$ is the primary Fe form in the soil. As $\text{Fe}^{3+}$ is insoluble in water (Korcak, 1987), most plants have to reduce $\text{Fe}^{3+}$ to $\text{Fe}^{2+}$ before Fe can be absorbed by the roots (Chaney et al., 1972). These two properties, rapid oxidation and insolubility of the oxidized species, are the key to the Fe deficiency chlorosis problem (Korcak, 1987).

Physiological Responses

There are two strategies that different plant species developed as a mechanism to acquire Fe under Fe deficiency (Römheld and Marschner, 1986). In gramineous
monocots, Strategy II is performed, in which roots release phytosiderophores under Fe deficient conditions (Crowley, 2001; Marschner, 1991; Römheld and Marschner, 1986). The phytosiderophores bind Fe$^{3+}$ in the rhizosphere and carry it into the roots via a specific transport system for Fe$^{3+}$ (Crowley, 2001; Römheld and Marschner, 1986). The release of phytosiderophores is not affected by the external pH (Römheld and Marschner, 1986). There is evidence that some graminaceous species may possess other Fe uptake mechanisms apart from the release of phytosiderophores. Recently, Wang and Peverly (1999) investigated Fe$^{3+}$ reduction on the root surfaces of common reeds (*Phragmites australis*), a graminaceous species, and observed the accumulation of Fe$^{2+}$ in the root apoplast. They concluded that common reed may have other mechanisms of Fe uptake besides the release of phytosiderophores.

Strategy I is the specific mechanism used by dicots, such as blueberry, and nongramineous monocots in response to Fe deficiency (Korcak, 1989; Marschner, 1991; Römheld and Marschner, 1986). The important components of this mechanism are the enhancement of proton (H$^+$) release, the increase of Fe$^{3+}$ reduction at the root plasma membrane and the release of phenolics and/or chelators (Römheld and Marschner, 1986). Iron-deficiency-induced H$^+$ release, which is likely due to the higher activity of the plasma membrane H$^+$-ATPase (Holden et al., 1994; Marschner, 1995), acidifies the rhizosphere and increases Fe$^{3+}$ solubility in both the root apoplast and the soil solution (Beinfait et al., 1985). The reduction of Fe$^{3+}$ to Fe$^{2+}$ is an obligatory step for Fe uptake by plants when supplied with chelated Fe (Chaney et al., 1972). Thus, increased Fe$^{3+}$ reduction enhances Fe uptake (Manthey et al., 1994; Römheld and Marschner, 1986; Zaharieva and Römheld, 2000). In the process of Fe$^{3+}$ reduction, Fe$^{3+}$-chelates are
cleaved and reduced via a plasma membrane-bound ferric chelate reductase (FCR) to form free Fe$^{2+}$, which is transported across the membrane by an uptake system specific for Fe$^{3+}$ (Fe$^{3+}$-transporter) (Zaharieva and Römheld, 2000). Two separate Fe$^{2+}$ uptake systems (high- and low-affinity) are reported, with the low-affinity uptake system supplying Fe under Fe sufficient conditions, while the high-affinity uptake system is operating under Fe deficiency (Zaharieva and Römheld, 2000). In the root xylem, the Fe$^{2+}$ is re-oxidized and chelated to form Fe$^{3+}$-citrate (Brown and Ambler, 1976; Marschner, 1991). Fe$^{3+}$-citrate is then transported in the xylem from root to shoot (Brown and Ambler, 1974; Marschner, 1991; Mengel, 1994). The transport of Fe$^{3+}$-citrate across the leaf plasma membrane is a crucial step in the Fe utilization in the leaf and is regulated by Fe$^{3+}$ reduction (Mengel, 1994) via the leaf FCR (Kosegarten et al., 1999).

**Optimum pH for FCR**

The activity of root and leaf FCR is pH dependent (Ao et al., 1985; Cohen et al., 1997; Holden et al., 1991; Moog and Braggemann, 1994; Romera et al., 1998; Susin et al., 1996). Moog and Braggemann (1994) summarized the characteristics of Fe-EDTA reduction in intact roots of several Strategy I plants and concluded that the optimum pH for FCR is around 5.5. Evidence shows that optimum pH for root FCR activity is likely to depend on the Fe status (sufficient vs deficient conditions) of the tissues in which FCR activity is determined. Under Fe deficiency, the optimum pH for root FCR activity of intact apple root was between 4 and 6. However, when there was sufficient Fe (90 µM FeDTPA), root FCR activity tended to increase as pH increased from 5 to 8, although differences were not statistically significant (Ao et al., 1985). The activity of excised root FCR of *Ficus* was similar at the pH range from 4.5 to 6.5. The activity then decreased sharply when pH was above 6.5 (Rosenfield et al., 1991). In hydroponically grown
sugarbeet (*Beta vulgaris*), Susin et al. (1996) found that intact root FCR activity of Fe-deficient plants decreased little from pH 3.0 to 6.0, but the activity decreased sharply when pH was higher than 6.0. In Fe-sufficient sugarbeet, FCR activity did not change significantly from pH 3.0 to 6.5, but decreased at higher pH. Cohen et al. (1997) examined the pH dependence of intact pea root FCR activity from pH 4.5 to 7.5. They reported that the optimum FCR activity in both Fe-sufficient and Fe-deficient pea seedlings occurred at pH 5.5 to 6.0. The activity decreased as pH increased to 7.5. In contrast, FCR activity of plasma membrane isolated from Fe-deficient tomato roots had an optimum pH of 6.5 (Holden et al., 1991).

Leaf FCR activity increases with decreased apoplastic pH (Kosegarten et al., 1999). In an experiment with young sunflower (*Helianthus annuus* L.) plants, Mengel et al. (1994) found that reduction of Fe$^{3+}$ in the leaf was greater at low leaf apoplastic pH (5.8), obtained from NH$_4^+$ supply, compared with high leaf apoplastic pH (6.3), obtained from NO$_3^-$ supply. Kosegarten et al. (1999) also found the highest leaf FCR activity in sunflower at apoplastic pH around 5.0, compared with pH ranging from 5.0 to 7.7.

**Iron-efficient and Fe-inefficient genotypes**

Genotypic difference in the ability to induce root FCR activity under Fe deficiency is used to characterize plant species and/or cultivars within a species into 2 categories, Fe-efficient or Fe-inefficient genotypes (Grusak et al., 1990; Marschner, 1991; Romera et al., 1991; Römheld and Marschner, 1990). In general, Fe-efficient genotypes exposed to Fe-deficient conditions are characterized by increased activity of root FCR and enhanced proton excretion from the roots compared with the same plant grown under Fe sufficient conditions (Marschner, 1991). Therefore, Fe-efficient genotypes can grow under Fe deficiency with less development of chlorosis. Fe-inefficient genotypes, on the other
hand, do not exhibit these responses under Fe deficiency and develop Fe chlorosis. The increase in root FCR activity in Fe-efficient genotypes exposed to Fe deficiency appears to be almost an universal response, and reductase activity is considered to be the rate limiting step in Fe acquisition (Grusak et al., 1993). Increased activity in response to Fe deficiency occurs within 3-10 days in many herbaceous annuals (Grusak et al., 1993; Moog and Bruggemann, 1994; Yi and Guerinot, 1996) but can take up to 7-8 weeks in Fe-efficient woody plants (Manthey et al., 1994; Moog and Bruggemann, 1994; Rosenfield et al., 1991).

**Morphological Responses**

The root-response mechanisms to Fe deficiency in Strategy I plants are located in apical and subapical root zones (Marschner, 1991; Grusak et al., 1993; Welch et al., 1993.) in association with morphological changes in roots (Brown and Ambler, 1974; Marschner, 1991). Typical morphological changes of Fe-efficient plants include the formation of cells, with a distinct wall labyrinth typical of transfer cells, in the rhizodermis (Marschner, 1991; Römheld and Marschner, 1986) or in the hypodermis (Marschner, 1995), enhancement of root hair formation (Egilla et al., 1994; Rosenfield et al., 1991) and the swelling of subapical root tip (Ao et al., 1985; Romera and Alcantara, 1994). The transfer cells are presumably the sites of Fe deficiency-induced root responses of Strategy I plants (Marschner, 1995). Egilla et al. (1994) reported differences in root morphology between tolerant and susceptible peach rootstocks under Fe deficient conditions. The tolerant genotype had the ability to sustain root growth better under Fe deficiency. They had more persistent lateral roots and root hairs compared to the susceptible genotype, which rapidly developed root necrosis. Egilla et al. (1994) concluded that the maintenance of a greater root surface area during Fe deficiency may
be important to sustain Fe$^{3+}$ reduction capacity under prolonged stress on a per plant basis.

**Responses of Blueberries to Iron Deficiency**

*Vaccinium* species and/or cultivars differ in their responses to Fe deficiency. Brown and Draper (1980) reported differences in 4 intra- and interspecific progenies (involving *V. ashei*, *V. corymbosum* and *V. darrowi*) in their ability to utilize Fe under relatively high (6.6) pH. Progeny derived from *V. ashei* x (*V. corymbosum* x *V. darrowi*) had the ability to decrease nutrient solution pH by releasing H$^+$ from its roots, making Fe more available for uptake and transport from roots to shoots. This progeny was considered to be an Fe-efficient genotype. On the other hand, progeny from *V. ashei* x *V. ashei* did not release H$^+$, developed Fe chlorosis and was considered to be an Fe-inefficient genotype. Brown and Draper (1980) concluded that blueberries could be bred for adaptation to soils with a wide range of pH. No information is available on the ability of blueberries to increase root and/or leaf FCR activity in response to Fe deficiency (Korcak, 1988).

**Nitrogen Nutrition**

Nitrogen is often the most limiting elements that plants have to acquire from the soil (Touraine et al., 2001) and it often determines the potential yield of crops (Le Bot et al., 1994; Touraine et al., 2001). Nitrogen can be taken up by the plant roots either in the form of NO$_3^-$ or NH$_4^+$.  

**Nitrate Uptake and Assimilation**

Nitrate is the major nitrogen source for most plants growing in well-aerated soils (Tischner, 1990; Wray and Abberton, 1994). It is taken up by plants through an active carrier system, involving the movement of two protons (H$^+$) and one NO$_3^-$ ion across the membrane (NO$_3^-/H^+$ symport) (Ullrich, 1992; Glass et al., 2002). Thus, NO$_3^-$ uptake
results in an increase in rhizosphere pH (Marschner, 1995; Touraine et al., 2001; Ullrich, 1992). There are 3 transport systems for NO\textsubscript{3}\textsuperscript{-} uptake across the plasma membrane of root cells (Glass et al., 2002; Touraine et al., 2001). A constitutive, high-affinity transport system (cHATS) allows entry of NO\textsubscript{3}\textsuperscript{-} at low external concentration (< 0.5 mM) (Glass et al., 2002; Touraine et al., 2001). The uptake rate of NO\textsubscript{3}\textsuperscript{-} is low within the first few hours after first exposure to NO\textsubscript{3}\textsuperscript{-}, then the rate increases rapidly in response to the induction of another high-affinity transport system (iHATS) (Glass et al., 2002; Touraine et al., 2001; Wray and Abberton, 1994). At NO\textsubscript{3} concentrations above 200 µM, the HATS plateau and the low-affinity transport system (LATS) becomes apparent (Glass et al., 2002; Touraine et al., 2001).

Once taken up, NO\textsubscript{3}\textsuperscript{-} can be reduced in the roots, translocated to the leaves for reduction, or stored in the vacuole of root cells (Sivasankar and Oaks, 1996; Zhang and MacKown, 1993). Nitrate reduction is a two-step process; NO\textsubscript{3}\textsuperscript{-} is first reduced to nitrite (NO\textsubscript{2}\textsuperscript{-}) by nitrate reductase (NR), followed by reduction of NO\textsubscript{2}\textsuperscript{-} to NH\textsubscript{4}\textsuperscript{+} by nitrite reductase (NiR) (Crawford, 1995; Marschner, 1995).

Activity of NR is considered to be the rate-limiting step of NO\textsubscript{3}\textsuperscript{-} uptake and assimilation and often gives a good estimate of the plant N status (Srivastava, 1980) and potential yield (Le Bot, et al., 1994; Touraine et al., 2001). Nitrate reductase is found either as a plasma membrane-bound enzyme (Berczi and Moller, 2000; Stohr, 1999; Ullrich et al., 1990) or as a cytosol localized enzyme in root epidermal and cortical cells and shoot mesophyll cells (Berczi and Moller, 2000; Crawford, 1995; Stohr, 1999). To reduce NO\textsubscript{3}\textsuperscript{-} to NO\textsubscript{2}\textsuperscript{-}, NR receives 2 electrons from either NADH or NADPH (Kaiser and Huber, 1994; Mengel and Kirkby, 2001) via three redox centers composed of two
prosthetic groups (flavin adenine dinucleotide (FAD) and heme) and a MoCo (molybdate and pterin complex) cofactor (Crawford, 1995). Three types of NR have been identified (Wray and Abberton, 1994). The most widely distributed is NADH-NR which has a pH optimum of 7.5. The second type of NR can use either NADH or NADPH as an electron donor. The third type was identified in nitrogen-fixing plants as a constitutive NADH-NR with a pH optimum of 6.5, rather than 7.5 (Rouzé and Caboche, 1992; Wray and Abberton, 1994).

The reduction of NO$_3^-$ may take place in the root, leaves or both, depending on external NO$_3^-$ concentrations and the plant species (Black et al., 2002; Gojon et al., 1991; Shaner and Boyer, 1976; Smirnoff and Stewart, 1985). At low external concentrations, NO$_3^-$ is preferentially reduced in the root, whereas at higher NO$_3^-$ concentrations it is transported in the xylem for reduction in the leaves (Gojon et al., 1991; Larsson and Ingemarsson, 1989; Le Bot et al., 1994; Lee and Titus, 1992). Both NR and NiR activities are induced by NO$_3^-$ and may be subjected to feedback inhibition by ammonium or amino acids or both (Le Bot et al., 1994; Mengel et al., 1983). Activity of NR also depends on environmental factors (Srivastava, 1980), such as light intensity, length of photoperiod and temperature (Hucklesby and Blanke, 1987; Kaiser et al., 1999; Naik et al., 1982). Nitrate reductase activity is greater in plants grown at higher irradiance compared with lower irradiance (Smirnoff and Stewart, 1985; Srivastava, 1980). Greater NR activity is also found in plants grown under long day compared with short day and/or short day with a night interruption (Black et al., 2002; Hucklesby and Blanke, 1987). In general, temperatures higher or lower than optimum growth temperatures of each plant species decreases NR activity (Srivastava, 1980, and references therein).
Ammonium Uptake and Assimilation

In well-aerated agricultural soils, $\text{NH}_4^+$ concentrations are often 10 to 1000 times lower than $\text{NO}_3^-$ (Glass et al., 2002; von Wiren et al., 2001). Nevertheless, $\text{NH}_4^+$ nutrition plays an important role in waterlogged and acid soils (Marschner, 1995). Two transport systems mediate $\text{NH}_4^+$ uptake (Forde and Clarkson, 1999; Glass et al., 2002; von Wiren et al., 2001). At external $\text{NH}_4^+$ concentrations below 1 mM, a saturable high-affinity transport system (HATS) is apparent (Forde and Clarkson, 1999; von Wiren et al., 2001). At $\text{NH}_4^+$ concentrations higher than 1 mM, a low-affinity transport system (LATS) has been identified (Forde and Clarkson, 1999; Glass et al., 2002; von Wiren et al., 2001). The uptake might occur through an $\text{NH}_4^+$ uniport (Engels and Marschner, 1995; von Wiren et al., 2001) or diffusion through a channel such as the $\text{K}^+$ channel (Forde and Clarkson, 1999; von Wiren et al., 2001). In rye roots, the $\text{K}^+$ channel was reported to be permeable to $\text{NH}_4^+$ (Forde and Clarkson, 1999 and references therein). In both transport systems, it is necessary for the plasma membrane H$^+$-ATPase (so called proton pump) to maintain the electrical gradient of the cell by releasing H$^+$ into the apoplast (Ullrich, 1992; von Wiren et al., 2001). Thus, uptake of $\text{NH}_4^+$ results in the acidification of the rhizosphere (Pilbeam and Kirkby, 1992; von Wiren et al., 2001). Ammonium is assimilated primarily in the root via the glutamine synthetase (GS) and glutamate synthase (GOGAT) pathway (Mengel and Kirkby, 2001). The end product of the GS/GOGAT pathway is glutamate. Glutamate either can be used directly for protein synthesis or can act as an amino group donor for various reactions which result in the synthesis of other amino acids (Iaccarino et al., 1990).
Effect of Nitrogen Form on Vaccinium Growth

The ability of blueberries to utilize N in the form of NH$_4^+$ and/or NO$_3^-$ is still controversial. In most studies, NH$_4^+$ has been found to be the preferential source of N for optimum blueberry growth (Finn et al., 1991; Korcak, 1989; Townsend, 1971); however, blueberries can be grown using NO$_3^-$ as the sole N source (Rosen et al., 1990; Sugiyama and Hanawa, 1992). Differences in the ability of blueberry to use NH$_4^+$ vs NO$_3^-$ may depend, in part, on pH.

Spiers (1978, 1979) reported higher fruit yield, greater shoot growth and higher leaf nutrient concentration of rabbiteye blueberries fertilized with NH$_4^+$-N compared with NO$_3^-$-N, at pHs ranging from 3.5 to 7.5. Rosen et al. (1990) reported that shoot growth and plant dry weight (DW) of blueberry hybrids (V. corymbosum x V. angustifolium) were greater at pH 4.5 compared with pH 6.5, regardless of N form. In rabbiteye blueberry, there was no difference in plant DW between NH$_4^+$ and NO$_3^-$ fertilized plants at pH 5 (Sugiyama and Hanawa, 1992). However, at lower pHs (3 and 4), DW of new leaves and stems were greater in plants fertilized with NH$_4^+$ compared with NO$_3^-$.

Townsend (1967), in agreement with Takamizo and Sugiyama (1991), reported greater plant fresh weight (FW) and DW in blueberries grown with NH$_4^+$ or a combination of NH$_4^+$ and NO$_3^-$ compared with NO$_3^-$.

Increased growth of blueberries under NH$_4^+$ conditions may be related to uptake differences between NH$_4^+$ and NO$_3^-$. Merhaut and Darnell (1995) reported that uptake of NH$_4^+$ was faster than NO$_3^-$ in southern highbush blueberry. The low uptake rate of NO$_3^-$ was associated with low activity of NR in roots and shoots, even in NO$_3^-$ grown plants. They concluded that southern highbush blueberry possessed low levels of NR which were not responsive to fluctuations in NO$_3^-$ availability.
Low NR activity is speculated to be the limiting factor for blueberry growth on high pH soils where NO$_3^-$ is the dominant N source (Korcak, 1988, 1989). Nitrate reductase activities in roots of lowbush (Townsend, 1970), highbush (Claussen and Lenz, 1999; Dirr et al., 1972; Wang and Korcak, 1995), rabbiteye (Wang and Korcak, 1995), and southern highbush blueberry (Merhaut and Darnell, 1995) are low, ranging from 25 to 390 nmol NO$_2^-$/g FW/h. These NR activities are lower than activities found in other woody (Bussi et al., 1997; Hucklesby and Blanke, 1987; Lee and Titus, 1992) and herbaceous plants (Hucklesby and Blanke, 1987). In addition, leaf NR activity in blueberry is low (Dirr et al., 1972; Townsend, 1970) or non-detectable (Claussen and Lenz, 1999; Merhaut, 1993). Identifying blueberry genotypes with high NR activity may be a useful tool in breeding blueberries for adaptation to high pH soils.

