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Review

Physiology of Nitrogen and Calcium Nutrition in Blueberry (*Vaccinium* sp.)

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Abstract: Sustaining the fourfold increase in blueberry (*Vaccinium* sp.) production witnessed during the previous two decades requires better understanding of its mineral nutrient physiology. The primary goals of this review are to evaluate our current understanding of the physiology of nitrogen (N) and calcium (Ca) nutrition in blueberry. Nitrogen concentration in blueberry ranges from 0.4% to >2% across organs. Blueberry uses N in various forms (organic and inorganic), but it appears to display preference for ammonium (NH_4^+) over nitrate (NO_3^-). The roles of N acquisition, translocation and assimilation in determining N-source preference in blueberry are evaluated. Calcium plays important roles in determining fruit quality owing to its function in maintaining cell wall and membrane integrity. It is unique in its translocation characteristics being transported primarily via the xylem. Fruit [Ca^{2+}] typically declines from around 0.2% during early development to <0.05% at ripening. Modes of Ca acquisition and transport to the fruit, and various approaches to improve fruit [Ca^{2+}] are discussed. Areas where further research is warranted to improve our understanding of N and Ca physiology in blueberry are identified. Such knowledge is essential for sustainable nutrient management, improving productivity, and enhancing fruit quality in blueberry.

Keywords: blueberry (*Vaccinium* sp.); calcium transport; calcium-related disorders; N-source preference; nitrogen assimilation; nitrogen uptake

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1. Introduction

Blueberry (*Vaccinium* sp. L.), a member of the economically important and geographically widely distributed family Ericaceae, has emerged as a major fruit crop. The rapid and extensive increase in its cultivation is related, in-part, to health-promoting, nutraceutical characteristics associated with blueberry consumption. Numerous studies have demonstrated antioxidant, anti-inflammatory and anti-tumorigenic properties (among others) of blueberry fruit and their extracts [1–3]. Continued interest in its potential health benefits and general adaptability of its cultivation to various temperate regions is likely to ensure sustained growth of blueberry production. Currently, world-wide blueberry cultivation stands at over 119,000 ha (2019), more than double that at the beginning of the current century [4]. Global production exceeded 82,000 metric tons in 2019, an increase of almost 4-fold over that in 2000 [4]. The United States of America is currently the leading producer of blueberries, accounting for about a third of global cultivation at more than 308,000 metric tons. Various types of blueberry are cultivated in the United States (US): northern highbush (*Vaccinium corymbosum* L.); rabbiteye (*V. virgatum* Aiton); southern highbush (an inter-specific hybrid of *V. corymbosum*); and lowbush blueberry (*V. angustifolium* Aiton). The blueberry genome was sequenced, and a draft version of a wild diploid species genome was released in 2015 [5]. This was further augmented by sequencing of a cultivated tetraploid highbush blueberry (*V. corymbosum* 'Draper') genome [6]. Sustaining current blueberry production trends requires such advances in our understanding of its genetics and physiology, and subsequently their application to cultural practices.

One area where considerable gaps in knowledge exist is in our understanding of mineral nutrient physiology in blueberry. Blueberry is adapted to acidic soils and has often been classified as a 'calcifuge', a lime-avoiding plant [7,8]. Blueberry plants often display optimal growth at lower pH, typically between 4.0 and 5.5 [8–11]. Under such conditions, availability of most nutrients is limited and consequently, it has long been described as a plant with relatively lower nutritional requirements and slower growth habit [7]. Blueberry plants generally display lower elemental composition in comparison to other major fruit crops [7,8]. For example, foliar nitrogen (N) concentration in blueberry is often under 2% during most of the growing season while that of calcium (Ca) does not typically exceed 1% [7,8,12]. However, this is substantially variable across different *Vaccinium* species and in response to fertilization [13–15]. Multiple aspects of blueberry morphology and physiology influence their ability to acquire and use nutrients. For example, blueberry roots lack root hairs, may display preference for certain forms of nutrients, and develop mycorrhizal associations with specific fungi [7,8]. These features can greatly impact their nutrient acquisition and use characteristics. The goal of this review is to evaluate the physiology of the macronutrient, N, and the secondary macronutrient, Ca.

2. Nitrogen Physiology in Blueberry

Nitrogen is a macronutrient with a multitude of functions and is often limiting in crop production. Nitrogen physiology in perennial plant systems involves its acquisition, translocation, assimilation (incorporation of N into organic molecules), storage, and re-mobilization [16,17]. All these processes influence N homeostasis and its use in blueberry (Figure 1). These processes also regulate steady-state N concentration in various tissues of the plant (Table 1).

Table 1. Nitrogen (N) concentration in different parts of a blueberry (*Vaccinium* sp.) plant.

Plant Part	N Concentration (% Dry Weight)	Notes	Source
Leaves	1.2–2.1		8
	1.85–2.95		13 [z]
	1.7–2.7	between leaf senescence and fruit harvest	15 [y]
Vegetative growth	2.1	typical period of fruit harvest	14 [x]
	1.1–4.7	During growing season	13
	1.5–2.5	between anthesis and ~80 d after anthesis	17 [w]
Woody canes	0.4–0.75	highest before anthesis; lowest during fruit development	17
	0.8–1.7	highest at dormancy; low at fruit harvest	14
	0.76–1.58	highest at dormancy; lowest at fruit harvest	13
Flower	2.1–2.2	anthesis	17
	5		14
	5.3		13
Fruit	1–1.1	at harvest	17
	1.37	at harvest	13, 14
Crown	1.2–1.75	at fruit harvest and at dormancy	14
	0.95–1.73	highest at dormancy and lowest during mid-fruit development	13
Root	1.1–1.6	Increasing towards end of fruit development	17
	1.2–1.6	At dormancy and at fruit harvest	14
	0.95–2.3	Low during early fruit development and highest during dormancy (following year)	13

[z] *V. corymbosum* 'Bluecrop'; data from 50 kg ha^{-1} N treatment. [y] *V. corymbosum* (interspecific hybrids; 'Emerald' and 'Farthing'); estimated from 168 kg ha^{-1} N fertigation treatment. [x] *V. corymbosum* 'Bluecrop'; data from 50 kg ha^{-1} N treatment. [w] *V. virgatum* ('Bonita' and 'Climax').

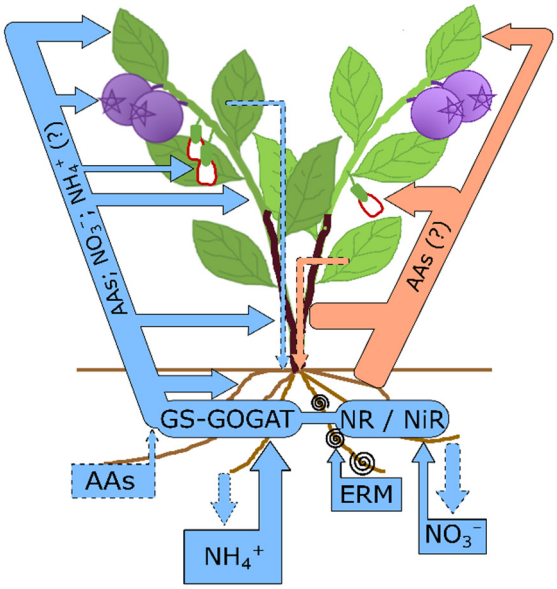

Figure 1. Nitrogen (N) homeostasis in blueberry. The size of arrows or boxes reflects relative size of flow or pool, respectively. Dotted outline indicates a potential mechanism for which evidence is not yet available in blueberry. Nitrogen can be acquired in the inorganic forms as ammonium (NH_4^+) or nitrate (NO_3^-), or in the organic form likely as amino acids (AAs). Inorganic N forms are subject to efflux, particularly at high external concentration. Specific ericoid mycorrhizae (ERM) aid in root N acquisition. Nitrate may be assimilated within roots by nitrate reductase (NR) and nitrite reductase (NiR). Ammonium acquired from soil or from NO_3^- assimilation can be incorporated into AAs by glutamine synthase (GS) and glutamine 2-oxoglutarate aminotransferase (GOGAT). Nitrogen can be translocated to leaves, flowers, fruit and shoots in the form of AAs, NO_3^- or NH_4^+. New N acquired from soil and assimilated or translocated is indicated in blue. Nitrogen recirculation contributes to its homeostasis but has not been investigated in blueberry. Nitrogen supply to new growth occurs largely through remobilization of stored N from roots and woody shoots. Nitrogen resorption from leaves prior to leaf fall likely contributes to storage N in woody canes and roots but has not yet been quantified. Nitrogen transport in the plant as remobilization and resorption is shown in orange.

2.1. Nitrogen Acquisition in Blueberry: Organic N

Plants acquire N in one of two main forms: organic and inorganic N (Figure 1). Organic N is derived from soil organic matter and is primarily present in the form of proteins, peptides and free amino acids which can be acquired by plants [18–20]. At least three sub-families of transporter genes, *AMINO ACID PERMEASE* (*AAP*), *LYSINE/HISTIDINE TRANSPORTER* (*LHT*) and *PROLINE TRANSPORTER* (*ProT*) within the larger *AMINO ACID/AUXIN PERMEASE* (AAAP) family, encode plant proteins directly involved in root uptake of amino acids [21,22]. In arabidopsis (*Arabidopsis thaliana* (L.) Heynh.), AAP1 and AAP5 facilitate root uptake of neutral and cationic amino acids, respectively [23,24]. LHT1 and LHT6 are expressed in roots and likely facilitate uptake of lysine/histidine, or neutral and acidic amino acids [24–27]. In arabidopsis, ProT1 and ProT2 aid in root uptake of proline and glycine-betaine from the rhizosphere [28,29]. Furthermore, the PEPTIDE TRANSPORTER (PTR) family transporter, PTR1, may be involved in the uptake of short-chain oligopeptides [30,31]. Additionally, proteins may be transported either as peptides/amino-acids following root exudate-mediated proteolysis or directly through endocytosis in arabidopsis and *Hakea actites* W.R. Barker, a plant adapted to low-fertility soils [32]. Considering their relatively conserved roles across multiple plants it may be speculated that such transporters play substantial roles in organic N acquisition in blueberry, but evidence for this is limited. In a recent transcriptome analysis of *V. corymbosum* and *V. arboreum* Marshall grown at pH 4.5 and 6.5, six *AAPs* were identified as substantially upregulated under high pH in *V. corymbosum* [33]. Additionally, a putative proline trans-

porter was upregulated in *V. corymbosum*, while a putative *LHT1* and an *OLIGO-PEPTIDE TRANSPORTER (OPT)* family gene were upregulated in *V. arboreum* under high pH (6.5) conditions. These data suggest that organic N transport mechanisms are expressed and functional in acquiring N at high pH in blueberry. However, the functional relevance of direct acquisition of amino acids to N nutrition needs further evaluation in blueberry.

Mycorrhizal associations can facilitate organic N acquisition in plants [34]. Ericaceae plants form specific mycorrhizal associations termed ericoid mycorrhizal (ERM) associations with certain genera of fungi such as *Hymenoscyphus*, *Pezizella* and *Oidiodendron* [35]. Cranberry (*V. macrocarpon* Aiton), a close relative of blueberry, formed ERM associations which enhanced their ability to use several N sources, including organic N [18,36,37]. When ^{15}N labeled ammonium (NH_4^+) sources were supplied to ERM-free and ERM-associated cranberry, shoot dry weight and total N concentration was enhanced in ERM-associated plants, but concentration of labeled ^{15}N was lower, suggesting enhanced uptake of unlabeled N-sources, potentially from organic soil N [36]. In closely related bilberry (*V. myrtillus* L.) plants with ERM associations, glycine uptake was a major fraction of N absorbed (up to 91%) when the labeled amino acid was injected into the organic (mor) layer of the soil in boreal forests [38]. Some ERM may also use organic sources of N as peptides of smaller chain lengths [39]. Blueberry plants establish similar ERM associations in native soils and under cultivation conditions [35,40–42]. In a survey of commercial blueberry orchards, up to 44% of total blueberry root length was associated with ERM, although this varied greatly depending on a range of factors such as cultivar, plant age, fruit maturity rate, soil pH and N fertilization [40]. *Hymenoscyphus ericae*, one of the ERMs, readily used organic N as short chain-length peptides and was able to improve N acquisition and plant dry weight when associated with *V. corymbosum* plants [39]. Recently, rhizosphere microbiomes of greenhouse grown southern highbush, rabbiteye and wild (*V. darrowi* Camp) blueberry were evaluated [42]. While a common core of the microbiome was apparent across different blueberry types, differences in abundance of fungal microbiomes was also revealed. Rabbiteye blueberry displayed greater abundance of specific ERM and dark septate endophytic taxa. Additionally, specific ERM taxa were associated with each of the blueberry types evaluated, emphasizing that ERM associations are species, and possibly genotype, specific. Whether ERM associations influence nutrient acquisition in agricultural soils, particularly that of N, and overall fitness of blueberry species needs to be resolved. Inoculation of blueberry plants with mycorrhizal peat or a mix of ERM isolates enhanced nutrient uptake (including N), growth and yield suggesting that these plants benefit in terms of N acquisition through ERM associations under production conditions [35,43]. However, such benefits were determined to be limited in other studies where only a minor increase in growth and no effects on N acquisition were reported [44].

2.2. Nitrogen Acquisition in Blueberry: Inorganic N

Plants use inorganic N as nitrate (NO_3^-) or ammonium (NH_4^+). Some plants display preference for an inorganic N source, which manifests as improved growth and biomass production, higher tissue N concentration, and/or yield in response to supply of a specific N-source. This parameter is often described as N-source preference [45,46]. For example, the conifer white spruce (*Picea glauca* (Moench) Voss) and the major agronomic crop, rice (*Oryza sativa* L.) display N-source preference for NH_4^+. Similarly, some plants within the Ericaceae family, including blueberry, are thought to display N-source preference for NH_4^+ [47]. Across different types of blueberry, multiple studies have suggested that plants display higher growth and/or greater N accumulation with NH_4^+ as the N-source (Table 2) [48–59]. Plant growth was enhanced, and N concentration increased when NH_4^+ was used as the N-source at low pH (4.5–4.9) in highbush and lowbush blueberry [9,50,51]. Similarly, greater growth in new leaves and stems and higher N concentration with NH_4^+ as the N-source under low pH (3.0–5.0) was noted in rabbiteye blueberry [11]. Greater shoot growth and increased chlorophyll and leaf N concentration in response to NH_4^+-based fertilization in comparison to NO_3^--based fertilization were reported in potted

southern highbush blueberry [52]. Consistently greater NH_4^+ uptake than that of NO_3^- over multiple weeks of cultivation in hydroponic culture was observed in *V. corymbosum* and *V. arboreum* [53]. Furthermore, uptake rates of labeled $^{15}NH_4^+$ were greater than that of $^{15}NO_3^-$ during a 24 h period, resulting in almost 2-fold greater N accumulation under $^{15}NH_4^+$ treatment in southern highbush blueberry [54]. However, it should also be noted that several other studies reported little or no N-source preference in blueberry (Table 2) [10,55–57,59].

Table 2. Summary of nitrogen (N)-source preference studies in blueberry (*Vaccinium* sp.).

Species	N Concentration (mM)	N-Source Preference	Suggested Mechanism/Notes	Study
V. corymbosum 'Jersey'	25 mM (250 mL per week); 2–8 mM as NH_4NO_3 or NH_4Cl	NH_4^+	pH: > 6.0; < 5.2	48
V. corymbosum 'Bluecrop'	5 mM	None	pH: 4.0, 6.0 and 8.0	55
V. angustifolium	1 mM and 10 mM	NH_4^+	pH: 4.9	50
V. corymbosum 'Berkley'	1.5 mM	NH_4^+	pH: 4.5; NH_4NO_3 displayed intermediate effects	51
V. angustifolium	2 mM	NH_4^+ at pH 4.5	pH: 4.5 and 6.0; pH and N-source may have independent effects	9
V. corymbosum 'Wolcott'	0.44 mM to 1.75 mM (combinations of NH_4^+ and NO_3^-	None	pH: 5.8–6.2; pH of eluent decreased with increasing NH_4^+	59
Interspecific hybrid clone of *V. corymbosum* and *V. angustifolium* 'Northblue'	2 mM	None	pH: 4.5 and 6.5; Plants displayed higher growth at lower pH; plants displayed similar N uptake rates regardless of N-source	10
V. virgatum 'Tifblue' and *V. corymbosum* 'Jersey	1 mM to 4 mM (combinations of NH_4^+ and NO_3^-; final N: 4 mM)	None	pH: 5.5, continually corrected; leaves accumulated greater free NH_4^+ with higher NH_4^+ supply	57
V. virgatum 'Tifblue'	1 mM	NH_4^+ (pH: 3.0; 4.0)	pH: 3.0, 4.0, 5.0	11
V. corymbosum 'Sharpblue'	Soil drench of 7.5 mmol ^{15}N in 500 mL	NH_4^+	pH: 6.5; Uptake rates higher for NH_4^+; translocation of N to shoots higher for NH_4^+	54
V. corymbosum '13-16-A'	6 mM	NH_4^+	pH: 3.5–4.2 for NH_4-N and 6.6-7.2 for NO_3-N	58
V. arboreum (Va) and *V. corymbosum (Vc)* 'Misty'	5 mM	NH_4^+ in *Vc*	pH: 5.5	53
V. virgatum 'Alapaha' and *V. corymbosum* 'Sweetcrisp'	5 mM	None: based on N uptake rates	pH: 5.0	56
V. corymbosum 'Emerald'	17.86 mmol N per week	NH_4^+	NH_4NO_3 displayed marginally better performance	52