**Effect of Nitrogen Form on Iron Availability**

The source of N plays an important role in Fe chlorosis of plants (Mengel, 1994; Mengel and Geurtzen, 1988). Under low pH soils, in which NH$_4^+$ is the predominant N source, uptake of NH$_4^+$ by plant roots leads to acidification of the rhizosphere (Marschner, 1995; von Wirén et al., 2001) and enhanced Fe solubility in soils. In contrast, under high pH, in which NO$_3^-$ is the predominant N source, uptake of NO$_3^-$ results in alkalization of the rhizosphere (Marschner, 1995; Touraine et al., 2001; Ullrich, 1992), which limits Fe availability (Lindsay, 1984). Nitrate nutrition not only increases rhizosphere pH but also induces high leaf apoplastic pH (Kosegarten and Englisch, 1994; Kosegarten et al., 1998 a,b; Kosegarten et al., 2001). The increased apoplastic pH reduces leaf FCR activity, causing Fe inactivation in leaf (Mengel, 1994) and Fe chlorosis. Thus, blueberries develop Fe chlorosis when supplied with NO$_3^-$-N compared with NH$_4^+$ (Korcak, 1987, 1988; Kosegarten et al., 1998b; Mengel and
Geurtzen, 1988), due to decreased Fe uptake from the soil and decreased Fe utilization in the leaf.

**Adaptation of Vaccinium in Mineral Soils**

Certain cultural practices can modify the adverse effects of limited Fe and/or N in the form of NO₃⁻ on growth of Vaccinium under high pH soil conditions, including incorporation of peatmoss and addition of mulch (Erb et al., 1993). However, as the cost of these management practices can be high, research has focused on selection of blueberry species adapted to sub-optimal soil conditions (low organic matter, high pH soils).

Korcak et al. (1982) studied growth and elemental composition of several blueberry genotypes on 5 unmulched soils. Three of the soil types were low in pH and fertility while the other two had high pH and high fertility. The results showed that all blueberry genotypes grown at high pH (pH 6.7) were stunted with tip burn, reddening of new growth, and chlorosis of old leaves, while those grown at low pH (pH 4.2) had normal growth and coloration. Considering overall performance of blueberry genotypes on various soil types in association with the germplasm source, Korcak et al. (1982) concluded that *V. ashei* seemed to be the best source of genetic variation for growth on mineral and limed soils.

In a later study, Korcak (1986) studied the adaptability of 5 blueberry crosses (ranging from pure highbush to interspecific hybrids) to four soil types (Berryland, Manor clay loam, Galestown sandy clay loam and Pope sandy clay loam) which possessed low pH and fertility. The Berryland soil, pH 3.9 and 1.8% organic matter, produced the greatest plant volume compared with the other soils. Progenies containing less highbush parentage produced more vigorous growth on all soil types (Korcak, 1986).
His study also showed the inconsistent effect of peatmoss on growth. Initially, blueberries grown on the peatmoss-amended soils produced significantly more growth compared with growth on non-amended soils. However, by the second year, little difference in growth between amended and non-amended soil was observed.

Finn et al. (1993) screened 33 seedling blueberry progenies from crosses among *V. corymbosum*, *V. angustifolium* and *V. corymbosum* x *V. angustifolium* hybrids, under 3 soil pH levels (5.0, 5.5 and 6.5). The plants grew well in the low pH regime and poorly in the high pH regime. They concluded that *V. angustifolium* was not a general source of tolerance to higher pH, but some populations derived from *V. angustifolium* were tolerant to high pH soils.

The wild blueberry, *V. arboreum*, has the ability to thrive on upland soils with pH up to 6.0 (Lyrene, 1993, 1997). This makes it a valuable source of genetic variation for increasing the soil adaptation of cultivated blueberry (Lyrene, 1997). The adaptation of *V. arboreum* to upland high pH soils may be due to an increased ability to assimilate NO$_3^-$ and/or utilize Fe under high pH compared with the cultivated blueberry. Understanding the physiological differences that contribute to the differences in soil adaptation between *V. arboreum* and cultivated blueberry species can aid in attempts to develop commercial cultivars with wider soil adaptation.
CHAPTER 3
EFFECT OF AMMONIUM AND NITRATE ON FERRIC CHELATE REDUCTASE AND NITRATE REDUCTASE IN BLUEBERRIES

Introduction

Blueberries have been grown commercially in Florida since the 1960s (Williamson and Lyrene, 1995). In the central region of Florida, southern highbush blueberry (Vaccinium corymbosum L. interspecific hybrid) acreage has increased 128% between 1989 and 2000 (Williamson et al., 2000). Blueberries grow best in acid soils (pH 4.0 to 5.5) where ammonium ($\text{NH}_4^+$) is the dominant nitrogen (N) form (Williamson and Lyrene, 1998). However, there is interest in expanding blueberry acreage to upland high pH soils, which are characterized by low iron (Fe) availability, and N primarily in the form of nitrate ($\text{NO}_3^-$) (Williamson and Lyrene, 1998). Thus, under upland high pH soils, blueberries may develop leaf Fe chlorosis (Brown and Draper, 1980), N deficiency, and suppression of growth (Korcak et al., 1982). In order to alleviate these symptoms, growers must amend the soils with peat and pinebark to maintain low pH (Williamson, personal communication), which significantly increases production costs. Breeding blueberry cultivars that are adapted to high pH soils, without developing Fe chlorosis and/or N deficiency, would be an advantage for the expansion of the blueberry industry.

Iron deficiency is characterized by an interveinal chlorosis of young leaves while the veins remain green (Korcak, 1987). Physiological responses of dicotyledonous plants to Fe deficiency include induction of plasma membrane-bound ferric chelate reductase (FCR) and an increase in proton ($\text{H}^+$) excretion (Marschner and Römheld, 1994). The
root FCR reduces ferric (Fe$^{3+}$)-chelate to ferrous (Fe$^{2+}$) before Fe$^{2+}$ is taken up into the cytoplasm (Chaney et al., 1972; Schmidt, 1999). The lower rhizosphere pH, resulting from the H$^+$ excretion, increases Fe solubility and makes it more available for the plant (Korcak, 1987; Römheld and Marschner, 1986). Plants that are able to induce root FCR activity and/or increase H$^+$ excretion under Fe deficiency, leading to an increase in Fe uptake, are classified as Fe efficient genotypes (Marschner, 1991; Romera et al., 1991; Römheld and Marschner, 1990). Plants that do not respond to Fe deficiency are Fe inefficient genotypes (Marschner, 1991). Differences among plant species and genotypes in the ability to increase FCR activity under Fe deficiency has been used to screen for genotypes that are considered Fe efficient (Marschner, 1991) in several fruit crops, such as apple (Ao et al., 1985), kiwifruit (Vizzotto et al., 1999) and peach (Egilla et al., 1994; de la Guardia et al., 1995). Some blueberries respond to Fe deficiency by decreasing rhizosphere pH and increasing Fe availability (Brown and Draper, 1980). There are no reports on the effects of Fe deficiency on FCR activity in blueberry.

Blueberry growth is greater when supplied with NH$_4^+$ compared with NO$_3^-$ (Townsend, 1967; Korcak, 1988). Lowbush (V. angustifolium Ait) and rabbiteye (V. ashei Reade) blueberries fertilized with NH$_4^+$ had greater fresh weight, longer shoots, more shoots per plant and increased flower bud number compared with plants fertilized with NO$_3^-$ (Townsend, 1969; Spiers, 1978). This greater growth appears to be related to increased uptake rates of NH$_4^+$-N compared with NO$_3^-$-N (Merhaut and Darnell, 1995; Spiers, 1978). In other work, however, shoot and root dry weight of blueberry hybrids (V. corymbosum L. x V. angustifolium Ait.) grown in nutrient solutions containing NH$_4^+$, NH$_4$NO$_3$ or NO$_3^-$, were not significantly different (Rosen et al., 1990). This suggests that
the response of blueberry to N-form depends on the medium in which they are grown or on genotype.

Assimilation of $\text{NO}_3^-$ is regulated by the activity of nitrate reductase (NR) (Crawford, 1995). This enzyme is found either as a plasma membrane-bound (Berczi and Moller, 2000; Stohr, 1999; Ullrich et al., 1990) or as a cytosol localized enzyme in root epidermal and cortical cells and shoot mesophyll cells (Berczi and Moller, 2000; Crawford, 1995; Stohr, 1999). Nitrate reductase catalyzes the reduction of $\text{NO}_3^-$ to nitrite ($\text{NO}_2^-$) as the first step in $\text{NO}_3^-$ assimilation (Mengel and Kirkby, 2001). Nitrate reductase activity has been detected in blueberry leaves (Dirr et al., 1972; Wang and Korcak, 1995) and roots (Townsend, 1970; Merhaut, 1993; Claussen and Lenz, 1999). However, NR activity in leaves is lower than in roots (Dirr et al., 1972). Root NR activity in southern highbush blueberry averaged 30 nmol $\text{NO}_2^-$/g FW/h (Merhaut, 1993), which is relatively low compared with other fruit crops such as apple (150-280 nmol $\text{NO}_2^-$/g FW/h) (Hucklesby and Blanke, 1987; Lee and Titus, 1992) and citrus (370 nmol $\text{NO}_2^-$/g FW/h) (Hucklesby and Blanke, 1987). The low NR activity found in blueberry may result in insufficient uptake and reduction of $\text{NO}_3^-$ (Spiers, 1978) and may lead to slower growth rates (Lee and Stewart, 1978) when blueberries are fertilized with $\text{NO}_3^-$ rather than $\text{NH}_4^+$. Thus, NR activity may be a limiting factor for blueberry growth under $\text{NO}_3^-$ conditions (Korcak, 1989).

*V. arboreum* or sparkleberry is a wild blueberry that is native to the southeastern U.S. (Lyrene, 1997) and typically grows on upland soils containing little organic matter (Lyrene, 1997). It has the ability to grow on relatively high pH ($\sim$ pH 6.0), in which southern highbush blueberry tolerates poorly (Brooks and Lyrene, 1995; Lyrene, 1997).
Under high pH soil conditions, N is primarily in the NO$_3^-$ form due to rapid nitrification and/or ammonia volatilization (Mengel, 1994). The ability of *V. arboreum* to grow under atypical blueberry soils suggests an increased efficiency for Fe and/or N assimilation compared with southern highbush blueberry grown on those soils. We hypothesized that *V. arboreum* is able to assimilate Fe and N more efficiently, resulting in higher Fe/N uptake compared with *V. corymbosum* under conditions that mimic upland soils. The objectives of the present study were to determine NR and FCR activity, N and Fe uptake and growth in two blueberry species, *V. corymbosum* and *V. arboreum*, grown under NO$_3^-$ or NH$_4^+$ and with or without Fe.

**Materials and Methods**

**Plant Culture**

Seeds of the wild species (open pollinated), *V. arboreum*, were collected from a natural habitat at Manatee Springs, Florida, during Fall 1998 and immediately germinated on the surface of Canadian peat under intermittent mist (4 hours/day) in a greenhouse, with average temperatures of 25°C and 500 µmol·m$^{-2}$·s$^{-1}$ Photosynthetic Photon Flux (PPF). In April 1999, shoot cuttings of the southern highbush cultivar, ‘Misty’, were rooted in peat:perlite (1:1 by volume) medium under intermittent mist in a greenhouse with average temperatures of 25°C and average PPF of 500 µmol·m$^{-2}$·s$^{-1}$. In August 1999, plants of both species were transplanted into 1-L pots containing pine bark and maintained in the greenhouse. On 3 March 2000, sixteen plants from each species were selected and whole plant fresh weights (FW) were determined. Plants were arranged in blocks by size before they were transferred to 2-L plastic bottles filled with a complete nutrient solution. Plastic bottles were wrapped with 2 layers of aluminum foil to eliminate
light infiltration. The nutrient solution contained the following composition (mM): 0.5 K$_2$HPO$_4$, 1.0 MgSO$_4$, 0.5 CaCl$_2$, 0.09 Fe-diethylenetriaminopentaacetic acid (Fe-DTPA), 0.045 H$_3$BO$_3$, 0.01 MnSO$_4$, 0.01 ZnSO$_4$, and 0.2 µM Na$_2$MoO$_4$. The nitrogen source was either 5.0 mM (NH$_4$)$_2$SO$_4$ or NaNO$_3$. The nutrient solutions were buffered at pH 5.5 with 10 mM 2-(4-Morpholino)-Ethane Sulfonic acid (MES). Eight plants of each species were acclimated in the nutrient solutions containing either (NH$_4$)$_2$SO$_4$ or NaNO$_3$ for 3 weeks. After acclimation (22 March 2000), the experimental treatments began by eliminating Fe from the solutions in half of the plants of each species and each nitrogen source (-Fe), while the other half continued to receive 90 µM Fe (+Fe). The pH of the nutrient solution in each bottle was monitored daily with a portable pH meter (Accumet 1001, Fisher, USA) and maintained at 5.5, using 0.1 N KOH or 0.1 N HCl. Aeration was provided to each bottle by an aquarium pump (Elite 801, Rolf C. Hagen, Mansfiled, MA), connected by tygon tubing, located at the bottom of the bottle. The airflow was adjusted to 1L/min. Nutrient solutions were changed weekly. The amount of solution left in each bottle was recorded and used to determine the water use in each bottle on a weekly basis. Plant water use was corrected for evaporative losses using aerated bottles containing nutrient solution without plants. Six weeks after treatments began (3 May 2000), Fe (90 µM) was resumed in all –Fe plants and plants were grown for an additional 7 weeks. The greenhouse conditions during the experimental period averaged 29/20°C day/night temperature, 90% relative humidity and averaged PPF of 540 µmol·m$^{-2}$·s$^{-1}$.

**Ammonium, Nitrate and Iron Uptake**

Ammonium, NO$_3^-$ and Fe uptake were determined weekly by measuring depletion from the nutrient solutions. For NH$_4^+$ uptake, 10 µl of the nutrient solution was taken
from each sample bottle before the solutions were changed each week. One hundred µl of
175 mM Na$_2$-EDTA, 200 µl phenolnitroprusside (744 mM phenol and 1.14 mM
nitroprusside) and 400 µl hypochlorite solution (0.37 M NaOH, 0.35 M Na$_2$HPO$_4$·7H$_2$O
and 1% NaOCl) were added to the samples. The samples were incubated in a shaking
water bath at 40°C for 30 min before reading spectrophotometrically (Shimadzu UV-160,
Japan) at 636 nm. The NH$_4^+$ uptake rate per day was determined by calculating the
amount of NH$_4^+$ depletion from the solution and dividing by the number of days between
nutrient solution changes.

For NO$_3^-$ uptake, 10 µl of the nutrient solution was taken from each sample bottle
before the solutions were changed each week and diluted with 1.5 ml distilled water.
Concentrated HCl (15 µl of 12.1 N) was added and samples were vortexxed before reading
spectrophotometrically (Shimadzu UV-160, Japan) at 210 nm. The NO$_3^-$ uptake rate per
day was determined by calculating the amount of NO$_3^-$ depletion from the solution and
dividing by the number of days between nutrient solution changes.

Iron concentration left in the solution was determined using atomic absorption
spectrophotometry (Perkin Elmer 3030B, Norwalk, Connecticut, USA) with a hallow
cathode lamp as a light source and air-acetylene flame. The depletion of Fe from the
nutrient solution was calculated and divided by the number of days between nutrient
solution changes to represent Fe uptake rate.

**Root Fe (III) Reducing Capacity**

Root FCR activities were quantified every two weeks. Root tips (1 cm long) were
cut, placed in a beaker filled with ice water and transferred to the lab. Roots were
weighed and about 100 mg tissue FW were placed in a test tube. Root tissue was rinsed in
0.2 mM CaSO₄ for 5 min before transferring to 2 mL assay solution, containing 10 mM CaSO₄, 5 mM MES (pH 5.5), 0.1 mM Fe-EDTA and 0.3 mM sodiumbathophenanthroine disulfonic acid (Na₂-BPDS). One test tube with 2 mL assay solution without root tissue was used as a control. Samples and control tubes were incubated for 1 h in a shaking water bath at 50 rpm and 23°C in the dark. After incubation, a 1 mL aliquot from each tube was transferred into a cuvette and read spectrophotometrically at 535 nm (Shimadzu UV-160, Japan). The concentration of Fe(II)-BPDS produced was calculated using the molar extinction coefficient of 22.14 mM/cm (Chaney et al., 1972).

**Root Nitrate Reductase**

Root NR activities were quantified every two weeks. Root tips (1 cm long) were cut, placed in a beaker filled with ice water, and transferred to the lab. Roots were weighed and about 100 mg tissue FW were placed in each test tube (2 tubes/trt/rep). Two mL of assay solution, composed of 2% 1-propanol, 100 mM KH₂PO₄ (pH 7.5) and 30 mM KNO₃ were added to each test tube. One tube/trt/rep was immediately filtered through Whatman No. 2 paper and used as the time 0 control. Samples were vacuum infiltrated for 5 min. and incubated in a shaking water bath at 31°C and 50 rpm for 1 h in the dark. After incubation, the assay solution with roots was filtered and a 1 mL aliquot from each sample was removed to a new tube. One-mL sulfanilamide (1% w/v in 1.5N HCl) and 1 mL N-(1-naphthyl)-ethylenediaminedihydrochloride (0.02% w/v in 0.2N HCl) were added. The samples were incubated at room temperature for 30 min. The absorbance at 540 nm was determined spectrophotometrically (Modified from Jaworski, 1971).
Plant Harvesting

Plants were harvested on 20 June 2000, separated into shoots (stems and leaves) and roots, and fresh weight of each part was determined. Dry weight was determined after oven drying at 70°C to constant weight.

Experimental Design

Treatments were arranged in a 2 x 2 x 2 factorial in a randomized complete block design with 4 replications, using a single plant per replication. The factors were plant species (V. corymbosum or V. arboreum), N forms ((NH₄)SO₄ or NaNO₃) and Fe concentrations (0 or 90 µM). Data were analyzed using SAS (SAS Institute, Cary, N.C., USA). Mean separation was performed using t-test on every pair of Least Square Means (LSM). Daily N and Fe uptake rates/plant and final FW and DW were analyzed using initial plant FW as a covariate to normalize for differences in initial plant size.

Results

Nitrogen Uptake

In general, the NH₄⁺ uptake rate was significantly higher than the NO₃⁻ uptake rate throughout the experiment, regardless of species (Fig. 3-1A). The average uptake rates for NH₄⁺ ranged from 0.18 to 0.57 mmol NH₄⁺/plant/day, while the uptake rates for NO₃⁻ ranged from 0.02 to 0.32 mmol NO₃⁻/plant/day. There was no interaction between species and N-form on N uptake rate, except at week 6 after treatment when the uptake rate of NH₄⁺ in V. corymbosum was significantly higher than the NO₃⁻ uptake rate of the same species and higher than the NH₄⁺ and NO₃⁻ uptake rates in V. arboreum (Fig. 3-1B). Throughout the experiment, NO₃⁻ uptake rates in V. arboreum were greater than in V. corymbosum, although significant differences were not manifested until 9 weeks after
treatment (Fig. 3-1B). Iron concentration did not significantly affect NH$_4^+$ or NO$_3^-$ uptake rate, regardless of species (data not shown).

**Root Nitrate Reductase Activity**

There was a significant interaction between species and N form on root NR activity (Fig. 3-2A). Under NO$_3^-$ conditions, NR activity in *V. arboreum* increased gradually from 240 to 540 nmol NO$_2^-$/g FW/h; however, in *V. corymbosum*, NR activity decreased from week 3 (350 nmol NO$_2^-$/g FW/h) until week 11 (170 nmol NO$_2^-$/g FW/h). Under NH$_4^+$ conditions, NR activity was relatively constant for *V. arboreum* throughout the experiment, averaging 90 nmol NO$_2^-$/g FW/h. In *V. corymbosum*, NR activity increased from 20 to 200 nmol NO$_2^-$/g FW/h between weeks 5 and 11.

Iron concentration had a significant effect on NR activity during the –Fe treatment period (Fig. 3-2B). NR activity was greater under Fe deficient compared with Fe sufficient conditions at week 3. However, by week 5, NR activity under Fe deficient conditions was lower than under Fe sufficient conditions. When Fe was resumed to –Fe plants at week 6, NR activities in these plants increased to rates similar to +Fe plants.

There were no interactions between species and Fe concentration or Fe concentration and N-form on NR activity.

**Iron Uptake**

There was no effect of species or N-form on Fe uptake. No Fe uptake was detected under Fe deficient conditions, while the average Fe uptake rate for Fe sufficient plants was 2.3 µmol/plant/day throughout the experiment (data not shown).

When Fe (90 µM) was resumed after week 6 in plants that were –Fe treated, there was a transient increase in Fe uptake rate in *V. corymbosum* at week 7 compared with +Fe plants of the same species (4.4 vs 3.6 µmol/plant/day, respectively). After week 7,
the Fe uptake rate in previously treated –Fe *V. corymbosum* decreased to rates similar to +Fe plants.

**Root Iron Reductase Activities**

There was a significant interaction between species and Fe concentration on root FCR. Under Fe sufficient conditions, root FCR activity was similar between species, averaging ~ 200 nmol Fe$^{2+}$/gFW/h by week 1 after treatment, then decreasing to ~ 65 nmol Fe$^{2+}$/gFW/h by the end of the experiment (Fig. 3-3). Under Fe deficient conditions, root FCR activity in *V. corymbosum* increased from 150 to 400 nmol Fe$^{2+}$/gFW/h during the first 3 weeks after treatment, while activity in *V. arboreum* remained fairly constant, averaging 70 nmol Fe$^{2+}$/gFW/h. By week 5 after treatment, root FCR activity in *V. corymbosum* under Fe sufficient conditions decreased slightly, but was still significantly higher than activity in *V. arboreum*. In general, root FCR activity in *V. arboreum* was not different between Fe sufficient and Fe deficient conditions. When Fe was resumed to –Fe plants at week 6 after treatment, root FCR activity in previously treated –Fe *V. corymbosum* plants decreased dramatically to the same level as the +Fe plants.

There was no significant effect of N-form on root FCR activities, which averaged 130 nmol Fe$^{2+}$/gFW/h for both N forms (data not shown). There was no significant interaction between species and N form or N form and Fe concentration on root FCR activity.

**Plant Growth**

Shoot FW and DW were significantly greater in *V. arboreum* compared with *V. corymbosum* (Table 3-1). There was no effect of species or N-form on root or whole plant FW or DW. Shoot DW was greater in Fe sufficient plants compared with Fe deficient
plants regardless of species or N-form. However, Fe concentration had no effect on shoot or root FW or root DW.

There was a significant interaction between species and N-form on shoot FW and DW. Shoot FW of NO₃⁻ treated V. arboreum was significantly greater than NH₄⁺ treated plants of the same species and either NO₃⁻ or NH₄⁺ treated V. corymbosum (Table 2). However, dry weight of shoots and whole plants were significantly greater in NO₃⁻ treated V. arboreum and NH₄⁺ treated plants of both species compared with NO₃⁻ treated V. corymbosum.

**Discussion**

*V. corymbosum* and *V. arboreum* were able to take up both NO₃⁻ and NH₄⁺; however, NH₄⁺ uptake rates were 1.7 to 8.5-fold greater than NO₃⁻ uptake rates in both species. This agrees with previous work in *Vaccinium* (Merhaut, 1993; Sugiyama and Hirooka, 1993; Sugiyama and Ishigaki, 1994). Low rates of NO₃⁻ uptake in *Vaccinium* are likely related to activity of root NR which is markedly lower in *Vaccinium* compared with many other woody (Bussi et al., 1997; Hucklesby and Blanke, 1987; Lee and Titus, 1992) and herbaceous plants (Hucklesby and Blanke, 1987). Low root NR activity in *Vaccinium*, coupled with lack of detectable leaf NR activity (Merhaut, 1993), could contribute to N deficiency and decreased growth on soils where the predominant N form was NO₃⁻.

Although NR activity in *Vaccinium* is generally lower than in other plants, the average root NR activity in *V. arboreum* grown in NO₃⁻ was 1.5 to 3.2-fold higher than *V. corymbosum* grown in NO₃⁻. However, the higher root NR activity in *V. arboreum* was not reflected in significant increases in NO₃⁻ uptake compared with *V. corymbosum* until 9 weeks after treatment, although there was a trend towards greater NO₃⁻ uptake in *V.
arboreum throughout the experiment. The depletion technique used to determine NO$_3^-$ uptake in this study may not have been sensitive enough to detect small differences in NO$_3^-$ uptake between species. Using labeled NO$_3^-$, either $^{13}$N or $^{15}$N, is a more sensitive method for determining NO$_3^-$ uptake, and would be more likely to detect small differences in NO$_3^-$ uptake (Tischner, 2000).

Root NR activity was detected in Vaccinium species under both NO$_3^-$ and NH$_4^+$ conditions, however, activity under NH$_4^+$ conditions was only 37% of the activity under NO$_3^-$ conditions, confirming that NR is NO$_3^-$-induced in Vaccinium as it is in other plants (Knoepp et al., 1993; Peuke and Tischner, 1991; Min et al., 1998). However, the observation of NR activity in V. arboreum and V. corymbosum under NH$_4^+$ conditions supports the idea that Vaccinium also has a constitutive NR system (Merhaut, 1993).

Nitrate reductase is a complex enzyme that contains Fe in one of its subunits (Crawford et al., 1992) and lack of Fe can reduce NR activity (Pandey, 1989; Smith, 1984; Marschner, 1995). In this present study, NR activity in Fe deficient plants was greater in week 3 but lower in week 5 compared with Fe sufficient plants. The greater NR activity in Fe deficient plants at week 3 may reflect the ability of NR to compete with FCR for the electron provided by NAD(P)H, resulting in increased NR activity (Brown and Jones, 1976; Campbell and Redinbaugh, 1984). However, the reduction of NR activity by week 5 may be the result of a decrease in ferrodoxin, the electron donor for the NO$_2^-$ reductase, under Fe deficiency (Marschner, 1995). The decrease in ferrodoxin may down-regulate NR activity in order to prevent NO$_2^-$ accumulation, which is toxic to the plant. The ferrodoxin content and NR activity are restored upon Fe resupply.
The increase in NR activity after Fe was resumed to nutrient solutions in the present experiment supports this idea.

*V. corymbosum* responded to Fe deficiency by increased root FCR activity. Root FCR activity increased about 1.5 to 1.8-fold under Fe deficient conditions compared with Fe sufficient conditions in *V. corymbosum*. The increased root FCR activity under Fe deficiency in *V. corymbosum* is similar to the increases reported in peach (de la Guardia et al., 1995) and citrus rootstocks (Manthey et al., 1994) under Fe deficient conditions. In contrast, root FCR activity in *V. arboreum* did not increase under Fe deficiency. Based on responses to Fe deficiency, *V. corymbosum* may be classified as an Fe-efficient genotype, while *V. arboreum* is an Fe-inefficient genotype.

The higher root FCR activity in *V. corymbosum* in response to Fe deficiency was not reflected in increased Fe uptake rate due to the lack of Fe in the nutrient solution during the first 6 weeks of treatment. However, there was a significant increase in Fe uptake rate in previously treated –Fe *V. corymbosum* after Fe was resumed at week 7. Thus, Fe availability in the solution was the limiting factor for Fe uptake in the present experiment.

The lower NO$_3^-$ uptake rate compared with NH$_4^+$ uptake rate was reflected in decreased plant DW in *V. corymbosum* grown under NO$_3^-$-N vs NH$_4^+$-N. This result is in agreement with Merhaut (1993) and Claussen and Lenz (1999) in highbush blueberry. However, no difference in plant DW was observed in *V. arboreum*. The greater growth of *V. arboreum* compared with *V. corymbosum* under NO$_3^-$ conditions indicates that *V. arboreum* may survive better than *V. corymbosum* in upland high pH soils, where NO$_3^-$ is the predominant N form.
The results from the present study indicate that *V. corymbosum* is more efficient in responding to Fe deficiency compared with *V. arboreum*, as indicated by an increase in root FCR activity in *V. corymbosum* under –Fe conditions. As a result, *V. corymbosum* would be considered an Fe-efficient genotype, while *V. arboreum* would be an Fe-inefficient genotype. The higher root NR activity in *V. arboreum* supports our hypothesis that this species is potentially more efficient than *V. corymbosum* in terms of NO$_3^-$ reduction. The ability of *V. arboreum* to utilize NO$_3^-$ efficiently would be an advantage in upland high pH soils where NO$_3^-$ is the dominant source of N.
Fig. 3-1. Nitrogen form (NH₄⁺ or NO₃⁻) (A) and interaction between species and nitrogen form (B) on nitrogen uptake rate. Values are adjusted means using initial whole plant FW as a covariate. Mean ± SE (n = 4).
Fig. 3-2. Species and N-form (A) and Fe concentration (B) effects on root nitrate reductase (NR) activity. 0 µM Fe preconditioning indicates the –Fe (0 µM) treatments after Fe was resumed at week 6. Means ± SE (n = 4).
Weeks after treatment

Fig. 3-3. Interaction between species and Fe concentration (0 or 90 µM) on root ferric chelate reductase (FCR) activity. Fe (90 µM) were resumed in Fe-deficient treatments (0 µM Fe) after week 6. Mean ± SE (n = 4).
Table 3-1. Effect of species, nitrogen form and iron concentration on final fresh weight (FW) and dry weight (DW) of shoot (stem + leaves) and roots. Values are adjusted means using initial whole plant FW as a covariate (n = 4).

<table>
<thead>
<tr>
<th></th>
<th>Initial FW (g)</th>
<th>FW (g)</th>
<th>DW (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot</td>
<td>Root</td>
<td>Plant</td>
</tr>
<tr>
<td><strong>V. arboreum</strong></td>
<td>12.2</td>
<td>38.2</td>
<td>19.8</td>
</tr>
<tr>
<td><strong>V. corymbosum</strong></td>
<td>18.1</td>
<td>22.0</td>
<td>20.6</td>
</tr>
<tr>
<td><strong>NS</strong>, <strong>Z</strong></td>
<td>*</td>
<td>NS</td>
<td>NS</td>
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<table>
<thead>
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<th>DW (g)</th>
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<td>NO₃⁻</td>
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<td>21.9</td>
</tr>
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<td><strong>NS</strong></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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</table>

<table>
<thead>
<tr>
<th>Iron concentration (µM)</th>
<th>Initial FW (g)</th>
<th>FW (g)</th>
<th>DW (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot</td>
<td>Root</td>
<td>Plant</td>
</tr>
<tr>
<td>0</td>
<td>15.4</td>
<td>27.8</td>
<td>21.3</td>
</tr>
<tr>
<td>90</td>
<td>14.9</td>
<td>32.4</td>
<td>19.1</td>
</tr>
<tr>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
</tr>
</tbody>
</table>

*NS, **, * = nonsignificant, significant at the 1% and 10% level by t-test.
Table 3-2. Interaction between species and nitrogen form on final fresh weight (FW) and dry weight (DW). Values are adjusted means using initial whole fresh weight as a covariate (n = 4).

<table>
<thead>
<tr>
<th>species x N form</th>
<th>FW (g)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot</td>
<td>Root</td>
<td>Plant</td>
<td>Shoot</td>
<td>Root</td>
</tr>
<tr>
<td><em>V. arboreum</em> x NH₄⁺</td>
<td>26.9 b</td>
<td>15.8</td>
<td>47.7</td>
<td>14.6 a</td>
<td>2.8</td>
</tr>
<tr>
<td><em>V. arboreum</em> x NO₃⁻</td>
<td>49.5 a</td>
<td>23.8</td>
<td>64.9</td>
<td>20.2 a</td>
<td>4.6</td>
</tr>
<tr>
<td><em>V. corymbosum</em> x NH₄⁺</td>
<td>24.0 b</td>
<td>21.2</td>
<td>60.4</td>
<td>14.1 a</td>
<td>4.0</td>
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<td><em>V. corymbosum</em> x NO₃⁻</td>
<td>20.0 b</td>
<td>20.1</td>
<td>43.1</td>
<td>8.2 b</td>
<td>3.7</td>
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<tr>
<td></td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
</tr>
</tbody>
</table>

*NS, * = nonsignificant or significant at the 10% level by t-test.
CHAPTER 4
EFFECT OF SPECIES AND PH ON FERRIC CHELATE REDUCTASE, NITRATE REDUCTASE, AND IRON AND NITRATE UPTAKE IN BLUEBERRIES

Introduction

Blueberries are acid loving plants or calcifuges (Korcak, 1989), based on their increased growth under acidic compared with alkaline soils (Williamson and Lyrene, 1995). The pH for optimum growth in blueberry ranges from 3.5 to 5.5, depending on genotype (Gough, 1996; Korcak, 1989; Townsend, 1971; Williamson and Lyrene, 1995). Fruit size and yield of rabbiteye blueberry (V. ashei Reade,) decreased with increasing soil pH from 5.1 to 6.3 (Austin et al., 1986). Shoot and root dry weights of hybrid blueberry (V. corymbosum x V. angustifolium ‘Northblue’) were higher at pH 4.5 compared with 6.5 (Rosen et al., 1990).

Leaf and root nutrient concentrations are also affected by rhizosphere pH and effects on growth may be mediated through effects on nutrient uptake and assimilation, especially iron (Fe) and nitrogen (N). In general, Fe uptake is restricted at high pH (Mengel and Kirkby, 2001), due to the decrease in Fe solubility as pH increases (Korcak, 1987; Lindsay, 1984). For example, Austin et al. (1986) found that as soil pH increased from 5.1 to 6.9, leaf Fe concentration in rabbiteye blueberry decreased.

The decrease in Fe uptake as soil pH increases may also be due to an inhibition of activity of ferric chelate reductase (FCR), the key enzyme in Fe assimilation in dicots and non-graminaceous monocots (Marschner, 1995; Mengel and Kirkby, 2001). Ferric chelate reductase is a plasma membrane-bound enzyme that reduces ferric (Fe$^{3+}$)-chelate
to ferrous (Fe$^{2+}$) (Holden et al., 1991; Mengel, 1994, Mengel and Kirkby, 2001). Ferrous then passes through a specific cation channel in the plasma membrane of the root cells (Mengel, 1994; Mengel and Kirkby, 2001). Once in the symplast, Fe$^{2+}$ is oxidized, complexed with citrate, and translocated to the upper plant parts via xylem (Mengel, 1994). Once in the shoot, Fe$^{3+}$ must again be reduced to Fe$^{2+}$ via a leaf FCR and taken up into the mesophyll cytoplasm by a cation channel (Schmidt, 1999). The activity of root FCR is pH dependent (Ao et al., 1985; Holden et al., 1991; Mengel, 1994; Moog and Bruggemann, 1994; Susin et al., 1996), with the optimum rhizosphere pH range from 5.0 to 6.5, depending on plant species (Moog and Bruggemann, 1994). As rhizosphere pH increases above 6.5, root FCR activity decreases markedly (Holden et al., 1991).

Rhizosphere pH also affects N uptake. As pH increases, nitrate (NO$_3^-$) uptake decreases due to the negative effect of high OH$^-$ concentration in the soil solution, which depresses the NO$_3^-$:H$^+$ cotransport system (Vaast et al., 1998). Additionally, there appears to be an interaction between Fe and NO$_3^-$ uptake. Nitrate uptake increases rhizosphere pH (Smolders et al., 1997; Kosegarten et al., 1998 a,b), due to NO$_3^-$:H$^+$ cotransport (Mengel and Kirkby, 2001; Ullrich, 1992). In response to the increased pH, Fe uptake may be inhibited (Mengel et al., 1994; Kosegarten et al., 1998 a,b).

Nitrate reductase (NR) is the regulatory enzyme responsible for NO$_3^-$ uptake and assimilation (Crawford, 1995). It is found either as a plasma membrane-bound (Berczi and Moller, 2000; Stohr, 1999; Ullrich et al., 1990) or as a cytosol localized enzyme in root epidermal and cortical cells and shoot mesophyll cells (Berczi and Moller, 2000; Crawford, 1995; Stohr, 1999). This enzyme transfers electrons either from NADH or NADPH to NO$_3^-$, reducing it to nitrite (NO$_2^-$) (Crawford, 1995; Srivastava, 1992; Wray
and Abberton, 1994). In blueberry, low activities of NR were detected in leaves (Dirr et al., 1972) and roots (Townsend, 1970; Merhaut, 1993) of NO$_3^-$-fed plants. Claussen and Lenz (1999) reported no NR activity was found in either leaves or stem of highbush blueberry plants fed with NO$_3^-$. Low activity of NR may limit the ability of blueberry to thrive on high pH soils (Korcak, 1989), where NO$_3^-$ is the predominant N form (Mengel and Kirkby, 2001).

There is increasing interest in expanding blueberry production to upland soils, characterized by low organic matter and high pH (Williamson and Lyrene, 1997). These soils also possess low Fe availability and high NO$_3^-$ due to increased rates of nitrification at high pH. Blueberries grown in soils such as these usually develop symptoms of leaf chlorosis (Brown and Draper, 1980), and suppression of growth (Korcak et al., 1982), due to decreased Fe uptake (Korcak, 1987). Blueberry may also exhibit N deficiency on these soils, due to the limited ability to assimilate NO$_3^-$.  

The success of expanding blueberry production to upland high pH soils will depend in part on developing suitable blueberry cultivars adapted to these soils. One of the characteristics that should be considered is the effect of high soil pH on Fe and N availability and growth of blueberry. There have been attempts to breed blueberries for wider soil adaptation (primarily higher pH soils) (Lyrene, 1993, 1997; Patel and Goerge, 1996). Recently, Lyrene (1997) reviewed the value of various blueberry species in the Florida breeding program. One of the species that has several desirable traits is _V. arboreum_ Marsh, commonly known as the sparkleberry or farkleberry (Brooks and Lyrene, 1995). The sparkleberry is native to the southeastern U.S. and has the ability to thrive on higher pH soils (up to pH 6.0), which highbush and southern highbush
blueberry tolerate poorly (Brooks and Lyrene, 1995; Lyrene, 1997). *V. arboreum* also does well in soils that are seasonally dry and low in organic matter (Brooks and Lyrene, 1995). The sparkleberry has a coarse root system with a tap root, which differs from the shallow, fibrous root systems of other *Vaccinium* species (Lyrene, 1997). The undesirable traits of sparkleberry are small, dark, and scarcely palatable berries, large seeds, abundant sclerids, low juice content and occasionally astringent flesh (Lyrene, 1997). However, its ability to grow in alkaline soils suggests that the mechanism and/or extent of Fe and/or NO$_3^-$ uptake on high pH soils may differ from that of the cultivated species, *V. corymbosum*.

The objectives of the present study were to compare the FCR activity, NR activity, and Fe and NO$_3^-$ uptake of two blueberry species, *V. corymbosum* L. interspecific hybrid (southern highbush blueberry) and *V. arboreum* Marsh (wild blueberry) grown at pH 5.5 and 6.5. We hypothesized that *V. arboreum* should be able to grow better than *V. corymbosum* under high pH (6.5) due to increased ability to take up and assimilate Fe and NO$_3^-$.