Nitrate is the predominant N-source in soils under high pH, while NH_4^+ is predominant at low pH. In soils, conversion of NH_4^+ to NO_3^- through nitrification, a two-step process, is mediated by microorganisms. Ammonium in soils is initially oxidized to hydroxylamine and then to NO_2^-, generally by ammonia-oxidizing bacteria (AOB). Subsequently, NO_2^- may be oxidized to NO_3^- via the action of nitrite-oxidizing bacteria (NOB). The AOB species such as *Nitrosomonas* sp. and *Nitrosococcus* sp. were considered as major soil microorganisms mediating the initial rate-limiting oxidation step, while *Nitrobacter* sp. were considered the major organisms facilitating conversion of NO_2^- to NO_3^- [60,61]. The growth and activity of nitrifying bacteria are substantially reduced at low pH, and as a result, little nitrification was expected to occur under low pH conditions such as that of blue-

berry cultivation [62]. Recently, multiple other organisms, primarily ammonia-oxidizing archaea (AOA), have been demonstrated to facilitate NH_4^+ oxidation to NO_2^- [63]. In fact, AOA may likely be the numerically predominant organisms capable of ammonia oxidation in most soils [64,65]. Furthermore, the ratio of AOA to AOB is higher at lower pH, and they likely compete for highly limited availability of ammonia [62]. Consequently, it has been proposed that AOA are likely to serve the primary role as nitrifiers in nutrient-poor soils, as well as acid soils supplemented with urea [62]. Together, these studies suggest that nitrification can occur and contribute to NO_3^- availability in acid soils, such as those used for blueberry production. For example, application of $(NH_4)_2SO_4$ to 'Bluecrop' grown in soils at pH 4.8 resulted in substantial nitrification which was significantly but only temporarily reduced by co-application of a nitrification inhibitor, dicyandiamide (DCD), which inhibits NH_4^+ oxidation to NO_2^- [66].

Nitrate is generally poorly retained in the soil and is prone to multiple losses including that through denitrification and leaching [67]. It may be hypothesized that inhibition of nitrification in the rhizosphere can limit such losses and allow for enhanced availability of NH_4^+. Consistently, in other fruit crops such as strawberry (*Fragaria* × *ananassa* (Weston) Duchesne ex Rozier (pro sp.), grape (*Vitis vinifera* L.) and citrus (*Citrus* sp. L.), application of NH_4^+-based fertilizers along with nitrification inhibitors enhanced plant growth, fruit yield and fruit quality characteristics [68–70]. Application of DMPP (3,4-dimethylpyrazole phosphate), an inhibitor of ammonia oxidation, along with $(NH_4)_2SO_4$ to potted 'Emerald' southern highbush blueberry plants enhanced leaf N and chlorophyll content indicating that inhibition of nitrification can increase NH_4^+ acquisition in blueberry [52].

Many plants, including rice, display an ability to specifically release biological nitrification inhibitors (BNIs) that typically inhibit ammonia-oxidation to NO_2^-, thereby limiting nitrification capacity within the rhizosphere [61,71]. Furthermore, release of BNIs as root exudates often increases in the presence of NH_4^+ [72]. Such reduction of nitrification through specific release of BNIs can enhance NH_4^+ uptake and may serve as an important component of NH_4^+-preference [71,73]. It would be of high practical value to determine if blueberry displays such capacity for exudation of BNIs in the rhizosphere.

Inorganic N Acquisition Mechanisms

In many plants, NO_3^- and NH_4^+ uptake follow bi-phasic patterns dependent on the concentration of the available substrate [74–77]. In the case of NO_3^-, uptake at low concentrations of available substrate (<0.5–1 mM) is mediated by a high-affinity transport system (HATS) that has constitutive (cHATS) and inducible (iHATS) components [74,75,78–81]. The HATS for NO_3^- uptake is saturable and follows Michaelis-Menten kinetics. At higher substrate concentrations (>1 mM and up to 50 mM), in addition to the HATS, NO_3^- uptake is mediated by constitutive or inducible linear uptake mechanisms which operate with lower affinity for NO_3^- and are referred to as the low affinity transport system (LATS) [74,75,79–81]. The above transport systems remain poorly characterized in blueberry. One study described NO_3^- uptake kinetics in 'Tifblue' rabbiteye blueberry [82]. Here, NO_3^- depletion from nutrient medium was monitored at 8 h and 24 h after provision of various concentrations of NO_3^- up to 0.2 mM. A saturable HATS which followed Michaelis-Menten kinetics with V_{max} and K_m of 1.75 µmol g^{-1} h^{-1} and 23 µM, respectively, was identified [82]. The LATS uptake kinetics of NO_3^- remain poorly characterized, even though most studies analyzing NO_3^- uptake in blueberry have used N concentrations within the LATS range (Table 2 and references therein).

Ammonium uptake also follows a bi-phasic pattern in plants [76,83]. At low available NH_4^+ concentration (<1 mM), a saturable HATS component following Michaelis-Menten kinetics is functional [83,84]. At higher concentrations (>1 mM and up to around 40 mM), non-saturable, linear uptake kinetics are evident, in addition to the HATS, as noted previously for NO_3^- uptake [76]. In rabbiteye blueberry, NH_4^+ uptake within the HATS range displayed saturation kinetics, similar to that noted in other plants. The LATS component of NH_4^+ uptake was not evaluated in this study [85].

Nitrogen source preference for the NH_4^+ form may manifest at the level of N uptake. In white spruce, a plant with known N-source preference for NH_4^+, its influx was at least 4-fold higher than that for NO_3^-, potentially owing to atrophied NO_3^- acquisition mechanisms [86]. Similarly, it may be hypothesized that N-source preference in blueberry occurs, at least in part, at the level of N-uptake. Comparison of NH_4^+ and NO_3^- uptake kinetic parameters in rabbiteye blueberry suggested that uptake was potentially higher for NH_4^+ [82,85]. Using labeled ^{15}N-sources as drenches, uptake rate for NH_4^+ was noted to be around 2-fold higher than that for NO_3^- in 'Sharpblue' southern highbush blueberry [54]. However, other studies have indicated little difference between N sources in N uptake (Table 2) [56]. Furthermore, it is unclear if such preference exists within both (HATS and LATS) ranges of N availability.

Four families of transporters are involved in NO_3^- transport in plants: NITRATE TRANSPORTER 1 (NRT1)/PEPTIDE TRANSPORTER family (NPF); NRT2; CHLORIDE CHANNEL family (CLC) and SLOWLY ACTIVATING ANION CHANNEL family (SLAC) [87,88]. Members of the NPF and NRT2 families have demonstrated roles in root uptake of NO_3^- from the rhizosphere, in addition to facilitating NO_3^- transport within the plant, while members of the CLC and SLAC families are potentially involved in vacuolar uptake and efflux from guard cells, respectively [87,89]. Nitrate uptake at high and low affinities is mediated by secondary active co-transport with H^+s [79]. High-affinity transport of NO_3^- is mediated by inducible NRT2 family members. In Arabidopsis, which contains seven members in this family, NRT2.1 facilitates the majority of iHATS in association with NAR2 (NITRATE ASSIMILATION RELATED 2) [88,90]. Low-affinity transport of NO_3^- is mediated by the larger family (53 members) of NPF, of which many have been characterized, at least partially [88]. Among these, NPF6.3 was the first characterized NO_3^- transporter and displays dual-affinity [91]. Depending on the phosphorylation status of a specific threonine residue (T101), a process that is in-turn dependent on external NO_3^- concentration, NPF6.3 can switch between high-affinity (phosphorylated) and low-affinity transport (dephosphorylated) [91]. Furthermore, NPF6.3 also functions as a nitrate-sensor and is therefore referred to as a transceptor [92]. Additionally, NPF4.6 is a constitutively expressed low-affinity transporter [93].