**Materials and Methods**

**Plant Culture**

Seeds of the wild species, *V. arboreum* Marsh, were collected from a natural habitat at Juniper Springs during Fall 1999 and immediately germinated on the surface of Canadian peat under intermittent mist (4 hours/day) in a greenhouse, with average temperatures of 25°C and 545 µmol·m$^{-2}$·s$^{-1}$ PPF. In April 2000, shoot cuttings of southern highbush blueberry ‘Misty’ were rooted in peat:perlite (1:1 by volume) medium under intermittent mist in a greenhouse with average temperatures of 25°C and average PPF of
545 μmol·m⁻²·s⁻¹. In March 2001, plants of both species were transplanted into 1-L pots containing pine bark and maintained in the greenhouse. On 1 June 2001, sixteen plants from each species were selected and transferred to 2-L plastic bottles filled with a complete nutrient solution. Plastic bottles were wrapped with 2 layers of aluminum foil to eliminate light infiltration. The nutrient solution contained the following composition (mM): 0.5 K₂HPO₄, 1.0 MgSO₄, 0.5 CaCl₂, 0.09 Fe-diethylenetriaminopentaacetic acid, 0.045 H₃BO₃, 0.01 MnSO₄, 0.01 ZnSO₄, and 0.2 μM Na₂MoO₄. The N source was 5.0 mM NaNO₃. The nutrient solutions were buffered at pH 5.5 with 10 mM MES. Plants were acclimated in the nutrient solutions for 2 weeks. After acclimation (15 June 2001), the nutrient solutions for 8 plants of each species were adjusted to pH 6.5, while the remaining plants were maintained at pH 5.5. The pH of nutrient solutions in each bottle were monitored daily with a portable pH meter (Accumet 1001, Fisher, USA). The pH was adjusted as needed to 5.5 or 6.5 using 0.1 N KOH or 0.1 N HCl. Aeration was provided to each bottle by an aquarium pump (Elite 801, Rolf C. Hagen, Mansfield, MA), connected by tygon tubing, located at the bottom of the bottle. The airflow was adjusted to 1L/min. Nutrient solutions were changed weekly during the first 4 weeks of the experiment and every 2 weeks thereafter. The amount of solution left in each bottle was recorded and used to determine the water use in each bottle on a weekly basis. Plant water use was corrected for evaporative losses using bottles containing nutrient solution without plants. Plants were grown for 12 weeks, from June through September 2001, under greenhouse conditions with 29/20°C day/night temperature, 80% relative humidity and average PPF of 545 μmol·m⁻²·s⁻¹. Root temperature was measured twice in August,
using the temperature sensor attached to a portable pH meter (Accumet 1001, Fisher, USA). The average root temperature was 28°C.

**Nitrate and Iron Uptake**

Nitrate and Fe uptake were determined on a weekly basis by measuring depletion from the nutrient solutions. For NO$_3^-$ uptake, 10 µl of the nutrient solution taken from each sample bottle before the solutions were changed each time was diluted with 1.5 ml distilled water and vortexed. Concentrated HCl (15 µl of 12.1 N) was added and the samples were vortexed before reading spectrophotometrically (Shimadzu UV-160, Japan) at 210 nm. The NO$_3^-$ uptake rate per day was determined by calculating the amount of NO$_3^-$ depletion from the solution and dividing by the number of days between nutrient solution changes.

Iron concentration left in the solution was determined using atomic absorption spectrophotometry (Perkin Elmer 3030B, Norwalk, Connecticut, USA) with a hallow cathode lamp as a light source and air-acetylene flame. The depletion of Fe from the nutrient solution was calculated and represented the rate of Fe uptake.

**Root and Leaf Fe (III) Reducing Capacity**

Root and leaf FCR activities were quantified every two weeks. For root FCR, root tips (1 cm long) were cut, placed in a beaker filled with ice water and transferred to the lab. Roots were weighed and about 100 mg tissue FW were placed in a test tube. Root tissue was rinsed in 0.2 mM CaSO$_4$ for 5 min before transferring to 2 mL assay solution, containing 10 mM CaSO$_4$, 5 mM MES (pH 5.5), 0.1 mM Fe-EDTA and 0.3 mM sodiumbathophenanthroinedisulfonic acid (Na$_2$-BPDS). One test tube with 2 mL assay solution without root tissue was used as a control. Samples and control tubes were
incubated for 1 h in a shaking water bath at 50 rpm and 23°C in the dark. After the incubation period, a 1 mL aliquot from each tube was transferred into a cuvette and read spectrophotometrically at 535 nm (Shimadzu UV-160, Japan). The concentration of Fe(II)-BPDS produced was calculated using the molar extinction coefficient of 22.14 mM/cm (Chaney et al., 1972).

For the leaf FCR assay, 4 mm diameter leaf discs were taken from each plant, placed in a beaker filled with ice water and transferred to the lab. Leaf discs were weighed and about 100 mg FW tissue was placed in test tubes containing 0.2 mM CaSO₄. After 10 min, the CaSO₄ was discarded and 2 mL of assay solution (described above) were added to the tubes. Samples were vacuum infiltrated for 10 min. before incubating in a shaking water bath at 50 rpm and 23°C for 1 hr in the dark. After incubation, 1 mL aliquot from each tube was transferred into a cuvette and read spectrophotometrically at 535 nm. The concentration of Fe(II)-BPDS was calculated using the same method as described above.

Root Nitrate Reductase

Root NR activities were quantified every two weeks. Root tips (1 cm long) were cut, placed in a beaker filled with ice water, and transferred to the lab. Roots were weighed and about 100 mg tissue FW were placed in each test tube (2 tubes/trt/rep). Two mL of assay solution, composed of 2% 1-propanol, 100 mM KH₂PO₄ (pH 7.5) and 30 mM KNO₃ were added to each test tube. One tube/trt/rep was immediately filtered through Whatman No. 2 paper and used as the time 0 control. Samples were vacuum infiltrated for 5 min. and incubated in a shaking water bath at 31°C and 50 rpm for 1 h in the dark. After incubation, the assay solution with roots was filtered and a 1 mL aliquot
from each sample was removed to a new tube. One-mL sulfanilamide (1% w/v in 1.5N HCl) and 1 mL N-(1-naphthyl)-ethylenediaminedihydrochloride (0.02% w/v in 0.2N HCl) were added. The samples were incubated at room temperature for 30 min. The absorbance at 540 nm was determined spectrophotometrically (Modified from Jaworski, 1971).

**Leaf Chlorosis**

Estimation of leaf chlorophyll concentration was performed using a chlorophyll meter (SPAD 502, Minolta Corp., Japan) at the beginning of the experiment and every 10 to 20 days after that. Five leaves (the 2nd or 3rd mature leaves from the lateral branches) from each plant were used for each measurement time. The readings were averaged and represented the leaf chlorophyll concentration. Leaf chlorophyll concentrations were determined using the methods described by Moran and Porath (1980) and were used to calibrate the SPAD reading index.

**Leaf Growth**

Leaf width and length were determined at the beginning and the end of the experiment, using three leaves (the 2nd or 3rd mature leaves from the lateral branches) from each plant. Leaf area were calculated by multiplying the average leaf width and leaf length.

**Tissue N and Fe Determination**

Plants were harvested on 6 September 2001 and washed with deionized (DI) water before they were separated into shoots and roots. Fresh weight (FW) of each part was determined. Dry weight (DW) was determined after oven drying at 70°C to constant weight. Dried samples were ground through a 20-mesh screen in a Wiley Mill (Arthur Thomas Co., Pennsylvania, USA) and analyzed for total Kjedahl N (TKN) and Fe. For
TKN, 100 mg sample was placed into 50 mL digesting tube. Popes Kjeldahl mixture (2.0 g) was added to each tube, followed by 2.5 mL sulfuric acid. Glass funnels were inserted in each tube before placing the tubes on a digestion block at 380°C for 8-10 hr. After cooling, the tubes were rinsed with 10-15 mL of DI water, vortexed, and brought to 50 mL with DI water. Samples were then filtered (Whatman No. 8 filter paper) and analyzed using the Kjeldahl method (Horneck and Miller, 1998).

For Fe concentration, a dry ash digestion of the tissue was performed. Dried tissue (500 mg) was placed in a muffle furnace at 500°C for 10-12 hours. After cooling, 50 mL of 1N HCl was added to each sample and the solutions were filtered (Whatman No. 8 filter paper). Iron concentration was determined using atomic absorption spectrophotometry as described earlier.

For NO₃⁻ concentration, dried tissue (500 mg), ground through 40-mesh screen, was placed in a 50 mL Erlenmeyer flask, 50 mL of DI water was added, and samples were incubated on a lateral shaker for 30 min. The samples were filtered through Whatman No. 8 filter paper. The extract was analyzed for NO₃⁻ concentration by Automated Cadmium Reduction Method using Rapid Flow Analyzer ALPKEM 300 (Alpkem Corporation, Oregon, USA) at 540 nm (Anderson and Case, 1999).

**Experimental Design**

Treatments were arranged in a 2 x 2 factorial in a randomized complete block design with 8 replications, using a single plant per replication. The factors were plant species (*V. corymbosum* or *V. arboreum*) and pH (5.5 and 6.5). Data were analyzed using SAS (SAS Institute, Cary, N.C., USA). Mean separation was performed using t-test on every pair of Least Square Means (LSM). Daily NO₃⁻ and Fe uptake rates/plant and final plant FW and DW were analyzed using initial plant FW as a covariate.
Results

Nitrate Uptake

During the first 4 weeks of the experiment, there was no significant difference in NO₃⁻ uptake between the two species, with uptake averaging 0.21 mmol NO₃⁻/plant/day (Fig. 4-1A). By 6 weeks after treatments began, NO₃⁻ uptake rate was greater in *V. arboreum* compared with *V. corymbosum*, averaging 0.23 and 0.17 mmol NO₃⁻/plant/day, respectively. The effect of pH on NO₃⁻ uptake was inconsistent; uptake rate was significantly higher at pH 5.5 compared with pH 6.5 only during the second week of the experiment (Fig. 4-1B). There was an interaction between species and pH only at week 4, with the highest NO₃⁻ uptake found in *V. arboreum* at pH 5.5.

Root Nitrate Reductase

In general, root NR activity in *V. arboreum* was higher than *V. corymbosum* by 5 weeks after treatment and remained higher throughout the experiment, ranging from 100 to 370 nmol NO₂⁻/gFW/h (Fig. 4-2A). NR activity of *V. corymbosum* was relatively constant throughout the experiment, averaging 140 nmol NO₂⁻/gFW/h. There was no significant effect of pH on root NR activities (Fig. 4-2B). The average NR activities at pH 5.5 and 6.5 were 215 and 195 nmol NO₂⁻/gFW/h, respectively, and there was no interaction between species and pH.

Iron Uptake

The rate of Fe uptake was similar between *V. arboreum* and *V. corymbosum* during the experiment, although the actual rates fluctuated as much as 4-fold (from 1.2 to 4.9 µmol Fe/plant/day) throughout the experiment (Fig. 4-3A). During the first two weeks of the experiment, Fe uptake rate was greater at pH 5.5 than pH 6.5, averaging 3.4 and 0.4 µmol Fe/plant/day, respectively (Fig. 4-3B). Uptake rate at pH 6.5 then began to increase...
and was greater than the uptake rate at pH 5.5 by week 4. Uptake rates at both pHs continued to increase through week 6 before decreasing. There was no interaction between species and pH on rate of Fe uptake.

**Root and Leaf Fe (III) Reducing Capacity**

The average root FCR activity was 5-7 times higher than the average leaf FCR activity over the entire experimental period. There was no significant difference in root FCR activity between the two species, with activities ranging from 50-160 nmol Fe$^{2+}$/gFW/h (Fig. 4-4A). In contrast, leaf FCR activity in *V. arboreum* was significantly higher than in *V. corymbosum* throughout the experimental period (Fig. 4-5). No significant effect of pH on root FCR activity was detected during the first 5 weeks of the experiment, however, by week 7, root FCR activity was significantly higher at pH 6.5 compared with pH 5.5 (Fig. 4-4B). There was no effect of pH on leaf FCR activity (data not shown). No interaction was found between species and pH on either root or leaf FCR activity.

**Leaf Chlorosis**

The chlorophyll concentration of mature healthy leaves of *V. corymbosum* was higher than *V. arboreum*, and these concentration differences were maintained even in chlorotic leaves, thus it was not suitable to compare between species for this parameter. In general, there was no effect of pH on leaf chlorophyll concentration (represented by the SPAD reading) in either blueberry species (Table 4-1). In this study, leaf chlorosis of *V. arboreum* and *V. corymbosum* occurred with an average SPAD reading of 33 and 48, respectively. Leaf chlorosis was observed at Day 10 (for both species) and Day 25 (for *V. corymbosum*), but it corresponded with leaf flushing in both species and was ameliorated
when the leaves matured. No symptom of leaf chlorosis was detected by the end of the experiment.

**Plant Growth**

Leaf area in both species was decreased by high pH by the end of the experiment. As pH increased from 5.5 to 6.5, leaf area of *V. arboreum* and *V. corymbosum* was reduced by 67% (from 7.7 to 4.6 cm$^2$) and 46% (from 11.4 to 7.8 cm$^2$), respectively.

*V. arboreum* had greater final shoot FW compared with *V. corymbosum*, regardless of pH (Table 4-2). Final shoot, root and plant DW of *V. arboreum* were also significantly greater than in *V. corymbosum*. There was no significant effect of pH on FW or DW in either species at the end of the experiment (Table 4-2). There was no interaction between species and pH on FW or DW.

**Plant Tissue Analysis**

*V. corymbosum* had higher shoot and root TKN, higher root NO$_3^-$ and root Fe concentrations than *V. arboreum* at the end of the experiment (Table 4-3). There were no significant difference in shoot NO$_3^-$ and shoot Fe concentration between species. Shoot TKN and root Fe concentrations were higher at pH 5.5 compared with pH 6.5. No significant differences between pH were found on root TKN, shoot and root NO$_3^-$ and shoot Fe concentration. There was no interaction between species and pH on tissue TKN, NO$_3^-$ or Fe concentration.

**Discussion**

In general, *V. arboreum* has the capacity for increased Fe and NO$_3^-$ assimilation compared with *V. corymbosum*, as indicated by higher leaf FCR and root NR activities. The average root NR activities of *V. corymbosum* in this study were about 3-fold lower than reported in *V. corymbosum* ‘13-16-A’ (390 nmol NO$_2$/gFW/h) (Claussen and Lenz,
However, activities were higher than in the southern highbush blueberry ‘Sharpblue’ (27 nmol NO\textsubscript{2}/gFW/h) (Merhaut, 1993), and the northern highbush blueberry ‘Jersey’ (48 nmol NO\textsubscript{2}/gFW/h) (Dirr et al., 1972). Nonetheless, NR activity of *V. corymbosum* in the present study was lower than that found in other fruit crops, such as apple (280 nmol NO\textsubscript{2}/gFW/h) (Lee and Titus, 1992) and citrus (370 nmol NO\textsubscript{2}/gFW/h) (Hucklesby and Blanke, 1987). On the other hand, NR activity of *V. arboreum* was similar or higher than NR activities in those fruit crops. These results clearly indicate the decreased ability of *V. corymbosum* to assimilate NO\textsubscript{3}\textsuperscript{-} compared with *V. arboreum*.

Higher NR activity in *V. arboreum* corresponded well with the higher NO\textsubscript{3}\textsuperscript{-} uptake rate, lower root NO\textsubscript{3}\textsuperscript{-} concentration and increased shoot, root and whole plant DW observed in this species compared with *V. corymbosum*. Although *V. arboreum* exhibited greater NR activities and NO\textsubscript{3}\textsuperscript{-} uptake than *V. corymbosum*, this was not reflected in increased shoot and root TKN concentrations. This was likely due to the increased shoot and root DW of *V. arboreum*, resulting in a dilution of the reduced N concentration. These data suggest that *V. arboreum* has the ability to assimilate NO\textsubscript{3}\textsuperscript{-} better than *V. corymbosum*. This may partially explain why this species is better able to survive under high pH soils, where NO\textsubscript{3}\textsuperscript{-} is the primary N form.

Neither root NR activity nor NO\textsubscript{3}\textsuperscript{-} uptake was consistently affected by solution pH. This was in contrast with several studies that indicate NR activity increases as pH increases. Townsend (1970) reported lower root NR activity in lowbush blueberry (*V. angustifolium* Ait.) at a nutrient solution pH of 4.0 compared with pH 6.0. Peuke and Tischner (1991) found that NR activity in both root and shoot of spruce increased as pH increased from 3.5 to 6.5; however, NR activity decreased drastically at pH 7.5. They
concluded that optimum pH for NR activity for spruce was 5.5. It is possible that the pH range in the present study was too close to distinguish differences in NR activity. Similarly, there was no significant difference in root NR activity in spruce grown at pH 5.5 vs 6.5 (Peuke and Tischner, 1991).

The lack of pH effect on NR activity and NO$_3^-$ uptake was reflected in similar shoot, root and whole plant DW, root TKN and root and shoot NO$_3^-$ concentrations at both pHs. This is in agreement with work by Rosen et al. (1990) who found no pH effect (4.5 or 6.5) on root TKN in *V. corymbosum* L. x *V. angustifolium* Ait. ‘Northblue’. However, Townsend (1969) reported higher root TKN at pH 4.5 compared with pH 6.0 in lowbush blueberry. It is likely that the effect of pH on root TKN depends on genotype (Townsend, 1971). The root TKN in this present study was about 2- to 3 fold lower than previous reports in other *Vaccinium* spp. (Rosen et al., 1990; Townsend, 1969,1971), but was in the same range as reported by Merhaut and Darnell (1996) in the southern highbush blueberry ‘Sharpblue’.

No differences in root FCR or Fe uptake were found between species. Nevertheless, root Fe concentration was higher in *V. corymbosum* compared with *V. arboreum*. It is possible that more Fe was precipitated on the roots of *V. corymbosum* than *V. arboreum* due to the more fibrous nature of the root system of *V. corymbosum*. Alternatively, the apparent contradiction between Fe uptake and Fe concentration may be due to the limitations in the method used to measure Fe uptake. Differences in Fe uptake would have to be relatively large in order to be detected by measuring depletion from the nutrient solutions. Longer term depletion studies or the use of radioisotopes would be necessary to determine the uptake rate more accurately. Nevertheless, root Fe
concentration in both *V. arboreum* and *V. corymbosum* were similar in magnitude and range to concentrations in other *Vaccinium* species (Korcak et al., 1982). The higher root Fe concentration compared with shoot Fe concentration observed in both species is in agreement with work in other *Vaccinium* species (Townsend, 1971; Rosen et al., 1990).

The increase in root FCR activity at pH 6.5 compared with pH 5.5 during the latter part of the experiment contrasts with other work indicating root FCR activity decreases as pH increases (Susin et al., 1996). Although in the present experiment sufficient Fe (90 µM) was initially added to all nutrient solutions, Fe solubility and therefore availability at pH 6.5 may have been lower compared with pH 5.5 (Townsend, 1971; Kosegarten et al., 1998 a,b). The decreased Fe availability at higher pH may have resulted in increased root FCR activity, as reported in other crops exposed to low Fe concentration (Jolley et al., 1996; Manthey and Crowley, 1997; Vizzotto et al., 1999).

Although root FCR activity increased at pH 6.5 compared with 5.5, this was not reflected in a difference in Fe uptake. This may be due to competition between NO$_3^-$ and Fe$^{3+}$ for the electron provided by FCR, which would result in NO$_3^-$ reduction to NO$_2^-$ instead of Fe$^{3+}$ reduction to Fe$^{2+}$. Since Fe$^{2+}$ is the Fe form taken up by blueberry, failure to reduce Fe$^{3+}$ would limit Fe uptake even under high FCR activities (Lucena, 2000). Alternatively, the lack of correlation between Fe uptake and FCR activity may be due to the method used to measure Fe uptake, which might not detect small differences in Fe uptake.

Shoot Fe concentration was not different between species, even though *V. arboreum* exhibited consistently higher leaf FCR activity compared with *V. corymbosum*. Iron concentrations in shoots of both species were similar to those reported for other
Vaccinium (Korcak et al., 1982; Rosen et al., 1990). The lack of pH effect on shoot Fe concentration is in agreement with Brown and Draper (1980) and Rosen et al. (1990) who found no difference in shoot Fe concentration of various blueberry progenies grown in nutrient solutions at pHs ranging from 3.5 to 6.5. However, analysis of total leaf Fe concentration does not reflect available Fe (Bavaresco et al., 1999; Morales et al., 1998). Sufficient leaf FCR activity must occur for the transported Fe $^{3+}$-citrate to be reduced to the available Fe $^{2+}$ form and used by leaf mesophyll cells. Thus, the increased leaf FCR in V. arboreum, compared with V. corymbosum, suggests an increased ability to utilize leaf localized Fe.

In the present study, solution pH did not affect leaf chlorosis in either species; however, leaf area of plants growing at pH 6.5 was reduced compared with leaf area of plants grown at pH 5.5. This finding is in agreement with Haynes and Swift (1986) who reported the depression of highbush blueberry leaf growth at pH 6.5 compared with pH 4.5, with no visual symptoms of leaf chlorosis. Reduction of leaf growth is one of the Fe deficiency symptoms (Kosegarten et al., 1998b). Hence, this may imply that both blueberry species under high pH condition suffered from Fe deficiency, even though no visual chlorosis was detected.

The results of the present study confirmed our hypothesis that V. arboreum possesses higher leaf FCR activity and NR activity than V. corymbosum, suggesting the capacity for increased assimilation of Fe and NO$_3^-$ . The potential for increased assimilation of Fe and NO$_3^-$ in V. arboreum was reflected in increased shoot, root and whole plant DW compared with V. corymbosum. However, growth of both species, at least as reflected in leaf area, was limited at high compare with low pH. Thus, the ability
of *V. arboreum* to tolerate high pH soils better than *V. corymbosum* may be due to an inherently higher growth rate, regardless of pH.
Fig. 4-1. Effect of species (A) and nutrient solution pH (B) on nitrate uptake rate of *Vaccinium*. Values are adjusted means ± SE (n=8), using initial whole plant FW as a covariate.
Fig. 4-2. Effect of species (A) and nutrient solution pH (B) on root nitrate reductase (NR) activity of *Vaccinium*. Means ± SE (n = 8).
Fig. 4-3. Effect of species (A) and nutrient solution pH (B) on Fe uptake rate. Values are adjusted means ± SE (n=8), using initial whole plant FW as a covariate.
Fig. 4-4. Effect of species (A) and nutrient solution pH (B) on root FCR activity of Vaccinium. Means ± SE (n = 8).
Fig. 4-5. Effect of *Vaccinium* species on leaf FCR activity. Means ± SE (n = 8).
Table 4-1. Effect of pH on leaf chlorophyll concentration (SPAD reading index) in *V. arboreum* and *V. corymbosum*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Days after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><em>V. arboreum</em></td>
<td></td>
</tr>
<tr>
<td>pH 5.5</td>
<td>46.7ab$^Z$</td>
</tr>
<tr>
<td>pH 6.5</td>
<td>49.5 a</td>
</tr>
<tr>
<td><em>V. corymbosum</em></td>
<td></td>
</tr>
<tr>
<td>pH 5.5</td>
<td>55.4 a</td>
</tr>
<tr>
<td>pH 6.5</td>
<td>56.0 a</td>
</tr>
</tbody>
</table>

$^Z$ = Mean separation across days after treatment and pH within species by t-test, P = 0.05, n = 8.
Table 4-2. Main effects of species and pH on final fresh weight (FW) and dry weight (DW) of shoots (stems + leaves), roots and whole plants. Values are adjusted means using initial whole plant FW as a covariate (n = 8)

<table>
<thead>
<tr>
<th>Species</th>
<th>Initial FW (g)</th>
<th>FW (g)</th>
<th></th>
<th>DW (g)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shoot</td>
<td>Root</td>
<td>Plant</td>
<td>Shoot</td>
</tr>
<tr>
<td>V. arboreum</td>
<td>49.6</td>
<td>36.1</td>
<td>31.0</td>
<td>67.1</td>
<td>16.5</td>
</tr>
<tr>
<td>V. corymbosum</td>
<td>40.5</td>
<td>21.3</td>
<td>31.1</td>
<td>52.4</td>
<td>9.7</td>
</tr>
<tr>
<td>NS</td>
<td>*Z</td>
<td>NS</td>
<td></td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH</th>
<th>Initial FW (g)</th>
<th>FW (g)</th>
<th></th>
<th>DW (g)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shoot</td>
<td>Root</td>
<td>Plant</td>
<td>Shoot</td>
</tr>
<tr>
<td>5.5</td>
<td>48.8</td>
<td>31.5</td>
<td>32.4</td>
<td>63.9</td>
<td>14.4</td>
</tr>
<tr>
<td>6.5</td>
<td>41.3</td>
<td>25.8</td>
<td>29.7</td>
<td>55.5</td>
<td>11.8</td>
</tr>
<tr>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Z NS, ** = Nonsignificant or significant at the 1% level by t-test.
Table 4-3  Main effects of species and pH on shoot (stem + leaf) and root total Kjedahl nitrogen (TKN), nitrate concentration and iron concentration.

<table>
<thead>
<tr>
<th>Species</th>
<th>TKN (mg/g DW)</th>
<th>NO$_3$-N (µg/g DW)</th>
<th>Fe (µg/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot</td>
<td>Root</td>
<td>Shoot</td>
</tr>
<tr>
<td><em>V. arboreum</em></td>
<td>5.5</td>
<td>7.5</td>
<td>47.2</td>
</tr>
<tr>
<td><em>V. corymbosum</em></td>
<td>8.0</td>
<td>12.1</td>
<td>44.3</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>pH</td>
<td>TKN (mg/g DW)</td>
<td>NO$_3$-N (µg/g DW)</td>
<td>Fe (µg/g DW)</td>
</tr>
<tr>
<td></td>
<td>Shoot</td>
<td>Root</td>
<td>Shoot</td>
</tr>
<tr>
<td>5.5</td>
<td>7.2</td>
<td>10.5</td>
<td>44.9</td>
</tr>
<tr>
<td>6.5</td>
<td>6.3</td>
<td>9.1</td>
<td>46.5</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

$^z$ NS, * = Nonsignificant or significant at the 5% level by t-test, n = 8.
CHAPTER 5
EFFECT OF IRON DEFICIENT AND IRON SUFFICIENT CONDITIONS ON FERRIC CHELATE REDUCTASE AND NITRATE REDUCTASE

Introduction

Blueberries (Vaccinium spp.) require low pH soils, which are characterized by high iron (Fe) availability and nitrogen (N) primarily in the ammonium (NH$_4^+$) form, for optimum growth (Korcak, 1989). There is an effort to expand blueberry production to sub-optimal blueberry soils (Korcak, 1989), which are characterized by pH higher than 5.5, low Fe availability and N primarily in the nitrate (NO$_3^-$) form (Williamson and Lyrene, 1997). Thus, one of the problems in growing blueberries on these sites is the development of Fe and/or N deficiency symptoms and suppression of growth (Korcak, 1989).

Plants can be classified as either Fe-efficient or Fe-inefficient, depending on their response to Fe deficiency (Grusak et al., 1990; Marschner, 1991; Römheld et al., 1982). Fe-efficient plants respond to Fe deficiency by induction of biochemical reactions that make Fe more available (Marschner, 1991). These reactions include the release of hydrogen ions (H$^+$) from roots, which decreases rhizosphere pH and increases Fe solubility (Römheld and Marschner, 1986), or release of reducing compounds to enhance reduction of ferric (Fe$^{3+}$) to ferrous (Fe$^{2+}$) (Jolley et al., 1996; Marschner, 1995; Manthey et al., 1994). In contrast, Fe-inefficient plants do not show these responses (Brown and Draper, 1980). Additionally, Fe-efficient plants normally have the ability to increase the Fe reduction capacity at the root plasma membrane, resulting in increased of Fe uptake.
(Marschner, 1995; Marschner and Römheld, 1995; Schmidt, 1999). The increase in reduction capacity is due to the induction of ferric chelate reductase (FCR) under Fe deficient conditions (Marschner, 1995; Marschner and Römheld, 1994; Schmidt, 1999). Root FCR reduces Fe$^{3+}$-chelate to Fe$^{2+}$ before Fe$^{2+}$ is taken up into the cytoplasm (Schmidt, 1999). The ability of Fe-efficient plants to increase the root FCR activity under Fe deficient conditions has been used as a tool to select for tolerance to Fe-deficiency in citrus (Manthey et al., 1994), kiwifruit (Vizzotto et al., 1999), pear and quince (Tagliavini et al., 1995a). Brown and Draper (1980) found that several blueberry genotypes tolerated Fe deficiency by releasing H$^+$ from their roots, lowering the pH and increasing Fe availability. However, other genotypes did not show this response and developed Fe deficiency symptoms. There are no reports on the effects of Fe deficiency on FCR activity in blueberry.

Symptoms of Fe deficiency are usually manifested as interveinal chlorosis of young leaves, while the veins remain green (Abadia, 1992; Korcak, 1987). This is due to the inability to reduce and transport Fe from the leaf apoplast into the symplast, and suggests that leaf FCR may also play a crucial role in Fe deficiency symptoms (Kosegarten et al., 1999, 2001; Mengel, 1994).

The ability of blueberries to utilize NO$_3^-$ and/or NH$_4^+$ for normal growth has been studied extensively. In general, plant fresh weight (FW) and dry weight (DW) is greater in blueberries grown with NH$_4^+$ or a combination of NH$_4^+$ and NO$_3^-$ compared with NO$_3^-$ uptake rates (Townsend, 1967; Takamizo and Sugiyama, 1991). These observations correlate with reports that NH$_4^+$ uptake rates in blueberry are significantly greater than NO$_3^-$ uptake rates (Merhaut and Darnell, 1995). The limitation in NO$_3^-$ uptake may be related to
insufficient activity of nitrate reductase (NR) in the root or shoot of blueberries (Merhaut and Darnell, 1995).

Nitrate reductase, the enzyme responsible for NO$_3^-$ assimilation, is found either as a plasma membrane-bound (Berczi and Moller, 2000; Stohr, 1999; Ullrich et al., 1990) or as a cytosol localized enzyme in root epidermal and cortical cells and shoot mesophyll cells (Berczi and Moller, 2000; Crawford, 1995; Stohr, 1999). This enzyme transfers electrons either from NADH or NADPH to NO$_3^-$, reducing it to nitrite (NO$_2^-$) (Campbell, 1999; Crawford, 1995; Srivastava, 1992; Wray and Abbington, 1994). In the process, NO$_3^-$ acts as a signal to increase the expression of NR (Crawford, 1995; Lee and Titus, 1992). Activity of NR may be depressed in both roots and leaves when Fe supply is low, even in the presence of NO$_3^-$ (Pandey, 1989), and is likely due to the influence of non-heme Fe proteins on electron transport (Pandey, 1989). This suggests that the inductive effect of NO$_3^-$ on NR activity requires a certain threshold supply of Fe (Pandey, 1989).

The wild blueberry species, *V. arboreum* Marsh, is able to grow on soils with average pH of 6.0, low Fe availability and N in the NO$_3^-$ form (Lyrene, 1997). The ability of *V. arboreum* to grow on high pH soils, in which cultivated *Vaccinium* species tolerate poorly, suggests a better utilization of Fe and/or NO$_3^-$ compared with the cultivated species. We hypothesized that *V. arboreum* possesses higher FCR and/or NR activity, and increased Fe and/or NO$_3^-$ uptake than *V. corymbosum* under limiting Fe concentration. The objectives of the present study were to determine the effect of Fe concentration on root and leaf FCR activity, root NR activity, and Fe and NO$_3^-$ uptake of two blueberry species, the southern highbush blueberry ‘Misty’ (*V. corymbosum* L.
interspecific hybrid) and the wild species, *V. arboreum*, grown in nutrient solutions with NO$_3^-$ as the N source.

**Materials and Methods**

**Plant Culture**

Seeds of the wild species, *V. arboreum*, were collected from a natural habitat at Manatee Springs State Park near Chiefland, Florida, during Fall 1999. Seeds were germinated on the surface of Canadian peat under intermittent mist (4 hours/day) in a greenhouse with average temperature of 25°C and average PPF of 545 µmol·m$^{-2}·$s$^{-1}$. In April 2000, shoot cuttings of the southern highbush blueberry ‘Misty’ were rooted in peat:perlite (1:1 by volume) medium under intermittent mist in a greenhouse with average temperature of 25°C and average PPF of 545 µmol·m$^{-2}·$s$^{-1}$. On March 2001, plants of both species were transplanted to 1-L pots containing pine bark and maintained in the greenhouse. On 17 September 2001, fifteen plants from each species were selected and initial FW were determined. Plants were blocked by size before transferring to 2-L plastic bottles filled with a complete nutrient solution. Plastic bottles were wrapped with 2 layers of aluminum foil to eliminate light infiltration. The nutrient solution contained the following composition (mM): 0.5 K$_2$HPO$_4$, 1.0 MgSO$_4$, 0.5 CaCl$_2$, 0.09 Fe-diyethylenetriaminopentaacetic acid (Fe-DTPA), 0.045 H$_3$BO$_3$, 0.01 MnSO$_4$, 0.01 ZnSO$_4$, and 0.2 µM Na$_2$MoO$_4$. The nitrogen source was 5.0 mM NaNO$_3$. The nutrient solutions were buffered at pH 5.5 with 10 mM MES. Plants were acclimated in the nutrient solutions for 3 weeks. After acclimation, plant roots were soaked in 1 mM NaEDTA for 5 min. and washed 3 times in deionized (DI) water to remove the apoplastic Fe (Rosenfield et al., 1991) before placing in nutrient solutions containing 0, 45 or 90 µM Fe-DTPA.
Nutrient solution pH was monitored daily with a portable pH meter (Accumet 1001, Fisher, USA) and maintained at 5.5, using 0.1 N KOH or HCl. Aeration was provided to each bottle by an aquarium pump (Elite 801, Rolf C. Hagen, Mansfiled, MA), connected by tygon tubing, located at the bottom of the bottle. The airflow was adjusted to 1L/min. Nutrient solutions were changed every 2 weeks. Deionized water was added to the solutions weekly to maintain a 2 L volume. Plants were grown for 19 weeks, from October 2001 through February 2002. On November 26, 2001, three 100 W incandescent light bulbs and 2 fluorescence light bulbs were installed over each bench to extend daylight to 14 hrs until the end of the experiment. The average greenhouse conditions were 22/18°C day/night, 90% relative humidity and average PPF of 360 µmol·m⁻²·s⁻¹. Root temperatures were determined at the beginning and the end of the experiment, using a temperature sensor attached to a portable pH meter (Accumet 1001, Fisher, USA). The average root temperature was 16°C.

Nitrate and Fe Uptake

Nitrate and Fe uptake were determined every 2 weeks by measuring depletion from the nutrient solutions just prior to solution replacement. For NO₃⁻ determination, 10 µl of each nutrient solution was taken from each sample bottle before solutions were changed, and diluted with 1.5 ml distilled water. Fifteen µl of 12.1 N HCl was added and the samples were vortexed before reading spectrophotometrically (Shimadzu UV-160, Japan) at 210 nm. The NO₃⁻ uptake rate per day was determined by calculating the amount of NO₃⁻ depletion from the solution and dividing by the number of days between nutrient solution changes.
The Fe concentration in the solutions was determined using atomic absorption spectrophotometry (Perkin Elmer 3030B, Norwalk, Connecticut, USA) with a hollow cathode lamp as a light source and air-acetylene flame. The depletion of Fe from the nutrient solution was calculated and divided by the number of days between the cycle of nutrient solution changed and represented the rate of Fe uptake.

**Root and leaf Fe (III) Reducing Capacity**

Root and leaf FCR activity were quantified every two weeks. For root FCR, root tips (1 cm long) were cut, placed in a beaker filled with ice water and transferred to the lab. Roots were weighed and about 100 mg tissue were placed in a test tube. Root tissue was rinsed in 0.2 mM CaSO$_4$ for 5 min before transferring to 2 mL assay solution, containing 10 mM CaSO$_4$, 5 mM MES (pH 5.5), 0.1 mM Fe-EDTA and 0.3 mM sodiumbathophenanthroinedisulfonic acid (Na$_2$-BPDS). One test tube with 2 mL assay solution without root tissue was used as a control. Samples and control tubes were incubated for 1 h in a shaking water bath at 50 rpm and 23°C in the dark. After incubation, a 1 mL aliquot from each tube was transferred into a cuvette and read spectrophotometrically at 535 nm (Shimadzu UV-160, Japan). The concentration of Fe (II)-BPDS produced was calculated using the molar extinction coefficient of 22.14 mM/cm (Chaney et al., 1972).

For the leaf FCR assay, 4 mm diameter leaf discs were taken from each plant in the greenhouse, placed in a beaker filled with ice water and transferred to the lab. Leaf discs were weighed and about 100 mg (FW) tissue were placed in test tubes containing 0.2 mM CaSO$_4$. After 10 min, the CaSO$_4$ was discarded and 2 mL of assay solution (described above) were added to the tubes. Samples were vacuum infiltrated for 10 min. before incubating in a shaking water bath at 50 rpm and 23°C for 1 hr in the dark. After
incubation, 1 mL aliquot from each tube was transferred into a cuvette and read spectrophotometrically at 535 nm. The concentration of Fe(II)-BPDS was calculated using the same method as described above.

**Root Nitrate Reductase**

Root NR activity was quantified every two weeks. Root tips (1 cm long) were cut, placed in a beaker filled with ice water, and transferred to the lab. Roots were weighed and about 100 mg FW were placed in each test tube (2 tubes/trt/rep). Two mL of assay solution, composed of 2% 1-propanol, 100 mM KH$_2$PO$_4$ (pH 7.5) and 30 mM KNO$_3$ were added to each test tube. One tube/trt/rep was immediately filtered through Whatman No. 2 paper and used as the time 0 control. Samples were vacuum infiltrated for 5 min. and incubated in a shaking water bath at 31º C and 50 rpm for 1 h in the dark. After incubation, the assay solution with roots was filtered through Whatman No. 2 paper and a 1 mL aliquot from each sample was removed to a new tube. One-mL sulfanilamide (1% w/v in 1.5N HCl) and 1 mL N-(1-naphthyl)-ethylenediaminedihydrochloride (0.02% w/v in 0.2N HCl) were added. The samples were incubated at room temperature for 30 min and absorbance at 540 nm was determined spectrophotometrically (modified from Jaworski, 1971).