Ammonium uptake at low external concentration in plants is facilitated by the AMT family of transporters of which six have been identified in arabidopsis, and four implicated in root acquisition [94–97]. Activity of AMT transporters appears to be additive and de-repressed by low NH_4^+ availability in arabidopsis. Furthermore, these transporters facilitate high-affinity transport of NH_4^+ [97].

In a study of the root transcriptome of *V. corymbosum* roots supplied with NO_3^-, eight transcripts of members from the NPF family (NPF2.7; NPF2.13; NPF3.1; NPF7.3; NPF8.1) were differentially expressed in response to change in pH from 4.5 to 6.5 [33]. Three transcripts coding for NPF2.7 were downregulated at pH 6.5. *NPF2.7* encodes a NITRATE EXCRETION TRANSPORTER 1 (NAXT1) that facilitates NO_3^- efflux from plant roots particularly in response to root zone acidification [98]. These data suggest greater NO_3^- efflux at lower pH (4.5) in *V. corymbosum*. Efflux of N and futile cycling is commonly associated with low affinity nutrient uptake [99]. As external nutrient concentration increases, particularly within the LATS range, the efflux: influx ratio increases, approaching 1 at high external ion concentrations [99]. Considering that the N concentration used in this study was 0.5 mM, it is likely that some futile cycling of NO_3^- occurred at pH 4.5. *NPF7.3* codes for a bi-directional transporter associated with xylem loading and was upregulated at pH 6.5 suggesting increased xylem loading of NO_3^- [100]. Some of the other *NPF* genes differentially expressed in this study are likely associated with hormone transport (NPF3.1), peptide transport (NPF8.1), and NO_3^- remobilization (NPF2.13) [88]. The *AMTs* were not identified as differentially expressed in this study, likely owing to the use of NO_3^- as the N-source. Further research is essential to identify and characterize NO_3^- and NH_4^+ transporters in blueberry, and to better understand their contributions to N acquisition and N-source preference. An initial approach in this context can involve characterization of

the blueberry root transcriptome in response to supply of different N-sources separately within the HATS- and LATS-related concentration ranges.

2.3. Nitrogen Translocation in Blueberry

Nitrogen translocation within the plant can occur in the form of NO_3^-, NH_4^+, or organic N compounds such as amino acids (Figure 1). In many plants, NO_3^- can be either assimilated within the roots following its acquisition or translocated from roots to shoots via the xylem following xylem-loading, and subsequently stored or assimilated in the shoots [101]. Often, NH_4^+ acquired from the soil is assimilated within roots rapidly as its build-up can be toxic to plant cells. The physiological basis of such toxicity is related to the effects of its influx on uptake of other cations, acidification of the rhizosphere, and increased metabolic demands owing to futile cycling, among others [102,103]. Hence, NH_4^+ has often been described as a N-form that is not substantially translocated via the xylem [104,105]. Rather, its assimilation products (amino acids, generally glutamine) are transported [101,104,105]. However, this has been disputed as studies with more sensitive analytical methods indicate substantial NH_4^+ transport, reaching 11% of total translocated N in the xylem sap of oilseed rape (*Brassica napus* L.) under NH_4^+ supply [106].

In blueberry, root to shoot transport of different N-forms has only been evaluated indirectly. In 'Sharpblue' southern highbush blueberry, supply of equimolar labeled ^{15}N-sources resulted in greater accumulation of NH_4^+ derived N than NO_3^- derived N, even after normalizing for differences in uptake, indicating lower capacity for NO_3^- translocation [54]. In another study, with *V. arboreum* and *V. corymbosum*, increasing NO_3^- supply from 1 mM to 5 mM did not alter shoot total Kjeldahl N or NO_3^- concentration but increased the root NO_3^- concentration, further indicating low capacity for translocation of this N form in blueberry [107]. Xylem sap NO_3^- concentration and NO_3^- flux in the xylem were not significantly affected by NO_3^- as the N-source, further supporting lower xylem loading or transport capacity for this form [56]. In other plants such as carob (*Ceratonia siliqua* L.), limitation in xylem loading of NO_3^- was associated with reduced capacity of shoot assimilation of NO_3^-, as its direct supply to the shoots through the stem enhanced assimilation capacity [108,109]. Furthermore, in lodgepole pine (*Pinus contorta* Dougl.) and other conifers, NO_3^- translocation to the shoots is limited in comparison to that in trembling aspen (*Populus tremuloides* Michx.), indicating diminished capacity for its loading into the xylem and/or higher capacity for root storage [110,111]. Together, these data suggest that in addition to N uptake, N loading into the xylem and its root-to-shoot translocation play important roles in N-source preference in blueberry. As indicated earlier, *V. corymbosum* roots displayed enhanced expression of NPF7.3, a NO_3^- transporter involved in xylem loading, at higher pH suggesting lower xylem-loading capacity under acidic pH conditions [33,100]. Further analysis of such transporter expression is essential to determine if limitations in their functions contribute to differential N loading in blueberry. Ammonium transport in the xylem sap has not been quantified in blueberry owing to the general assumption that it is assimilated prior to translocation. However, higher free NH_4^+ in leaves was reported in response to increasing root supply of NH_4^+ [57]. Considering the potential for N transport in xylem sap in this form [106], it is worth re-evaluation particularly under different levels of NH_4^+ supply. Additionally, the capacity of, and forms in which amino-acid transport occurs in the xylem, need to be determined in blueberry. Another aspect of N translocation in plants involves its re-circulation via the phloem (Figure 1). However, such transport in blueberry remains currently un-investigated.

2.4. Nitrogen Assimilation in Blueberry

Ammonium is directly assimilated into amino acids, while NO_3^- is reduced to NH_4^+ prior to its assimilation. Ammonium is assimilated mainly through the action of glutamine synthetase (GS), which uses glutamate (Glu) and NH_4^+ as substrates to generate glutamine (Gln). Glutamine subsequently reacts with 2-oxoglutarate to generate two molecules of Glu, a reaction catalyzed by glutamine 2-oxoglutarate aminotransferase/glutamate synthase

(GOGAT) [16,96,112]. The GS-GOGAT cycle can often be localized to the plastids in the shoots where it is operational for NH_4^+ derived from NO_3^- assimilation (following its reduction), or from other metabolic pathways such as photorespiration [16]. Glutamine synthetase can also be localized to the cytosol where it contributes to primary NH_4^+ assimilation. For example, in rice, loss of *OsGS1;2* leads to a reduction of multiple free amino acids and an increase in free [NH_4^+] in roots and xylem sap indicating that it is a critical component of primary NH_4^+ assimilation [113]. Considering that NH_4^+ may be the preferred source of N in blueberry and that NH_4^+ translocated to the shoots likely represents a small proportion, it may be expected that a significant proportion of acquired NH_4^+ is primarily assimilated within roots. However, this has not been extensively tested in blueberry. In *V. corymbosum*, GS activity was measurable in the shoots, stem and roots in response to different forms of N-supply [58]. However, it was not significantly enhanced by N-supply as NH_4^+ in comparison to that as NO_3^-, even if greater overall dry weight accumulation occurred under NH_4^+ nutrition [58]. At least three genes potentially coding for GS and one encoding a glutamate synthase were downregulated in *V. corymbosum* roots at pH 6.5 in comparison to that at 4.5, suggesting lower root NH_4^+ assimilation under higher pH [33]. However, two glutamate synthases were also upregulated under these conditions, suggesting that additional characterization is necessary to determine their contribution to root NH_4^+ assimilation in blueberry.

Nitrate reduction to NH_4^+ is a two-step process that is spatially separated in the cell between the cytoplasm and the plastid. Nitrate is initially reduced to nitrite (NO_2^-) in the cytoplasm by nitrate reductase (NR) and requires NADH/NADPH. This reaction is often considered to be rate-limiting for NO_3^- assimilation. Nitrite is subsequently transported to the plastid where it is rapidly reduced to NH_4^+ by nitrite reductase (NiR). Plants can either reduce NO_3^- within roots following its acquisition, or in shoots following its translocation [16,101]. Shoot NO_3^- reduction derives input of reductants and ATP from photosynthesis and may be favorable when light is not limiting [114]. Many herbaceous species perform NO_3^- reduction within shoots following its translocation. In contrast, it was thought that in woody plants much of this occurred in roots [115]. However, following extensive analyses of leaf NR activity in multiple plant taxa, Smirnoff et al. (1984) concluded that woody plant species also display considerable leaf NO_3^- reduction, particularly when families adapted to low NO_3^- soils, including Ericaceae members (*Erica* sp. and *Vaccinium* sp.), were excluded from the analysis. Ericaceae members evaluated in this study displayed very low or undetectable levels of NR activity in the shoots [116]. Consistently, many studies in blueberry have indicated undetectable levels of NR activity in the shoots (leaves) [53,58]. However, several studies have also reported measurable but relatively low leaf NR activity in blueberry [56,107,117]. As such, NR activity and NO_3^- reduction capacity in the shoots (leaves) is lower than that in many other species and may therefore limit the ability of blueberry plants to use NO_3^-, thereby serving as an additional factor contributing to N-source preference [53,58]. Consequently, much of the NO_3^- acquired may be assimilated within roots in blueberry [53]. Consistent with this idea, root NR activity is substantially higher than that in shoots [53,56,58]. Nitrate assimilation is inducible by the substrate, NO_3^-, as NR and NiR activity are up-regulated in the presence of μM concentrations of NO_3^- in plants such as barley (*Hordeum vulgare*) [118–120]. In *V. corymbosum*, root NR activity is detectable with NO_3^- as the N-source but not with NH_4^+ [58]. Similarly, NR activity and *NR* transcript abundance in roots are enhanced with NO_3^- as the N-source [56]. However, increasing root supply of NO_3^- (beyond 0.5 mM) does not increase root NR activity [107,121], suggesting that blueberry root NR activity is saturated at these levels of NO_3^-. Furthermore, continued supply of NO_3^- over several weeks is often associated with an initial increase followed by decline in root NR activity in *V. corymbosum* and *V. virgatum* [53,56,107].

It may be likely that limited shoot NR activity observed in blueberry is associated with limited availability of the substrate. Consistently, direct supply of NO_3^- to shoots through foliar applications of KNO_3 transiently increased leaf NR activity [56]. Additionally, NO_3^-

supply to the cut end of the stem increased *NR* and *NiR* transcript abundance by more than 10-fold and 195-fold, respectively, and resulted in an increase in NR activity [56]. These data indicate that blueberry shoot N assimilation system is responsive to NO_3^- availability and that limitations in root uptake and root-to-shoot translocation of NO_3^- limit its assimilation capacity in the shoots. However, even with such induction, shoot NR activity was substantially lower than that in many other non-Ericaceous plants and that in herbaceous species [58,116]. Together, these data indicate that in addition to limitations in NO_3^- uptake capacity and translocation, limitations in shoot assimilation capacity also contribute to N-source preference in blueberry.

2.5. Nitrogen Storage and Remobilization in Blueberry

In perennial fruit species, N storage and remobilization play important roles in overall N nutrition [122–125]. In addition to N acquired during the current season, N compartmentalized to storage during previous year(s) contributes to new growth and development, particularly in early spring [124]. In deciduous species, such remobilization of N reserves determines early spring growth even when N is available for uptake in the current season [124,126]. Nitrogen storage can occur in multiple organs with roots and stems being significant storage organs [122,124]. Long-term N storage can occur in the form of proteins and/or amino acids [123,126]. The relative significance of each pool varies based on species and extent of N destined for storage [122,127]. For example, N storage in apple (*Malus* ×*domestica* Borkh.) occurs primarily as proteins but can also occur as free amino acids, the proportion of which increases as N availability increases [127].

Sources of N contributing to the storage pool are primarily two-fold: N uptake during the growing season and N resorption from leaves. In the deciduous *V. myrtillus* L., new N taken up later in the season (after cessation of vegetative growth) was allocated mainly to roots and woody stems [128]. In the evergreen *V. vitis-idaea* L., new N taken up during this period was primarily allocated to new leaf growth. These data indicate that in deciduous species N uptake later in the season is largely allocated to storage, a feature also noted in other perennial fruit crops such as nectarine (*Prunus persica* (L.) Batsch var. *nectarina*) [129]. Resorption of nutrients from senescing leaves in deciduous plants contributes to N storage. Generally, around 20–50% of leaf N may be resorbed prior to leaf fall and dormancy [122]. In mature field-grown peach (*Prunus persica*) trees, N resorption from leaves in the fall (assuming 50%) was calculated to represent about 80% of the storage N pool and likely sufficient to support N requirements of early spring growth [130]. In pistachio (*Pistacia vera* L.), a biennial bearing fruit crop, around 30% of leaf N was resorbed, and in the 'On' year contributed to 100% of N in the storage N pool [131]. In blueberry, it is likely that similar resorption may occur and contribute substantially to N nutrition in the spring particularly in field-grown mature plants of deciduous *Vaccinium* species (Figure 1). However, this value has not yet been accurately determined for blueberry.

A few studies have attempted to determine the contribution of remobilization to N nutrition using N budgets and labeled ^{15}N in *Vaccinium* species. Relative contributions of N from remobilization and current supply were assessed in two cultivars of rabbiteye blueberry (*V. virgatum*) that differed in synchrony of vegetative and floral bud break in the spring, by labeling the storage N pool with ^{15}N supplied as NO_3^- [17]. In both cultivars, loss of shoot and root dry weights and storage N was observed before anthesis and during early spring growth indicating that both organs serve as storage units. However, as decline in storage N and protein concentration was greater in roots, they are likely to serve as the primary storage organs supporting early spring growth. Remobilization contributed around 50–80% to the N requirements of early fruit and vegetative growth but contributed lesser at later stages when N requirements were primarily met through current acquisition of new N by roots. The contributions of remobilization and current year N uptake to plant growth in *V. myrtillus* and *V. vitis-idaea* were evaluated by providing ^{15}N labeled NH_4NO_3 during the current year [128]. New leaf and shoot growth, particularly the first flush, was supported by remobilization of N (55–80%). The source organ differed based on the

species: roots and woody stem in deciduous species, and old leaves and green stems in evergreen species. These studies data clearly demonstrate the significance of remobilization in meeting N demands during early spring growth in blueberry.

3. Calcium Physiology in Blueberry

Foliar Ca^{2+} concentration, [Ca^{2+}], in blueberry is typically lower than that in other fruit crops such as apple [8], although, at below 1%, it is still comparable to that noted in other herbaceous plants [7,14,132]. Consequently, blueberry plants and other calcifuges have been described as having higher efficiency of Ca^{2+} uptake and/or use [8,133]. Calcium availability in soil is generally considered sufficient and its deficiency is not often reported in the field, but plant [Ca^{2+}] below 0.2% may lead to deficiency-related issues in production [8,134,135]. Importantly, even with high foliar concentrations, other organs such as fruit may yet display localized Ca^{2+} deficiency [8,133,136]. Such characteristics of Ca^{2+} homeostasis and relative importance for fruit make understanding of its physiology essential for fruit production in blueberry.

3.1. Calcium Acquisition in Blueberry

Calcium is taken up by plants as the divalent cation, Ca^{2+}. Soil factors such as inadequate supply, very low pH, and excess availability of other cations, particularly magnesium (Mg^{2+}), NH_4^+, potassium (K^+) and sodium (Na^+), affect Ca^{2+} uptake and induce its deficiency [137–140]. Calcium moves in the soil to roots via mass flow and subsequently enters the apoplastic space within the root from where it can be acquired by root cells and/or be transported to the xylem. The apparent free space (AFS) in the root wherein Ca^{2+} movement occurs can be divided into the Donnan Free Space (DFS) and the Water Free Space (WFS) [141,142]. In the DFS, negative charges primarily associated with cell wall pectins contribute to the cation exchange capacity (CEC) of the root apoplastic space, and influence movement and distribution of cations such as Ca^{2+} [142,143]. While only a part of the CEC associated with the DFS may be accessible in vivo, it is of substantial and specific importance to Ca^{2+} movement and distribution, owing to its valence and hydrated size [142,143]. Calcium can be cross linked through ionic interactions with carboxylic groups of pectic material such as homogalacturonans, thereby strongly influencing the free apoplastic [Ca^{2+}] and Ca^{2+} transport (Figure 2) [144]. In the WFS, lack of such interactions allows for less restricted cation movement including that of Ca^{2+} [142].

In addition to its role as a macronutrient, Ca^{2+} performs a vital function as a second messenger in plant cells. In this role, it aids in regulating growth and development, relaying external biotic or abiotic stress signals, and eliciting cellular responses [133,145]. This role is manifested as an increase in cytosolic Ca^{2+} concentration, $[Ca^{2+}]_{cyt}$, the amplitude and frequency patterns of which serve as a 'Ca signature' to relay specificity of the message [145]. This function of Ca^{2+} requires steady state $[Ca^{2+}]_{cyt}$ in un-elicited cells to be in the sub-micromolar range (~0.1 µM; Figure 2) [143,146]. Additionally, at higher $[Ca^{2+}]_{cyt}$, Ca^{2+} can precipitate with phosphates. The required balance between dual functions of Ca^{2+} greatly influences its uptake and transport within roots.