**Tissue N and Fe Determination**

Plants were harvested on 21 February 2002, separated into stem, leaf, and root, and FW of each part was determined. Dry weight (DW) was determined after oven drying at 70ºC to constant weight. Dried samples were ground through a 20-mesh screen in a Wiley Mill (Arthur Thomas, Pennsylvania, USA) and were analyzed for total Kjedahl nitrogen (TKN) and Fe. For TKN, 100 mg tissue was placed into 50 mL digesting tube. Popes Kjeldahl mixture (2.0 g) was added to each tube, followed by 2.5 mL sulfuric acid.
Glass funnels were inserted in each tube before placing the tubes on a digestion block at 380°C for 8-10 hr. After cooling, the tubes were rinsed with 10-15 mL of DI water, vortexed, and brought to 50 mL with DI water. Samples were then filtered (Whatman No. 8 filter paper) and analyzed using the Kjeldahl method (Horneck and Miller, 1998).

For Fe concentration, a dry ash digestion of the tissue was performed. Dried tissue (500 mg) was placed in a muffle furnace at 500°C for 10-12 hours. After cooling, 50 mL of 1N HCl was added to each sample and the solutions were filtered (Whatman No. 8 filter paper). Iron concentration was determined using atomic absorption spectrophotometry as described earlier.

For NO\textsubscript{3}\textsuperscript{-} concentration, dried tissue (500 mg), ground through 40-mesh screen, was placed in a 50 mL Erlenmeyer flask, 50 mL of DI water was added, and samples were incubated on a lateral shaker at 100 rpm for 30 min, then filtered with Whatman No. 8 filter paper. The extract was analyzed for NO\textsubscript{3}\textsuperscript{-} concentration by Automated Cadmium Reduction Method using Rapid Flow Analyzer ALPKEM 300 (Alpkem Corporation, Ore., USA) at 540 nm (Anderson and Case, 1999).

**Experimental Design**

Treatments were arranged as a 2 x 3 factorial in a randomized complete block design with 5 replications, using a single plant per replication. The factors were plant species (*V. corymbosum* or *V. arboreum*) and iron concentration (0, 45 and 90 µM). Data were analyzed using SAS (SAS Institute, Cary, N.C., USA). Mean separation was performed using t-test on every pair of Least Square Means (LSM). Daily NO\textsubscript{3}\textsuperscript{-} and Fe uptake rates/plant and final plant FW and DW were analyzed using initial plant FW as a covariate to normalize for differences in initial plant size.
Results

Root Nitrate Reductase

Root NR activity in *V. corymbosum* was significantly higher than in *V. arboreum* from week 3 to 7 after treatments began (60 vs 17 nmol NO$_2^-$/gFW/h, respectively) (Fig. 5-1A). By week 9, NR activity in *V. arboreum* increased to the same level as *V. corymbosum*, but decreased again by week 13. The average NR activity for *V. corymbosum* during the experiment was 66 nmol NO$_2^-$/gFW/h, while the activity in *V. arboreum* ranged from 15 to 57 nmol NO$_2^-$/gFW/h.

Iron concentration had no consistent significant effect on NR activity (Fig. 5-1B), and the activities ranged from 20 to 87 nmol NO$_2^-$/gFW/h. There was no significant interaction between species and Fe concentration on root NR activity.

Nitrate Uptake

In general, NO$_3^-$ uptake rates were not significantly different between the two species (Fig. 5-2A). Average uptake rates ranged from 0.03 to 0.21 mmol NO$_3^-$/day.

During the first 5 weeks after treatment, there was no difference in NO$_3^-$ uptake at various Fe concentrations (Fig. 5-2B). By week 7, NO$_3^-$ uptake rates at 0 and 45 µM Fe were significantly higher than at 90 µM Fe. However, uptake rates increased at both 0 and 90 µM Fe by week 11 and were significantly higher than at 45 µM Fe. The NO$_3^-$ uptake rates at all Fe concentrations then decreased to less than 0.1 mmol NO$_3^-$/day for the reminder of the experiment. In general, there was no interaction between species and Fe concentration on the NO$_3^-$ uptake.

Root Fe (III) Reducing Capacity

There was no significant difference in root FCR activity between species from week 2 to week 12 after treatment began (Fig. 5-3A), with average activities ranging
from ~10 to 30 nmol Fe$^{2+}$/gFW/h. Root FCR activity in *V. arboreum* increased between week 12 and week 19, and was significantly greater than activity in *V. corymbosum* from week 15 to the end of the experiment.

Iron concentration did not affect root FCR activity during the first 4 weeks of the experiment (Fig. 5-3B), with activity averaging 11 nmol Fe$^{2+}$/gFW/h. From week 6 until the end of the experiment, plants treated with 45 and 90 µM Fe had significantly higher root FCR activities compared with plants receiving no Fe. Root FCR activity at 0 µM Fe was relatively constant throughout the experiment, averaging 8 nmol Fe$^{2+}$/gFW/h, while root FCR activities at 45 and 90 µM Fe increased from ~15 to 60 nmol Fe$^{2+}$/gFW/h. There was no interaction between species and Fe concentration on root FCR activity.

**Leaf Fe (III) Reducing Capacity**

During the experimental period, most of the plants did not produce new leaves. Therefore, leaf FCR was determined only 3 times; before the treatments started, and at weeks 2 and 4 after treatments began. Leaf FCR activity in *V. arboreum* was significantly greater than in *V. corymbosum* (Fig. 5-4). Activities in *V. arboreum* leaves ranged from 17 to 31 nmol Fe$^{2+}$/gFW/h, while activities in *V. corymbosum* ranged from 7 to 19 nmol Fe$^{2+}$/gFW/h. There was no effect of Fe concentration and no interaction between species and Fe concentration on leaf FCR activity.

**Iron Uptake**

The Fe uptake rate was significantly greater in *V. corymbosum* compared with *V. arboreum* at 7, 9 and 13 weeks after treatments began (Fig. 5-5A). Iron concentration significantly affected Fe uptake rate throughout the experimental period (Fig. 5-5B). No Fe uptake was detected at 0 µM Fe concentration. During the first 3 weeks after treatments began, the Fe uptake rate was similar at external Fe concentrations of 45 and
90 µM. By week 5, Fe uptake rate increased as Fe concentration in the nutrient solutions increased, except for week 13 when Fe uptake was higher at 45 µM Fe compared with 90 µM Fe. The uptake rate ranged from 0 to 2.1 µmol/plant/day and 0 to 4.0 µmol/plant/day for external Fe concentration of 45 and 90 µM, respectively.

**Plant Growth**

Plant FW in both species decreased from the beginning of the experiment (October) to the end (February) (Table 5-1). In general, new shoots were not produced by *V. arboreum* plants during this period. In addition, leaf abscission and root tissue death was observed. *V. corymbosum* plants, on the other hand, produced flowers, which were removed regularly, and some new leaves during the experiment. Thus, the growth reduction was more pronounced in *V. arboreum* compared with *V. corymbosum*, as indicated by the relative reduction in whole plant FW during the experiment (50 vs 10% for *V. arboreum* and *V. corymbosum*, respectively) (Table 5-1). This resulted in significantly greater final plant FW in *V. corymbosum* compared with *V. arboreum*. However, this difference was not reflected in plant DW, which was similar between species. *V. corymbosum* had greater stem DW but lower root DW compared with *V. arboreum* at the end of the experiment. No significant effect of Fe concentration was found on either plant FW or DW.

**Plant Tissue Analysis**

*V. corymbosum* exhibited greater stem and root TKN and root NO$_3^-$ concentrations compared with *V. arboreum* (Table 5-2). Due to limited leaf samples, leaf TKN and leaf NO$_3^-$ concentrations were not determined. There was no significant effect of species on stem or leaf Fe concentration.
There was no effect of Fe concentration on root or stem TKN, root NO$_3^-$, or stem and leaf Fe concentrations. There was an interaction between species and Fe concentration on stem NO$_3^-$-N and root Fe concentration (Table 5-3). Stem NO$_3^-$ concentration in *V. arboreum* at 0 µM Fe was greater than at 45 or 90 µM Fe, while stem NO$_3^-$ concentration in *V. corymbosum* was similar at all external Fe concentrations. In general, root Fe concentration increased as external Fe concentration increased; however, the difference in root Fe concentration between 45 and 90 µM Fe was not significant. Root Fe concentration in *V. corymbosum* was greater than *V. arboreum* at 45 and 90 µM Fe.

**Discussion**

Root NR activities of *V. arboreum* and *V. corymbosum* were about 5- and 2-fold lower, respectively, than in the previous experiments (Chapter 3 and 4). This may have been due to the short days and low light conditions that occurred during the present study. Previous experiments were conducted between March and September when daylengths were more than 12 h and light intensities averaged 540 µmol·m$^{-2}$·s$^{-1}$. The duration and intensity of light influence NR activity (Smirnoff and Stewart, 1985; Srivastava, 1980). Greater NR activity is found in plants grown at higher irradiance compared with lower irradiance (Srivastava, 1980 and references therein). Recently, Black et al. (2002) reported a decrease in NR activity in poplar (*Populus tremula* L. x *P. alba* L.) under an 8 h photoperiod and PPF of 400 µmol·m$^{-2}$·s$^{-1}$ compared with an 8 h photoperiod with a 30 minute night interruption during the middle of the dark cycle. This suggests that NR activity is phytochrome-mediated with increased activity under long days/short nights. The low NR activities in the present study may also be the consequence of the growth reduction of both blueberry species resulting from the short day and low light conditions.
The relatively low root temperature in the present study (~ 16ºC) compared with average root temperatures of ~ 28ºC recorded in the previous experiment (Chapter 4) may also have contributed to the restriction of growth in both blueberry species. The reduction of growth under low root temperature (10-15ºC vs 20-25ºC) has been reported in sesame (*Sesamum indicum* L.) (Ali et al., 2000) and *Eucalyptus* (Garnett and Smethurst, 1999). Thus, it is likely that *Vaccinium* species exhibit lower NR activity in winter compared with other times of the year, due to low light intensity, decreased photoperiod, and decreased root temperature.

In the present study, root NR activity was greater in *V. corymbosum* compared with *V. arboreum*. This result is in contrast with the previous experiments (Chapter 3 and 4), in which *V. arboreum* exhibited greater root NR activity compared with *V. corymbosum*. The decrease in NR activity in *V. arboreum* compared with *V. corymbosum* coincided with the much greater growth reduction in *V. arboreum*. The lower NR activity in *V. arboreum* compared with *V. corymbosum* was reflected in the lower stem and root TKN concentrations in *V. arboreum*.

The greater root NR activity in *V. corymbosum* was not reflected in a higher NO$_3^-$ uptake rate compared with *V. arboreum*. This result contradicts work with many species, which indicates that the rate of NO$_3^-$ reduction is primarily controlled by the rate of NO$_3^-$ uptake in order to prevent the excess accumulation of either NO$_2^-$ or ammonia in plant (Imsande and Touraine, 1994 and references therein). However, cells of the NR-deficient (*nia*) mutant in *Nicotiana* can take up NO$_3^-$ at the same rate as wild-type cells (Muller and Mendel, 1989), indicating that NR activity may not always regulate NO$_3^-$ uptake (Larsson and Ingemarsson, 1989). The lack of correlation between NR activity and NO$_3^-$ uptake in
the present study may be due to the method used to measure uptake, which may not be sensitive enough to detect differences in uptake rate, especially at high external NO$_3^-$ concentration (Glass et al., 2002). Although differences in uptake rates were not observed between species, *V. corymbosum* exhibited higher stem and root TKN compared with *V. arboreum*, suggesting the increased NR activity did result in increased NO$_3^-$ uptake.

In general, external Fe concentration had no significant effect on NR activity. This was in contrast to work by Pandey (1989) who reported the depression of root NR activity in sorghum if external Fe concentration was below 3.6 µM. It is possible that there were sufficient Fe reserves in *Vaccinium* tissues, compared with an annual such as sorghum, to maintain normal NR activity even under conditions of 0 µM Fe supply in the nutrient solution. The lack of Fe effect on NR activity was reflected in similar whole plant FW and DW, tissue TKN and tissue NO$_3^-$ concentrations under different external Fe concentrations.

*V. arboreum* showed consistently higher leaf FCR activity than *V. corymbosum*. This is in agreement with results from the previous experiment (Chapter 2) and confirms that *V. arboreum* has the ability to better utilize Fe once it is transported to the leaf compared with *V. corymbosum*.

Root FCR activity in the present study was 4-fold lower than the activity measured in the previous experiments (Chapter 3 and 4). Low root temperature (10-15°C vs 20-25°C) normally decreases nutrient uptake rates and restricts plant growth (Ali et al., 2000; Garnett and Smethurst, 1999). The relatively low root temperature in the present study may have played a key role in limiting root growth, resulting in decreased root FCR activity and Fe uptake (Welkie, 1995). The greater root FCR activity in *V. arboreum* at
the end of the experiment was not reflected in greater Fe uptake rates compared with *V. corymbosum*. This may be due to the method used to measure uptake, which may not be able to detect small differences in Fe uptake rates between species. However, Bavaresco et al. (1991) were unable to detect differences in Fe uptake rates in grapevine genotypes that differed in root FCR activity, even with the use of $^{59}$Fe, which is a much more sensitive method of determining Fe uptake. The lack of difference in stem and leaf Fe concentrations between species in the present study supports the lack of difference in Fe uptake rates. On the other hand, higher root Fe concentration in *V. corymbosum* compared with *V. arboreum* suggests that Fe uptake may have been greater in *V. corymbosum*. However, the increased Fe concentration may be due to more Fe precipitation on *V. corymbosum* roots, which are more fibrous than roots of *V. arboreum*, and therefore have greater surface area for adsorption.

Root FCR activity was greater under Fe sufficient (45 or 90 µM) compared with Fe deficient (0 µM) conditions. This finding is in contrast to reports in other fruit crops such as apple (Ao et al., 1985), citrus (Manthey et al., 1994), avocado (Manthey and Crowley, 1997), kiwifruit (Vizzotto, et al., 1999) and papaya (Marler et al., 2002), in which plants responded to Fe-deficient conditions by inducing higher root FCR activity compared with Fe-sufficient conditions. In contrast, Fe-deficient conditions led to decreased root FCR activity in peach (Romera et al., 1991; Gogorcena et al., 2000), pear and quince (Tagliavini, et al., 1995b) compared to Fe sufficient conditions. The differences in response under Fe-deficient conditions may be due to the genotypic differences in the ability to induce FCR (Tagliavini, et al., 1995b). Alternatively, the use of Fe-free nutrient solutions in the present study may have prevented activation of root FCR. Some work
suggests that increased ethylene production is a prerequisite for stimulating root FCR activity (Romera and Alcantara, 1994; Romera et al., 1996), and Fe is required to activate 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase required for ethylene biosynthesis (Romera et al., 1996). An alternative explanation for activation of FCR by a low concentration of Fe comes from Grusak et al. (1990), who speculated that plants grown with a small amount of Fe were healthier than plants grown without Fe, and might be able to generate more cytoplasmic NAD(P)H to serve as an electron donor for the Fe reduction process. The minimum amount of external Fe concentration required to activate root FCR depends on plant species, and ranges from 0 in sugarbeet (Susin et al., 1996), to 0.32–2 µM in bean (Chaney et al., 1972), to 20 µM in pea (Romera et al., 1996).

Greater root FCR activity under Fe-sufficient (45 and 90 µM) conditions was reflected in increased root Fe concentrations compared with Fe deficient (0 µM) conditions. However, the stem and leaf Fe concentrations were not higher at higher external Fe concentration. The stem Fe concentrations in the present study were in the same range as reported in other Vaccinium species (Rosen et al., 1990). Leaf Fe concentrations (226-251 µg/g DW); however, were greater than the maximum range recommended for highbush blueberry (200 µg/g DW) (Williamson and Lyrene, 1995). It is possible that little root Fe was translocated to the shoot, as plant growth was restricted in the present study, and therefore the demand for shoot Fe would be minimal.

The results from the present study indicate that V. arboreum has the ability to better utilize Fe compared with V. corymbosum, as illustrated by higher leaf FCR activity. The lack of species difference in root FCR activity and Fe uptake rates suggests that Fe uptake and translocation to the shoot may not be the cause of Fe chlorosis in Vaccinium.
Instead, utilization of Fe in the leaf may play the key role in development of leaf Fe chlorosis. Under the conditions of the present experiment, *V. corymbosum* has the potential for increased assimilation of NO$_3^-$ compared with *V. arboreum*, as suggested by higher root NR activity, although this was not reflected in NO$_3^-$ uptake. The greater growth reduction experienced by *V. arboreum* compared with *V. corymbosum* under the present experimental conditions may have decreased its ability to assimilate NO$_3^-$ efficiently. The responses to Fe deficiency may be different under the slow growth conditions elicited by low temperatures and low light, compared with the rapid growth that would occur under higher temperatures and light conditions.
Fig. 5-1. Effect of species (A) and iron concentration (B) on root nitrate reductase (NR) activity. Means ± SE (n=5). * New root growth in V. arboreum was insufficient for NR activity assays after 13 weeks.
Fig. 5-2. Effect of species (A) and iron concentration (B) on nitrate uptake rate. Values are adjusted means ± SE (n=5), using initial whole plant FW as a covariate.
Fig. 5-3. Effect of species (A) and iron concentration (B) on root ferric chelate reductase (FCR) activity. Means ± SE (n=5).
Fig. 5-4. Effect of species on leaf ferric chelate reductase (FCR) activity. Means ± SE (n=5).
Fig. 5. Effect of species (A) and iron concentration (B) on iron uptake rate. Values are adjusted means ± SE (n=5), using initial whole plant FW as a covariate.
Table 5-1. Effect of species and iron concentration on final fresh weight (FW) and final dry weight (DW) of stems, leaves, roots and whole plants. Values are adjusted means using initial whole plant FW as a covariate (n = 5).

<table>
<thead>
<tr>
<th>Species</th>
<th>Initial FW (g)</th>
<th>FW (g)</th>
<th>DW (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stem</td>
<td>Leaf</td>
</tr>
<tr>
<td><em>V. arboreum</em></td>
<td>62.81</td>
<td>8.4</td>
<td>1.2</td>
</tr>
<tr>
<td><em>V. corymbosum</em></td>
<td>43.69</td>
<td>13.2</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td><em>Z</em></td>
<td>*</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>iron conc.</th>
<th>Initial FW (g)</th>
<th>FW (g)</th>
<th>DW (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stem</td>
<td>Leaf</td>
</tr>
<tr>
<td>0 µM</td>
<td>54.12</td>
<td>10.4</td>
<td>1.8</td>
</tr>
<tr>
<td>45 µM</td>
<td>53.18</td>
<td>10.5</td>
<td>1.4</td>
</tr>
<tr>
<td>90 µM</td>
<td>52.44</td>
<td>11.5</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Z* NS, * = nonsignificant or significant at the 5% level by t-test.
Table 5-2. Effect of species and iron concentration on stem and root total Kjedahl nitrogen (TKN), root nitrate concentration and stem and leaf iron concentrations (n = 5).

<table>
<thead>
<tr>
<th>Species</th>
<th>TKN (mg g(^{-1}) DW)</th>
<th>NO(_3)(^-) (µg g(^{-1}) DW)</th>
<th>Fe (µg g(^{-1}) DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stem</td>
<td>Root</td>
<td>Root</td>
</tr>
<tr>
<td>V. arboreum</td>
<td>12.3</td>
<td>12.5</td>
<td>149.3</td>
</tr>
<tr>
<td>V. corymbosum</td>
<td>19.5</td>
<td>20.1</td>
<td>236.2</td>
</tr>
</tbody>
</table>

Iron concentration(µM) | TKN (mg g\(^{-1}\) DW) | NO\(_3\)\(^-\) (µg g\(^{-1}\) DW) | Fe (µg g\(^{-1}\) DW) |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stem</td>
<td>Root</td>
<td>Root</td>
</tr>
<tr>
<td>0</td>
<td>14.8</td>
<td>16.9</td>
<td>249.0</td>
</tr>
<tr>
<td>45</td>
<td>17.8</td>
<td>16.0</td>
<td>183.0</td>
</tr>
<tr>
<td>90</td>
<td>15.1</td>
<td>16.0</td>
<td>146.2</td>
</tr>
<tr>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(Z\), NS, * = nonsignificant or significant at the 5% level by t-test.
Table 5-3. Effect of species and iron concentration on stem nitrate concentration and root iron concentration (n = 5).