Calcium uptake by root cells is electrochemically favorable, due in part to low $[Ca^{2+}]_{cyt}$, and is facilitated by Ca^{2+}-permeable channels [147,148]. These include CYCLIC NUCLEOTIDE GATED CHANNELS (CNGCs), GLUTAMATE RECEPTOR LIKE PROTEINS (GPRs), ANNEXINS, TWO-PORE CHANNELS (TPCs) and MECHANOSENSITIVE-LIKE Ca^{2+}-permeable channels [147–149]. Following its acquisition by cells, Ca^{2+} is rapidly compartmentalized into the vacuole, the lumen of the endoplasmic reticulum, into other organelles, or effluxed into the apoplast to ensure that its function as a second messenger is not compromised [150]. This is mediated against the electro-chemical gradient by Ca^{2+}-ATPases (CALCIUM TRANSPORTING ATPases; ACAs) that pump cytosolic Ca^{2+} into various compartments (or efflux it) and display high-affinity but low-capacity transport, or by Ca^{2+}/H^+-antiporters (exchangers) that facilitate such transport using the proton motive force (PMF) and display low affinity and high-capacity transport [149].

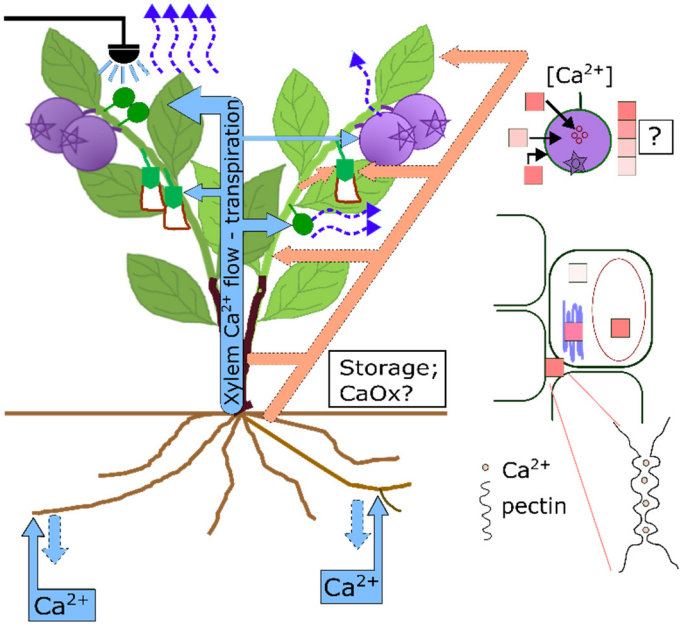

Figure 2. Calcium (Ca^{2+}) homeostasis in blueberry. Calcium is acquired primarily along root tips or regions where apoplastic barriers are interrupted (emerging lateral roots). Efflux of Ca^{2+} may contribute significantly to its homeostasis. Translocation of acquired Ca^{2+} from roots to shoots occurs in the xylem and may be largely driven by transpiration. Wavy blue arrows above organs indicate transpiration. Organs with higher transpiration rates (indicated with more arrows), such as leaves, may have greater xylem supply of Ca^{2+} compared to fruit (indicated with fewer arrows). Blue boxes and arrows indicate transport of Ca^{2+} acquired from roots via the xylem. Remobilization of Ca^{2+} via phloem is likely minimal. Calcium sources and forms (CaOx: Ca oxalate) involved in supporting new growth such as that of the leaves, flowers and new shoots in the spring are not clear. Here, orange boxes and arrows with dashed outline indicate potential remobilization-related movement of Ca^{2+} via the xylem. The dashed outline on boxes and arrows indicates putative processes for which evidence is not yet available in blueberry. Foliar applications may serve as an additional source of Ca^{2+} and may be more effective in increasing fruit [Ca^{2+}] when applied during early fruit development. Top right inset demonstrates spatial [Ca^{2+}] in the fruit (seeds, pulp and peel). Higher intensity of color in box represents higher relative [Ca^{2+}]. A putative proximal to distal gradient in [Ca^{2+}] is often seen in fruits but remains to be evaluated in blueberry. The bottom right inset depicts [Ca^{2+}] in cellular compartments (cytosol, vacuole and endoplasmic reticulum) and the cell wall space. Within the cell wall, Ca^{2+} mainly binds to pectic polymers and may aid in regulating cell wall properties. Higher intensity of color in the box indicates higher [Ca^{2+}].

Radial transport of Ca^{2+} in roots toward the xylem can potentially follow symplastic or apoplastic routes [146]. A largely symplastic route is likely countered by the critical necessity to maintain sub-micromolar $[Ca^{2+}]_{cyt}$ to support its role as a second messenger. Symplastic transport may still occur in regions where significant barriers for apoplastic transport, such as the Casparian band, exist. In regions of the root where the Casparian band is well developed, Ca^{2+} may enter endodermal cells, likely through Ca^{2+}-permeable channels [143,146,148,151]. Subsequently, Ca^{2+} may exit into the xylem apoplast through efflux by Ca^{2+}-ATPases and Ca^{2+}/H^{+}-antiporters [133,151]. In regions of roots where the Casparian band is not sufficiently developed, the pathway is largely apoplastic [146]. Consequently, substantial Ca^{2+} uptake occurs from apical regions of the root where the Casparian band is not yet well developed, or other regions where apoplastic barriers are interrupted (Figure 2) [133,146]. When suberin (a key component of the Casparian band) accumulation in roots is enhanced in an arabidopsis mutant, *esb1*, Ca^{2+} content in shoots declines, supporting the concept of a largely apoplastic route for Ca^{2+} flow in roots [152].

However, at least in some plants such as onion (*Allium cepa*), symplastic routes may yet play important roles in radial root Ca^{2+} transport [153].

A survey of the root transcriptome data indicated differential expression of multiple transcripts coding for Ca^{2+} transporters in *V. corymbosum* and *V. arboreum* in response to changes in pH [33]. In *V. corymbosum*, increasing media pH from 4.5 to 6.5 resulted in a consistent decline in transcript abundance of approximately eight *Ca^{2+}-TRANSPORTING ATPases (ACAs)*. The proteins coded by many of these are potentially localized to the plasma-membrane (based on homology to arabidopsis ACA8, 9 and 10 proteins). Consistently higher expression of multiple putatively PM-localized *ACAs* at lower pH (4.5) suggests that under these conditions *V. corymbosum* root cells actively efflux Ca^{2+}. Some of the differentially expressed *ACAs* code for proteins potentially localized to the ER (*ECAs*), suggesting increased compartmentation of Ca^{2+} in root cells at low pH. Potentially, higher influx of Ca^{2+} into root cells at lower external pH requires such efflux or compartmentalization to maintain $[Ca^{2+}]_{cyt}$. Furthermore, if these ACAs are localized to the endodermis, these results imply enhanced xylem-loading of Ca^{2+} under low pH conditions. Growth of *V. corymbosum* at higher pH (6.5) was associated with highly reduced abundance of three *ALIPHATIC SUBERIN FERULOYL TRANSFERASE* (*ASFT*) transcripts and five *CYTOCHROME P450 A86* (*CYP86*) transcripts associated with suberin biosynthesis [33,154,155]. To some extent, this was also noted in *V. arboreum* (for *ASFTs*). Together these data suggest greater apoplastic barriers to solute movement in blueberry at low pH (4.5). Under such conditions, it may be inferred that part of the radial Ca^{2+} transport to the xylem is symplastic and involves influx into endodermal cells through Ca^{2+}-permeable channels and exit through Ca^{2+}-ATPases or Ca^{2+}/H^{+} antiporters. The relatively higher expression of several *ACAs* indicated above supports such a mechanism at low pH. It must be emphasized that these interpretations are preliminary considering that they are-based solely on transcript abundance. Further analysis of physiological and molecular mechanisms of Ca^{2+} transport and root uptake are clearly essential to better understand Ca^{2+} acquisition by blueberry roots.