<table>
<thead>
<tr>
<th>Species x iron concentration</th>
<th>Stem NO$_3^-$ (µg g$^{-1}$ DW)</th>
<th>Root Fe (µg g$^{-1}$ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V.$ arboreum x 0 µM</td>
<td>167.0 a</td>
<td>123.0 c</td>
</tr>
<tr>
<td>$V.$ arboreum x 45 µM</td>
<td>66.6 b</td>
<td>199.2 bc</td>
</tr>
<tr>
<td>$V.$ arboreum x 90 µM</td>
<td>57.5 b</td>
<td>251.6 b</td>
</tr>
<tr>
<td>$V.$ corymbosum x 0 µM</td>
<td>54.6 b</td>
<td>144.2 c</td>
</tr>
<tr>
<td>$V.$ corymbosum x 45 µM</td>
<td>61.0 b</td>
<td>470.2 a</td>
</tr>
<tr>
<td>$V.$ corymbosum x 90 µM</td>
<td>100.8 ab</td>
<td>468.6 a</td>
</tr>
</tbody>
</table>

*Z* = Mean follows by the same letter are not significant at the 5% level by t-test.
CHAPTER 6
EFFECT OF IRON CONCENTRATION ON FERRIC CHELATE REDUCTASE, NITRATE REDUCTASE AND NUTRIENT UPTAKE IN BLUEBERRIES

Introduction

Cultivated blueberries (*Vaccinium* spp.) have strict soil requirements for optimal growth. In general, blueberry production is limited to acidic, high organic matter soils, where ammonium (NH$_4^+$) is the predominant nitrogen (N) form (Korckak, 1989; Williamson and Lyrene, 1995). There is an effort to expand blueberry production to sub-optimal blueberry soils, which are characterized by low organic matter, high pH (higher than 5.5), low iron (Fe) availability and N primarily in the nitrate (NO$_3^-$) form (Korckak, 1988). Leaf chlorosis usually develops when blueberries are grown in mineral soils (Brown and Draper, 1980) and growth is suppressed, possibly due to decreased Fe and/or N uptake or utilization (Korckak, 1987).

The ability of a plant to take up Fe from the rhizosphere depends on the activity of ferric chelate reductase (FCR), the key plasma membrane-bound enzyme in Fe assimilation in dicots and non-graminaceous monocots (Marschner, 1995; Mengel and Kirkby, 2001). Ferric chelate reductase reduces and cleaves the ferric (Fe$^{3+}$)-chelate to ferrous (Fe$^{2+}$) (Holden et al., 1991; Mengel, 1994, Mengel and Kirkby, 2001). Ferrous then passes through a specific cation channel in the plasma membrane of the root cells (Mengel, 1994; Mengel and Kirkby, 2001). In the symplast, Fe$^{2+}$ is oxidized, complexed with citrate, and translocated to the upper plant parts via the xylem (Mengel, 1994). Once
in the shoot, Fe$^{3+}$ must again be reduced to Fe$^{2+}$ via a leaf FCR and taken up into the mesophyll cytoplasm (Schmidt, 1999).

The ability to induce root FCR activity under Fe deficient conditions, thereby increasing Fe uptake, has been used as a tool to screen many fruit crops for tolerance to Fe deficiency (Manthey and Crowley, 1997; de la Guardia et al., 1995). Plants that are able to induce root FCR in response to Fe deficient conditions are classified as Fe efficient genotypes. In contrast, plants that are not able to induce root FCR under Fe deficient conditions are Fe inefficient genotypes (Brown and Jones, 1976). However, the lack of correlation between leaf Fe concentration and leaf Fe deficiency symptoms led Mengel (1994) to speculate that the leaf FCR activity could play a crucial role in Fe deficiency as well. The high Fe concentration in chlorotic leaves indicates that Fe transport from the leaf apoplast across the plasma membrane into the cytosol is restricted (Mengel, 1994), especially under calcareous or NO$_3^-$ fertilization conditions (Kosegarten et al., 1999, 2001). The increased leaf apoplast pH under such conditions (Mengel, 1994; Kosegarten et al., 1999, 2001) decreases leaf FCR activity, resulting in Fe accumulation in the apoplast (Tagliavini et al., 2000). Although leaf chlorosis is a common problem in blueberry grown on high pH soils, there has been no work on the effects of Fe deficiency and/or NO$_3^-$-N on blueberry root or leaf FCR activity.

Nitrate reductase (NR) is the regulatory enzyme responsible for NO$_3^-$ uptake and assimilation (Crawford, 1995). Nitrate reductase is found either as a plasma membrane-bound (Berczi and Moller, 2000; Stohr, 1999; Ullrich et al., 1990) or as a cytosol-localized enzyme in root epidermal and cortical cells and shoot mesophyll cells (Berczi and Moller, 2000; Crawford, 1995; Stohr, 1999). This enzyme transfers electrons either
from NADH or NADPH to NO$_3^-$, reducing it to nitrite (NO$_2^-$) (Crawford, 1995; Srivastava, 1992; Wray and Abberton, 1994). In cultivated blueberry, low activities of NR have been detected in leaves (Dirr et al., 1972; Wang and Korcak, 1995) and roots (Merhaut, 1993; Townsend, 1970). The low activity of NR may limit the ability of blueberry to grow well on high pH soils (Korcak, 1989), where NO$_3^-$ is the dominant N form (Mengel and Kirkby, 2001). Determination of differences in root NR activities among blueberry species and/or cultivars may result in the selection of species and/or cultivars that possess high root NR activity and therefore better adaptation to high pH, mineral soils (Korcak, 1988, 1989).

*V. corymbosum* L. interspecific hybrid cv Misty is a commercial southern highbush blueberry cultivar and normally shows symptoms of leaf chlorosis when grown on high pH soils. *V. arboreum* Marsh, a wild blueberry genotype native to the southeastern U.S., has the ability to thrive on soils with high pH (up to 6.0) (Brooks and Lyrene, 1995) and NO$_3^-$ as the sole source of N. The ability of *V. arboreum* to grow better than *V. corymbosum* on high pH soils suggests that the mechanism and/or extent of Fe and/or NO$_3^-$ uptake of this species may differ from that of the cultivated species. The present study was the second experiment in the series to study the effect of Fe concentration on *Vaccinium* species. In the previous experiment, neither *V. corymbosum* nor *V. arboreum* increased root FCR activity in response to Fe stress conditions (0 µM) and there was no difference in root FCR activity between Fe concentrations of 45 or 90 µM. As a result, Fe concentration was adjusted to 2, 22.5 and 45 µM in the present study. We hypothesized that *V. arboreum* is able to take up and utilize Fe and NO$_3^-$ more efficiently than *V. corymbosum*, especially under low Fe concentration conditions. The objectives of the
present study were to determine the effect of Fe concentration on root and leaf FCR activity, root NR activity, and Fe and \( \text{NO}_3^- \) uptake of two blueberry species, *V. corymbosum* ‘Misty’ and *V. arboreum* under \( \text{NO}_3^- \)N conditions.

**Materials and Methods**

**Plant Culture**

Seeds of the wild species, *V. arboreum*, were collected from a natural habitat at Manatee Springs, Florida during Fall 2000. Seeds were germinated on the surface of Canadian peat under intermittent mist (4 hours/day) in a greenhouse with average temperatures of 25ºC and average PPF of 545 \( \mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \). In March 2001, plants of *V. arboreum* were transplanted to 1-L pots containing pine bark and maintained in the greenhouse. In August 2001, shoot cuttings of *V. corymbosum* ‘Misty’ were rooted in peat:perlite (1:1 by volume) medium under intermittent mist in a greenhouse with average temperatures of 25ºC and average PPF of 545 \( \mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \). In January 2002, plants were transplanted to 1-L pots containing pine bark and maintained in the greenhouse. On 1 March 2002, fifteen plants from each species were selected and plant fresh weights were determined prior to transfer to 2-L plastic bottles filled with a complete nutrient solution. The nutrient solution contained the following composition (mM): 0.5 \( \text{K}_2\text{HPO}_4 \), 1.0 \( \text{MgSO}_4 \), 0.5 \( \text{CaCl}_2 \), 0.45 \( \text{Fe-diethylenetriaminopentaacetic} \) acid (Fe-DTPA), 0.045 \( \text{H}_3\text{BO}_3 \), 0.01 \( \text{MnSO}_4 \), 0.01 \( \text{ZnSO}_4 \), and 0.2 \( \mu \text{M} \) \( \text{Na}_2\text{MoO}_4 \). The nitrogen source was 5.0 mM \( \text{NaNO}_3 \). The nutrient solutions were buffered at pH 5.5 with 10 mM MES. The bottles were wrapped with 2 layers of aluminum foil to eliminate light infiltration and plants were acclimated in the nutrient solutions for 3 weeks. After acclimation, plant roots were soaked in 1 mM NaEDTA for 5 min. and washed 3 times in deionized (DI) water to remove the apoplastic Fe (Rosenfield et al., 1991) before placing
in nutrient solutions containing 2, 22.5 or 45 µM Fe-DTPA. The pH of nutrient solutions in each bottle was monitored daily with a portable pH meter (Accumet 1001, Fisher, USA), and maintained at 5.5 using 0.1 N KOH or HCl as needed. Aeration was provided to each bottle by an aquarium pump (Elite 801, Rolf C. Hagen, Mansfield, MA), connected by tygon tubing, located at the bottom of the bottle. The airflow was adjusted to 1 L/min. Nutrient solutions were changed every 2 weeks. Between each solution change, DI water was added to the solutions to maintain a 2 L volume. At each solution change, the amount of solution left in each bottle was recorded and used to determine the Fe and NO$_3^-$ uptake rate. Plants were grown for 16 weeks, from March through July 2002. The average greenhouse conditions were 27/20°C day/night temperature, 90% relative humidity and average PPF of 520 µmol·m$^{-2}$·s$^{-1}$. Root temperature was monitored weekly in April, using a temperature sensor attached to a portable pH meter (Accumet 1001, Fisher, USA). The average root temperature in this study was 24°C.

**Nitrate and Fe Uptake**

Nitrate and Fe uptake were determined every 2 weeks by measuring depletion from the nutrient solutions just prior to solution replacement. For NO$_3^-$ determination, 10 µl of each nutrient solution was diluted with 1.5 ml distilled water and vortexed. Fifteen µl of 12.1 N HCl was added and the samples were vortexed before reading spectrophotometrically (Shimadzu UV-160, Japan) at 210 nm. The NO$_3^-$ uptake rate per day was determined by calculating the amount of NO$_3^-$ depletion from the solution and dividing by the number of days between nutrient solution changes.

Iron concentration left in the solution was determined using atomic absorption spectrophotometry (Perkin Elmer 3030B, Norwalk, Connecticut, USA) with a hallow
cathode lamp as a light source and air-acetylene flame. The depletion of Fe from the nutrient solution was calculated and divided by the number of days between nutrient solution changes to represent the rate of Fe uptake per day.

**Root and Leaf Fe (III) Reducing Capacity**

Root and leaf FCR activities were quantified every two weeks. For root FCR, root tips (1 cm long) were cut, placed in a beaker filled with ice water and transferred to the lab. Roots were weighed and about 100 mg tissue FW were placed in a test tube. Root tissue was rinsed in 0.2 mM CaSO_4 for 5 min before transferring to 2 mL assay solution, containing 10 mM CaSO_4, 5 mM MES (pH 5.5), 0.1 mM Fe-EDTA and 0.3 mM sodium-bathophenanthroinedisulfonic acid (Na_2-BPDS). One test tube with 2 mL assay solution without root tissue was used as a control. Samples and control tubes were incubated for 1 h in a shaking water bath at 50 rpm and 23°C in the dark. After the incubation period, a 1 mL aliquot from each tube was transferred into a cuvette and read spectrophotometrically at 535 nm (Shimadzu UV-160, Japan). The concentration of Fe(II)-BPDS produced was calculated using the molar extinction coefficient of 22.14 mM/cm (Chaney et al., 1972).

For the leaf FCR assay, 4 mm diameter leaf discs were taken from the 2nd or 3rd mature leaves of the lateral branches of each plant, placed in a beaker filled with ice water and transferred to the lab. Leaf discs were weighed and about 100 mg FW tissue was placed in test tubes containing 0.2 mM CaSO_4. After 10 min, the CaSO_4 was discarded and 2 mL of assay solution (described above) were added to the tubes. Samples were vacuum infiltrated for 10 min before incubating in a shaking water bath at 50 rpm and 23°C for 1 hr in the dark. After incubation, 1 mL aliquot from each tube was
transferred into a cuvette and read spectrophotometrically at 535 nm. The concentration of Fe (II)-BPDS was calculated using the same method as described above.

**Root Nitrate Reductase**

Root NR activities were quantified every two weeks. Root tips (1 cm long) were cut, placed in a beaker filled with ice water, and transferred to the lab. Roots were weighed and about 100 mg tissue FW were placed in each test tube (2 tubes/trt/rep). Two mL of assay solution, composed of 2% 1-propanol, 100 mM KH$_2$PO$_4$ (pH 7.5) and 30 mM KNO$_3$ were added to each test tube. One tube/trt/rep was immediately filtered through Whatman No.2 paper and used as the time 0 control. Samples were vacuum infiltrated for 5 min. and incubated in a shaking water bath at 31ºC and 50 rpm for 1 h in the dark. After incubation, the assay solution with roots was filtered and a 1 mL aliquot from each sample was removed to a new tube. One-mL sulfanilamide (1% w/v in 1.5N HCl) and 1 mL N-(1-naphthyl)-ethylenediaminedihydrochloride (0.02% w/v in 0.2N HCl) were added. The samples were incubated at room temperature for 30 min. The absorbance at 540 nm was determined spectrophotometrically (Modified from Jaworski, 1971).

**Leaf Chlorosis**

Estimation of leaf chlorophyll concentration was performed using a chlorophyll meter (SPAD 502, Minolta Corp., Japan) at the beginning of the experiment and every 2 weeks throughout the experimental period. Five fully expanded leaves (the 2$^{nd}$ or 3$^{rd}$ leaf from each lateral branch of each plant) were measured at each time and readings were averaged to give the mean leaf chlorophyll concentration. Visual ratings for leaf chlorosis were also performed during the same period. Rating scores ranged from 1 to 5, with 1 = green and 5 = severe chlorosis with necrosis (Chaney et al., 1992).
Tissue N and Fe Determination

Plants were harvested on 12 July 2002, washed with DI water before separated into stems, leaves, and roots, and fresh weight (FW) of each part was determined. Dry weight (DW) was determined after oven drying at 70°C to constant weight. Dried samples were ground through a 20-mesh screen in a Wiley Mill (Arthur Thomas, Pennsylvania, USA) and were analyzed for total Kjedahl nitrogen (TKN) and Fe. For TKN, 100 mg tissue was placed into 50 mL digesting tubes. Popes Kjeldahl mixture (2.0 g) was added to each tube, followed by 2.5 mL sulfuric acid. Glass funnels were inserted in each tube before placing the tubes on a digestion block at 380°C for 8-10 hr. After cooling, the tubes were rinsed with 10-15 mL of DI water, vortexed, and brought to 50 mL with DI water. Samples were then filtered (Whatman No.8 filter paper) and analyzed using the Kjeldahl method (Horneck and Miller, 1998).

For Fe concentration, a dry ash digestion of the tissue was performed. Dried tissue (500 mg) was placed in a muffle furnace at 500°C for 10-12 hours. After cooling, 50 mL of 1N HCl was added to each sample and the solutions were filtered (Whatman No. 8 filter paper). Iron concentration was determined using Inductively Coupled Argon Plasma Spectrophotometry (ICAP, Spectro-CIROS CCD, FTCEA000, Germany).

For NO$_3^-$ concentration, dried tissue (500 mg), ground through 40-mesh screen, was placed in a 50 mL Erlenmeyer flask, 50 mL of DI water was added, and samples were incubated on a lateral shaker for 30 min. The samples were filtered with Whatman No. 8 filter paper. The extract was analyzed for NO$_3^-$ concentration by Automated Cadmium Reduction Method using Rapid Flow Analyzer ALPKEM 300 (Alpkem Corporation, Oregon, USA) at 540 nm (Anderson and Case, 1999).
Experimental Design

Treatments were arranged as a 2 x 3 factorial experiment in a randomized complete block design with 5 replications, using a single plant per replication. The factors were plant species (V. corymbosum or V. arboreum) and iron concentration (2, 22.5 and 45 μM). Data were analyzed using SAS (SAS Institute, Cary, N.C., USA). Mean separation was performed using t-test on every pair of Least Square Means (LSM). Daily NO$_3^-$ and Fe uptake rates/plant and final plant FW and DW were analyzed using initial plant FW as a covariate to normalize for differences in initial plant size.

Results

Root Nitrate Reductase

Root NR activity in V. arboreum was higher than V. corymbosum for the entire experimental period (Fig. 6-1). The activity increased in both species from week 2 until week 10, then fluctuated through the end of the experiment. Root NR activity ranged from 45 to 525 nmol NO$_2^-$/gFW/h in V. arboreum and 14 to 380 nmol NO$_2^-$/gFW/h in V. corymbosum.

In general, there was no significant effect of Fe concentration and interaction between species and Fe concentration on root NR activity (data not shown).

Nitrate Uptake Rate

In general, there was no effect of species on NO$_3^-$ uptake rate (Fig. 6-2). The uptake rate in V. corymbosum increased steadily from 0.06 mmol NO$_3^-$/plant/day at week 4 to 0.34 mmol NO$_3^-$/plant/day by week 10, before decreasing to 0.30 mmol NO$_3^-$/plant/day by week 12. Nitrate uptake rate in V. arboreum increased rapidly from 0.05 to 0.22 mmol NO$_3^-$/plant/day from week 4 to week 6 and remained fairly constant until week 14 before
the uptake rate increased to the same level as in *V. corymbosum* by the end of the experiment.

There was no significant effect of Fe concentration on NO$_3^-$ uptake rate (data not shown). There was no significant interaction between species and Fe concentration on NO$_3^-$ uptake rate (data not shown).

**Root Fe (III) Reducing Capacity**

*V. corymbosum* had greater root FCR activity than *V. arboreum* throughout most of the experiment (Fig. 6-3). Root FCR was relatively constant in *V. corymbosum* during the first week, then increased more than 2-fold from week 1 to week 5 (31 to 72 nmol Fe$^{2+}$/gFW/h). Activity then decreased gradually, reaching 40 nmol Fe$^{2+}$/gFW/h by the end of the experiment. In contrast, root FCR activity in *V. arboreum* decreased from 55 nmol Fe$^{2+}$/gFW/h at the beginning of the experiment to reach the minimum activity (16 nmol Fe$^{2+}$/gFW/h) at week 3 after treatment. Root FCR activity increased by week 5 (46 nmol Fe$^{2+}$/gFW/h) before decreasing to 28 nmol Fe$^{2+}$/gFW/h by the end of the experiment.

External Fe concentration had no effect on root FCR activity during the first three weeks after treatment began (Fig. 6-4). From week 5 through the end of the experiment, root FCR activity was significantly greater at high Fe (22.5 and 45 µM) compared with low Fe (2 µM). At both 22.5 and 45 µM Fe, root FCR activity averaged ~ 72 nmol Fe$^{2+}$/gFW/h at week 5, then decreased to ~ 40 nmol Fe$^{2+}$/gFW/h by the end of the experiment. In general, there was no interaction between species and Fe concentration on root FCR activity.

**Leaf Fe (III) Reducing Capacity**

Leaf FCR activity in *V. arboreum* ranged from 1.6 to 3.9-fold greater than activity in *V. corymbosum* (Fig. 6-5). The activities were relatively stable from week 6 to12,
averaging 21 and 9 nmol Fe\(^{2+}\)/gFW/h in *V. arboreum* and *V. corymbosum*, respectively. The activity increased to the maximum level at week 14 in both species before decreasing by week 16. Leaf FCR activity ranged from 18 to 34 nmol Fe\(^{2+}\)/gFW/h in *V. arboreum* and 5 to 19 nmol Fe\(^{2+}\)/gFW/h in *V. corymbosum* between week 6 and 16.

Iron concentration had no effect on leaf FCR activity and there was no significant interaction between species and Fe concentration on leaf FCR activity (data not shown).

**Iron uptake**

Iron uptake rate was not affected by species, ranging from 0.33-2.11 µmol Fe/plant/day in *V. arboreum* and 0.35-1.48 µmol Fe/plant/day in *V. corymbosum* (data not shown). However, uptake rate increased as external Fe concentration increased (Fig. 6-6). Uptake rates at 22.5 µM Fe were significantly higher than at 2 µM Fe for the entire experimental period, while uptake rates at 45 µM Fe were significantly higher than at 2 µM Fe from week 6 through the end of the experiment. Uptake rates at 22.5 and 45 µM Fe increased steadily from week 4 and reached the highest rate at week 8 (1.5 and 1.9 µmol Fe/plant/day, respectively). The uptake rates at 22.5 µM Fe then declined at week 10, and remained relatively constant until the end of the experiment. Uptake rates at 45 µM Fe decreased after week 8 and fluctuated through the end of the experiment. The Fe uptake rate was steady throughout the experiment at external Fe concentration of 2 µM, averaging 0.2 µmol Fe/plant/day. In general, there was no interaction between species and Fe concentration on Fe uptake rate.

**Leaf Chlorosis and Tissue Analysis**

Some chlorosis symptoms appeared in young, developing *V. arboreum* leaves at 2 µM Fe, but the chlorosis was mild and disappeared when leaves were mature. No
chlorosis was observed in *V. corymbosum* leaves during the experiment. There was no effect of species or Fe concentration on leaf chlorosis (data not shown).

There was no effect of species or Fe concentration on FW or DW of plant parts (Table 6-1). However, there was a significant effect of species on stem, leaf and root TKN concentration and leaf and root Fe concentration (Table 6-2). *V. corymbosum* tissues had greater TKN concentration and greater leaf and root Fe concentrations than *V. arboreum* by the end of the experiment. There was no effect of species on tissue NO$_3^-$ concentration and no effect of Fe concentration on tissue TKN, NO$_3^-$, or Fe concentration.

**Discussion**

*V. arboreum* is potentially more efficient in NO$_3^-$ assimilation than *V. corymbosum*, as indicated by greater root NR activity. The average root NR activity in *V. arboreum* (305 nmol NO$_2$/gFW/h) was about 1.6-fold greater than *V. corymbosum* (180 nmol NO$_2^-$ /gFW/h). These activities were greater than root NR activities in other blueberry cultivars, including the southern highbush ‘Sharblue’ (27 nmol NO$_2$ /gFW/h) (Merhaut, 1993) and the northern highbush ‘Jersey’ (48 nmol NO$_2$ /gFW/h) (Dirr et al., 1972). However, root NR activities were much lower than root NR activities found in many other woody (Bussi et al., 1997; Hucklesby and Blanke, 1987; Lee and Titus, 1992) and herbaceous plants (Hucklesby and Blanke, 1987). Nonetheless, the higher root NR activity in *V. arboreum* compared with *V. corymbosum* may partially explain why *V. arboreum* can thrive in high pH soils, where NO$_3^-$ is the predominant N form, while *V. corymbosum* cannot.

The greater root NR activity in *V. arboreum* was not reflected in increased NO$_3^-$ uptake or increased tissue TKN concentration. The NO$_3^-$ uptake rate is often correlated with NR activity, as shown in oak (*Quercus robur*) (Thomas and Hilker, 2000) and
spruce (*Picea abies*) (Peuke and Tischner, 1991). The lack of correlation in the present study may be due to the method used to measure uptake, which may not have been sensitive enough to detect small differences in NO$_3^-$ uptake, especially at high external NO$_3^-$ concentration (Glass et al., 2002). Labeled nitrate, either as $^{13}$N or $^{15}$N, has been used successfully to study NO$_3^-$ uptake and is a much more sensitive method of determining total NO$_3^-$ uptake than is the depletion method, which measures net uptake (influx + efflux) (Tischner, 2000). Alternatively, NO$_3^-$ uptake in *Vaccinium* may not be correlated with NR activity. In a study of the NR-deficient *Nicotiana* mutant, *nia*, NO$_3^-$ uptake in mutant and wildtype cells was similar, even though NR activity was significantly different (Muller and Mendel, 1989). Thus, NR activity does not always correlate with NO$_3^-$ uptake (Larsson and Ingemarsson, 1989). However, if this were the situation in the present study, one might expect to find increased NO$_3^-$ concentration in *V. corymbosum* tissues compared with *V. arboreum*, and this was not observed. Nor were increased TKN concentrations observed in *V. arboreum*, which would support the idea that the NO$_3^-$ uptake measurement method was insensitive. However, the lower TKN in *V. arboreum* compared with *V. corymbosum* may simply reflect genotypic differences, which have been reported for other blueberry genotypes (Korcak, 1992; Merhaut, 1993).

Neither *V. arboreum* nor *V. corymbosum* exhibited increased root FCR activity under low Fe concentration (2 µM). In fact, both root FCR activity and Fe uptake increased as external Fe concentration increased. This suggests that both *V. arboreum* and *V. corymbosum* are Fe inefficient genotypes, and lack the ability to increase root FCR under Fe deficiency. This is similar to results found for *Vitis* spp. (Brancadoro et al.,
1995), in which some genotypes had lower root FCR activity under Fe deficient compared with Fe sufficient conditions.

On the other hand, the lack of leaf chlorosis under Fe deficiency treatment may indicate that plants were, in fact, not Fe deficient. The acclimation of plants under high Fe concentration (45 µM) before the experiment started may have supplied sufficient Fe for the entire experimental period and it is possible that Fe deficient conditions were not induced. In both woody and herbaceous plants, Fe can be stored in the root apoplast (Mengel, 1994). Thus, there may have been sufficient Fe stored in plants to prevent Fe deficiency, even after 16 weeks of low Fe treatment. The lack of differences in tissue Fe concentrations among the Fe treatments supports this possibility.

The greater root FCR activity in *V. corymbosum* compared with *V. arboreum* was not reflected in increased Fe uptake. This is in agreement with Bavaresco et al. (1991), who found no differences in Fe uptake in grapevine genotypes that differed in root FCR activity. In their study, Fe uptake was assessed using $^{59}$Fe, which is a more sensitive method compared with the depletion method used in the present study. This suggests that root FCR activity may not always correlate with Fe uptake.

Leaf FCR was greater in *V. arboreum* than in *V. corymbosum*. This supports results from the previous experiments (Chapter 4 and 5). Leaf FCR plays a crucial role in the uptake of Fe from the xylem across the leaf plasma membrane into the cytoplasm of the mesophyll cells (Mengel, 1994). Even though leaf chlorosis was not observed in the present study and leaf Fe concentration in all Fe treatments was within the recommended range for blueberry (Williamson and Lyrene, 1995), the greater activity of FCR in *V.
*V. arboreum* leaves suggests that the ability to utilize Fe in the leaf is greater in this species compared with *V. corymbosum*.

The higher root FCR activity and Fe uptake rate at 22.5 and 45 µM Fe was not reflected in higher Fe concentration in plant tissues compared with 2 µM Fe. It is possible that Fe adsorbed on the root surface and root apoplastic Fe were not fully removed before the Fe tissue determination, and this resulted in similar root Fe concentrations. The high root Fe concentration found at all 3 Fe concentrations supports this. Even though root Fe concentrations were 2-3 fold higher than Fe concentrations found in the previous experiments using 45 and 90 µM Fe, they were similar to those reported for other *Vaccinium* (Korčak, 1992).

The higher root Fe concentration in *V. corymbosum* compared with *V. arboreum* suggests the possibility of more Fe$^{3+}$ storage in the root apoplast (Mengel, 1994; Kosegarten et al., 1998b). Alternatively, there may have been greater precipitation of Fe on *V. corymbosum* roots. The fine root system in *V. corymbosum* may contribute to high Fe adsorption on the root compared with the coarse root system in *V. arboreum*.

Results from the present study indicate that *V. arboreum* has the potential for greater NO$_3^-$ assimilation compared with *V. corymbosum*, as shown by higher root NR activity, even though this was not reflected in either increased NO$_3^-$ uptake or tissue N concentration. However, *V. corymbosum* is potentially more efficient in Fe assimilation than *V. arboreum*, as indicated by the greater root FCR activity. Neither species increased root FCR under low Fe concentration, suggesting both are Fe-inefficient genotypes. The high leaf FCR activity in *V. arboreum* suggests that *V. arboreum* is more efficient in using leaf Fe compared with *V. corymbosum*. The increased efficiency in leaf Fe
assimilation and NO₃⁻ assimilation may explain why *V. arboreum* can grow better than *V. corymbosum* on high pH soils, where Fe availability is low and N is predominantly present as NO₃⁻.
Fig. 6-1. Effect of species on root nitrate reductase activity. Mean ± SE (n = 5)

Fig. 6-2. Effect of species on nitrate uptake rate. Values are adjusted means using initial whole plant FW as a covariate. Mean ± SE (n = 5)
Fig. 6-3. Effect of species on root ferric chelate reductase activity. Mean ± SE (n = 5)

Fig. 6-4. Effect of iron concentration on root ferric chelate reductase activity. Mean ± SE (n = 5)
Fig. 6-5. Effect of species on leaf ferric chelate reductase activity. Mean ± SE (n = 5)

Fig. 6-6. Effect of iron concentration on iron uptake rate. Values are adjusted means using initial whole plant FW as a covariate. Mean ± SE (n = 5)
Table 6-1. Effect of species and iron concentration on final fresh weight (FW) and dry weight (DW) of stems, leaves and roots. Values are means adjusted using initial whole plant FW as a covariate.

<table>
<thead>
<tr>
<th>Species</th>
<th>Initial FW (g)</th>
<th>FW (g) Stem</th>
<th>FW (g) Leaf</th>
<th>FW (g) Root</th>
<th>FW (g) Plant</th>
<th>DW (g) Stem</th>
<th>DW (g) Leaf</th>
<th>DW (g) Root</th>
<th>DW (g) Plant</th>
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<td>6.4</td>
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<td>32.3</td>
<td>44.3</td>
<td>101.4</td>
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**Z**

<table>
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<th>Iron concentration</th>
<th>Initial FW (g)</th>
<th>FW (g) Stem</th>
<th>FW (g) Leaf</th>
<th>FW (g) Root</th>
<th>FW (g) Plant</th>
<th>DW (g) Stem</th>
<th>DW (g) Leaf</th>
<th>DW (g) Root</th>
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<td>23.7</td>
<td>22.9</td>
<td>38.7</td>
<td>87.4</td>
<td>10.9</td>
<td>8.4</td>
<td>9.3</td>
<td>29.2</td>
</tr>
<tr>
<td>22.5 µM</td>
<td>33.6</td>
<td>20.5</td>
<td>25.4</td>
<td>36.3</td>
<td>86.1</td>
<td>10.1</td>
<td>11.4</td>
<td>7.7</td>
<td>28.5</td>
</tr>
<tr>
<td>45 µM</td>
<td>34.7</td>
<td>19.5</td>
<td>26.4</td>
<td>38.5</td>
<td>87.1</td>
<td>8.8</td>
<td>9.9</td>
<td>8.3</td>
<td>26.2</td>
</tr>
<tr>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Z**, NS = significant at the 1% level or nonsignificant by t-test, n = 5.
Table 6-2. Main effects of species and iron level on stem, leaf and root total Kjeldahl nitrogen (TKN), nitrate concentration and iron concentration.

<table>
<thead>
<tr>
<th>Species</th>
<th>TKN (mg g(^{-1}) DW)</th>
<th>NO(_3)-N (µg g(^{-1}) DW)</th>
<th>Fe (µg g(^{-1}) DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stem</td>
<td>Leaf</td>
<td>Root</td>
</tr>
<tr>
<td><strong>V. arboreum</strong></td>
<td>4.9</td>
<td>9.9</td>
<td>7.4</td>
</tr>
<tr>
<td><strong>V. corymbosum</strong></td>
<td>7.7</td>
<td>12.0</td>
<td>12.4</td>
</tr>
<tr>
<td><strong>Z</strong>, * or NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Iron concentration</th>
<th>TKN (mg g(^{-1}) DW)</th>
<th>NO(_3)-N (µg g(^{-1}) DW)</th>
<th>Fe (µg g(^{-1}) DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stem</td>
<td>Leaf</td>
<td>Root</td>
</tr>
<tr>
<td>2 µM</td>
<td>6.2</td>
<td>10.8</td>
<td>9.9</td>
</tr>
<tr>
<td>22.5 µM</td>
<td>6.7</td>
<td>11.1</td>
<td>9.8</td>
</tr>
<tr>
<td>45 µM</td>
<td>6.2</td>
<td>10.8</td>
<td>9.9</td>
</tr>
<tr>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Z*, **, * or NS = significant at the 1%, 5% level or non-significant by t-test, n = 5.
Blueberries grow well in acid soils, with pH ranging from 3.5 to 5.5. These soils are characterized by high iron (Fe) availability and nitrogen (N) predominantly in the ammonium (NH$_4^+$) form. Because suitable soil conditions are limited, growers need to expand production to areas containing sub-optimal soil types, such as upland soil conditions with low organic matter and pH higher than 5.5 (Korcak, 1989). Associated with the high pH are low Fe availability and N primarily in the form of nitrate (NO$_3^-$). Blueberries grown on high pH soils may develop leaf chlorosis and exhibit decreased growth compared with the typical acid soils where blueberries are usually grown. The limitation to blueberry growth on high pH soils may be due to decreased Fe availability and/or the inability to assimilate NO$_3^-$.

In this research, two blueberry species, which differ in their adaptation to high pH soils, were compared in their ability to take up and assimilate Fe and NO$_3^-$ under a variety of conditions that mimic high pH soils. The species used were the cultivated southern highbush blueberry, *V. corymbosum* L. interspecific hybrid ‘Misty’, which develops leaf chlorosis and grows poorly on high pH soils, and a wild species, *V. arboreum* Marsh, which can grow normally on pH up to 6.0. Both blueberry species were able to take up NH$_4^+$ and NO$_3^-$; however, the uptake rate of NH$_4^+$ was much greater than NO$_3^-$. Dry weight (DW) gain of *V. corymbosum* was greater under NH$_4^+$ compared with NO$_3^-$ conditions. In contrast, plant DW of *V. arboreum* was similar under both NH$_4^+$ and NO$_3^-$ conditions.
*V. corymbosum* and *V. arboreum* contained both a constitutive and inducible nitrate reductase (NR) system, but activity of the constitutive NR was much lower than that of the inducible NR. Under NO$_3^-$ conditions, *V. arboreum* had greater root NR activity than *V. corymbosum*, indicating a greater potential to assimilate NO$_3^-$. The greater root NR activity in *V. arboreum* was not always reflected in higher NO$_3^-$ uptake and/or greater N tissue concentration. The lack of correlation among these parameters may not only due to the depletion method used to determine NO$_3^-$ uptake rate which may lack sufficient sensitivity to measure small differences in uptake, especially at high external NO$_3^-$ concentration, but may also due to the variability among plants. Alternatively, NO$_3^-$ uptake may not be correlated with NR activity, as shown in other crops. Short term studies on NO$_3^-$ uptake in *Vaccinium*, using $^{13}$N or $^{15}$N, would provide a more sensitive method of determining whether NO$_3^-$ uptake and NR activity are correlated in *Vaccinium*.

Neither root NR activity nor NO$_3^-$ uptake were affected by solution pH (5.5 vs 6.5). The optimum pH for root NR activity is likely to depend on plant species and ranges between 6.5 to 7.5 (Wray and Abberton, 1994). The optimum pH for NR activity in spruce is 5.5 (Peuke and Tischner, 1991), while NR activity in lowbush blueberry is highest at pH 6.0 (Townsend, 1970). It is possible that the pH range in the present study was too close to distinguish differences in NR activity. The lack of pH effect on NR activity and NO$_3^-$ uptake was reflected in similar plant DW (both shoot and root), root total Kjedahl N (TKN), and root and shoot NO$_3^-$ concentration.

Neither *V. corymbosum* nor *V. arboreum* exhibited increased root ferric chelate reductase (FCR) activity under Fe deficient conditions in the present study. In contrast, leaf FCR activity was always greater in *V. arboreum* compared with *V. corymbosum*,
regardless of pH and/or external Fe concentrations. The efficient utilization of Fe in the leaf may play a more important role in the development of Fe chlorosis in *Vaccinium* compared with Fe uptake and assimilation in the root.

Root FCR activity increased in both species as nutrient solution pH increased from 5.5 to 6.5. The decrease Fe availability at higher pH (6.5) compared with lower pH (5.5) may induce the root response to Fe deficiency in these two blueberry species by increasing root FCR activity. However, this was not reflected in a difference in Fe uptake. The lack of correlation between Fe uptake and root FCR activity may be due to the method used to measure Fe uptake, which might not be sensitive enough to detect small differences in Fe uptake. The reduction of leaf size is considered to be one of the Fe deficiency symptoms (Kosegarten et al., 1998b). In the present study, leaf size in both blueberry species decreased at pH 6.5 compared with pH 5.5. This implies that both species suffered from Fe deficiency at pH 6.5, even though shoot Fe concentration were similar at both pHs and there were no symptoms of leaf chlorosis.

The ability of *V. arboreum* to utilize NO$_3^-$ and Fe more efficiently than *V. corymbosum*, as indicated by higher root NR activity and leaf FCR activity, may be an advantage in upland high pH soils, where NO$_3^-$ is the dominant source of N and Fe availability is low. Due to the poor fruit quality, *V. arboreum* does not have economical value, however, it may be used as a rootstock for commercial blueberry cultivars in order to expand the blueberry production area to upland high pH soils. In addition, determining root NR and/or leaf FCR activity may be used as a tool to screen for adaptation potential of blueberry genotypes to high pH soils.
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BIOGRAPHICAL SKETCH

Umpika Poonnachit was born in Nakornsawan, Thailand. She received the Bachelor and Master of Science degrees from the Horticultural Science Department, Kasetsart University, Bangkok, Thailand. She worked for the Horticultural Research Institute, Department of Agriculture, from 1984 to 1987. She was awarded a scholarship from the New Zealand Government for a post-graduate study at Massey University, Palmerston North, New Zealand, where she received her Diploma in Fruit Production in 1989. After returning from New Zealand, she worked as a researcher at the Chanthaburi Horticultural Research Center and has conducted several experiments aiming to improve the yield and quality of tropical fruit crops. She was also responsible for creating training programs for fruit growers, agricultural extension staffs, and visiting scientists from other Asian countries.

She was awarded a scholarship from the Royal Thai Government to study at the Department of Horticultural Sciences, University of Florida, starting in January 1998. Upon graduation she will go back to Thailand to pursue her career as a researcher.