3.2. Calcium Translocation in Blueberry

Supply of Ca^{2+} to organs via phloem is minimal at best, indicating that its internal distribution is predominantly xylem dependent [134,143]. Once Ca^{2+} is loaded into the root xylem, it follows an apoplastic route for transport to shoot, leaves and fruit. This flow is often dependent on the transpiration rate, particularly in organs with higher rates of transpiration [133]. Consequently, higher transpiration rates in leaves result in xylem flow and Ca^{2+} allocation to these organs rather than the fruit (Figure 2) [136,156,157]. In lettuce (*Lactuca sativa*), increasing transpiration rates by blowing air directly at the meristem increased Ca^{2+} supply to this region and prevented development of deficiency symptoms [158]. Additionally, treatments that affect transpiration, such as vapor pressure deficit (VPD) reduction, increased humidity, ABA treatments, and application of foliar anti-transpirants reduce symptoms of Ca^{2+} deficiency in organs/regions with limited xylem transport of Ca^{2+} [140,157,159–161]. In addition to transpiration, Ca^{2+} movement in the xylem is influenced by CEC of xylem cell walls. Calcium movement within the xylem may involve adsorption and desorption from these exchange sites, potentially reducing the extent of free xylem $[Ca^{2+}]$, and at least partially uncoupling its transport from bulk flow in the tissue [134,162]. There is increasing evidence of transpiration-independent mechanisms, such as root pressure, in determining Ca^{2+} transport to shoot organs including the fruit [163–165]. Additionally, interaction between polar auxin transport and Ca^{2+} transport may influence Ca^{2+} delivery into organs, independent of transpiration [166].

3.2.1. Calcium Transport to the Fruit and Its Distribution

A key structural function of Ca^{2+} in plants is maintenance of membrane integrity and cell wall strength [143,144,167]. Calcium can cross-link cell wall polysaccharides such as pectins and influence cell wall extensibility (Figure 2) [144]. Excess Ca^{2+} can increase

rigidity of the cell wall and limit expansion while low Ca^{2+} can lead to weakened cell walls and membrane breakdown [144,167]. This in turn influences growth, firmness, and susceptibility to plant pathogens. Consequently, multiple fruit disorders are associated with sub-optimal [Ca^{2+}] or Ca^{2+} distribution in fruits [162,167,168]. In many fruits, localized Ca^{2+} deficiency symptoms appear within a few weeks after anthesis, mainly at the distal end of the organ [167,168].

The rate of fruit growth appears to be inversely correlated with Ca^{2+} content and with incidence of deficiency symptoms [169]. This may occur owing to an increase in supply of photo-assimilates without concomitant increase in xylem transport of Ca^{2+}. Similarly, correlations between fruit size and Ca^{2+} deficiency symptom development have been observed in other fruits such as apple [169–172]. Phytohormones may also influence growth and Ca^{2+} deficiency symptom development. Often, higher gibberellin (GA) levels in organs are correlated with higher growth and lower [Ca^{2+}], leading to deficiency symptoms [162]. Consistently, GA applications increase deficiency symptoms, while GA biosynthesis inhibitors increase pericarp [Ca^{2+}] and decrease symptoms in tomato. Treatments with GA increase expression of *Ca^{2+}-ATPases* that presumably localize Ca^{2+} to organelles, thereby reducing the $[Ca^{2+}]_{apo}$ required to stabilize cell walls and membranes. Auxin transport inhibitor treatments also decrease Ca^{2+} in apple and tomato fruits and increase deficiency symptoms [134,162]. Relationships among rate of fruit growth, phytohormone signaling and fruit [Ca^{2+}] have not yet been explored in blueberry.

In most fruits, majority of fruit Ca^{2+} is translocated into the organ within a few weeks after anthesis [162,164,173]. For example, in melons (*Cucumis melo* L.), 80% of the fruit Ca^{2+} accumulates during early fruit development [174]. In *V. corymbosum*, fruit [Ca^{2+}] declines steadily due to a dilution effect during fruit development [175]. Calcium content per fruit increases during early fruit development but fruit Ca^{2+} intake declines during mid-fruit development and is negligible at later stages in multiple *V. corymbosum* cultivars [176]. Thus, like in many other fruits, bulk of Ca^{2+} entry into blueberry fruit appears to occur during early development.

In blueberry, fruit stomatal conductance is 3-fold lower during later stages compared to that at earlier stages of fruit development (petal fall). This is associated with a decline in stomatal density and localization primarily to the distal end of the fruit. Furthermore, at later stages of fruit development, stomates are largely covered with cuticular waxes, contributing to temporal decline in stomatal conductance. Higher stomatal density and conductance observed during early fruit development are still approximately 5-fold and 2-fold lower, respectively, than in leaves at the same period [176]. These data support a role for low transpiration in reducing Ca^{2+} intake into fruit during blueberry fruit growth.

Distribution of Ca^{2+} within fruit is a significant factor affecting development of related disorders such as blossom-end-rot in tomato [167,168]. Fruit pedicel and tissues internal to fruit such as seeds or placenta influence Ca^{2+} delivery to other tissues such as the pericarp [177,178]. The majority of apoplastic Ca^{2+} is bound to the cell wall, and a decrease in this fraction can result in excessive cell enlargement leading to deficiency-related disorders [167]. Calcium localized to the apoplast also influences membrane integrity. In transgenic tomato with down-regulated pectin methylesterase activity, a decrease in cell wall-bound, and an increase in free apoplastic Ca^{2+} fractions were associated with reduced membrane leakage, likely due to increased association of free Ca^{2+} with the membrane [168]. In blueberry, higher [Ca^{2+}] was noted in the peel and seed tissues, while the lowest was observed in the pulp (Figure 2) [176]. Owing to relatively greater dry matter associated with the pulp, the highest Ca^{2+} content was noted in this tissue. Higher resolution information on Ca^{2+} distribution in blueberry fruit is currently lacking. Such information, particularly during progression of fruit growth is needed to better understand its roles in fruit development.

Xylem distribution and functionality in the fruit can influence internal distribution of Ca^{2+} leading to localized deficiencies and related fruit disorders [167,168]. Lower density of xylem is observed in distal parts of tomato fruit, particularly the placental tissue, and

may contribute to development of deficiency symptoms in this region [179]. In many fruits, xylem functionality is highest during early fruit development and progressively decreases during later stages, potentially owing to growth-related damage of xylem vessels, among other factors [180,181]. Analyses across eight fruit species indicated that decrease in xylem conductance and functionality at later stages of fruit development is a conserved phenomenon [178]. Entry of water and most nutrients into fruit at later stages is consequently supported through phloem transport [162,182]. Considering the relative immobility of Ca^{2+} in the phloem, it is likely that little, if any, Ca^{2+} is delivered to the fruit through this route. In addition, phytohormones such as auxin, abscisic acid (ABA) and gibberillins (GA) affect vascular development, and thereby uptake and distribution of Ca^{2+}. In tomato fruit GA biosynthesis inhibitor treatments increased the extent of functional xylem [168]. Additionally, application of ABA increased functional xylem vessel number, flow rate of xylem sap, and Ca^{2+} transport to fruit, thereby reducing deficiency symptom development in tomato [161]. Information on xylem distribution and functionality, and associated changes during development of the blueberry fruit is currently lacking. Such information, along with an understanding of its responses to plant growth regulator applications, is essential to better comprehending Ca^{2+} transport and accumulation in blueberry.

3.2.2. Approaches to Improve Fruit [Ca^{2+}]

An important potential benefit of higher Ca^{2+} in fruit is enhanced firmness, especially due to Ca-pectin interactions that may limit pectin depolymerization and hydrolysis, which may in turn contribute to extended postharvest storability (Figure 2). Thus, a focus of Ca^{2+} nutrition in blueberry has been on applications to improve postharvest fruit quality. Multiple efforts have been made in blueberry to achieve the goal of increasing fruit [Ca^{2+}] but have often yielded inconsistent results (Table 3). Effects of soil applications of $CaSO_4$ on fruit quality and biochemical attributes were determined in 'O'Neal' and 'Bluecrop' [183]. Applications made in the previous fall were associated with decreased softening and postharvest fruit weight loss, more evidently at 21 d after cold storage than immediately after harvest. Cell wall associated fruit Ca^{2+} content increased by 10% at harvest in both cultivars. While hemicellulose components of the cell wall did not change, tightly bound pectins increased and loosely bound pectins decreased suggesting lower pectin solubilization, thus affecting cell wall disassembly. These data led the authors to conclude that soil Ca^{2+} applications can increase fruit shelf life by decreasing postharvest water loss and softening. However, in a multi-year study, soil applied lime or gypsum resulted in inconsistent effects on fruit characteristics [184]. Both treatments increased soil pH, and leaf [Ca^{2+}] after three years of application, but higher fruit [Ca^{2+}] was noted only in one year. The treatments did not consistently affect concentrations of other elements, yield, fruit firmness during postharvest storage, or incidence of common pathogens.

Alternatively, foliar applications of several Ca^{2+} formulations have been evaluated. In a two-year study $CaCl_2$ was sprayed multiple times between petal fall and harvest in *V. corymbosum* 'Bluecrop' (Table 3) [185]. None of the treatments affected fruit [Ca^{2+}], fruit firmness or percentage of sound fruit, including after storage, but increased leaf [Ca^{2+}] on a dry weight basis. Multiple formulations of Ca^{2+} and its combination with Boron (B) were evaluated as foliar applications, beginning from early fruit development (green fruit) in four *V. corymbosum* cultivars [186]. None of the treatments affected fruit [Ca^{2+}], firmness, or postharvest storage (up to 20 d after harvest). Similarly, pre-harvest foliar applications of Ca^{2+} either as $Ca(NO_3)_2$ or chelated-Ca, multiple times during 'O'Neal' fruit development did not increase fruit [Ca^{2+}], or firmness during postharvest storage for up to 28 d after harvest [187]. Pre-harvest applications (30 and 15 d before harvest) of various Ca^{2+} formulations had inconsistent effects on fruit firmness and weight in two rabbiteye blueberry cultivars over two years [188]. For example, $CaCO_3$ increased firmness at harvest in 'Powderblue' in one year by 5% but decreased it in the following year by over 15%. Calcium applications performed prior to fruit set did not affect fruit [Ca^{2+}], fruit set or firmness at later stages [189]. Additionally, in related species such as *V. macrocarpon*, foliar

Ca^{2+} applications did not improve fruit [Ca^{2+}] or firmness [190]. Increasing application rates alone may not be a solution to increasing tissue [Ca^{2+}] with pre-harvest applications, at least with $CaCl_2$, as it results in a linear increase in leaf injury [185]. A potential factor affecting efficacy of foliar Ca^{2+} applications may be the timing of application in relation to fruit development. Foliar applied Ca^{2+} is likely to enter fruit tissue through stomates, via the cuticle or through cracks in the cuticle. As indicated earlier, stomatal density and conductance decline sharply with progression of fruit development, while the extent of fruit cuticle increases, thereby limiting penetration of foliar Ca^{2+} at later stages [176,191]. In this context, applications performed during the early stages and using suitable surfactants may improve Ca^{2+} penetration. Consistently, early (around fruit set) foliar applications of Ca^{2+} to 'Liberty' blueberry enhanced fruit [Ca^{2+}] and increased fruit firmness in comparison to control and late applications [192]. Furthermore, these applications decreased membrane damage as determined by membrane lipid peroxidation, and enhanced antioxidant response through increased superoxide dismutase and radical species scavenging activities. Additionally, inclusion of non-ionic surfactants may contribute to higher [Ca^{2+}] in treated fruit [193]. Together, these data suggest that fine-tuning foliar Ca^{2+} applications by enhancing fruit physiological accessibility can allow for enhanced fruit [Ca^{2+}] and quality responses in blueberry. Additional work is needed to evaluate such possibilities.

Table 3. Summary of approaches for altering fruit calcium concentration [Ca^{2+}] in blueberry (*Vaccinium* Sp.).

Type of Application	Application Details	Concentration of Applied Ca	Leaf [Ca] and Treatment Effect	Fruit [Ca] and Treatment Effect	Source
Soil	Calcitic lime; $CaSO_4$; *V. corymbosum*;	1100 and 550 kg ha^{-1} per year; four-year applications	0.2–0.45%; increased during later years	0.03–0.07%; inconsistent increase	184
	$CaSO_4$; previous season application; *V. corymbosum*	600 kg ha^{-1}	NA	Cell wall [Ca^{2+}] increased by >10%; firmness increased	183
Foliar	$CaCl_2$; Nutrical; *V. corymbosum*	1–24.2 kg ha^{-1};	0.25–0.44%; increased at higher rates	0.03–0.04%; NS	185
	$CaCl_2$; Ca silicate; Ca chelate; Ca acetate; *V. corymbosum*	0.34–0.67 kg ha^{-1}	0.6–1.8%; NS	0.02–0.06%; NS	186
	$Ca(NO_3)_2$; chelate Ca-oxide; *V. corymbosum*	0.36–0.78 kg ha^{-1}; applied four times during fruit development	0.64%	0.11% (at harvest); NS; firmness increased but inconsistent	187
	$Ca(NO_3)_2$; neutralized CaCo3; chelated Ca; *V. virgatum*	0. 65 kg ha^{-1}; 0.1 kg ha^{-1}; 0.56 kg ha^{-1}; applied twice	Inconsistent change (18% increase and 26% decrease)	NS; inconsistent change in firmness	188
	$CaCl_2$ and $CaSO_4$; *V. corymbosum*	750–1500 ppm; 150 ppm, respectively; applied six times during fruit development	0.6–0.9%; NS	0.04–0.06%; NS	189
	$CaCl_2$; Ca phosphite; Ca thiosulfate solution; *V. corymbosum*	0.63 kg ha^{-1}; 0.2 kg ha^{-1}; and 0.42 kg ha^{-1}; up to 2.5 kg ha^{-1} and up to 3 times	0.57–0.75%	negative correlation with fruit drop; 0.11–0.19% in early fruit and 0.04–0.06% in ripe fruit; increased with high rates	193
	$CaCl_2$; *V. corymbosum*	0.4–0.8 kg ha^{-1}		Increase in firmness with increasing [Ca^{2+}]	192
Postharvest dip	$CaCl_2$ immersion; *V. corymbosum*	0–4%	NA	Linear increase in fruit firmness with increasing [Ca^{2+}] in dip; objectionable taste	194

Postharvest applications of Ca^{2+} ($CaCl_2$) have been evaluated to determine their effects on fruit quality (Table 3) [194]. A positive correlation between Ca^{2+} application rate, and fruit texture and undamaged berries was noted, indicating that a concentration of 2–4% of $CaCl_2$ results in reduction of physical injury to fruit. However, at these application rates, fruit retained residue on the surface and taste panelists rated the fruit as salty [194]. Potentially, if fruits are washed immediately after treatment, residue and taste effects may be minimized, but this may occur at the expense of treatment efficacy.

Specific disorders associated with Ca^{2+} deficiency have not yet been clearly defined in blueberry fruit. Green fruit drop (GFD) is potentially one such disorder associated with Ca^{2+} deficiency. It has been noted in *V. corymbosum* 'Draper' in British Columbia, Canada, and in Washington, USA, and has been compared to bitter pit as excised fruit show interior browning while external tissues appear normal [193]. Approximately 5–50% of the crop can be lost to this physiological disorder which manifests immediately prior to ripening-associated change in fruit color. A negative correlation between [Ca^{2+}] in ripe fruit and GFD was observed. Furthermore, higher incidence of GFD was associated with enhanced plant vigor owing to greater N availability. As noted previously, greater shoot vigor may limit Ca^{2+} availability/flux to low transpiring organs such as the fruit. Foliar Ca^{2+} applications, particularly as $CaCl_2$ with a surfactant applied several times during early stages of fruit development, were effective in limiting GFD in 'Draper' (Table 3).

3.3. Calcium Storage and Remobilization in Blueberry

The function of Ca^{2+} as a second messenger requires that Ca^{2+} in the cytoplasm is rapidly compartmentalized into cell organelles from where it can be released back to the cytosol in response to a stimulus [145,195]. The role of compartmentalized Ca^{2+} as a functional reserve may be limited considering that it has limited phloem mobility. In fact, Ca^{2+} in the vacuole may have very limited remobilization capacity [143]. The majority of Ca^{2+} in tissues may be present in the bound fraction of the cell wall and the extent to which it functions as a re-mobilizable reserve is not clear. However, a few cases of Ca^{2+} remobilization have been described. During senescence, Ca^{2+} from older leaves was remobilized in barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*), and substantial remobilization of Ca^{2+} from root to shoot occurred in *Brassica napus* subjected to Ca^{2+} deficiency [196]. Additionally, in response to extreme stress, Ca^{2+} remobilized from older leaves [197]. It should be noted that in all such cases, remobilization likely occurred primarily via the xylem [198].

Calcium storage and remobilization potentials, and their forms are relatively unknown in blueberry and warrant investigations. As much of early spring growth is supported by reserves from previous year(s), it is important to determine if Ca^{2+} requirements during this period are met through remobilization via the xylem. This may be particularly important for two reasons: 1. Maximizing Ca^{2+} entry during early growth is essential as it is the primary period for its accumulation in the developing fruit [191]; and 2. Lack of sufficient foliage immediately following floral budbreak may limit transpiration, thereby limiting Ca^{2+} entry into shoot organs through new uptake. In the absence of new uptake, three potential sources may be speculated to support Ca^{2+} requirements of early growth: buds, woody stems, and roots (Figure 2). If Ca^{2+} stored in buds is the primary source, a sharp reduction in [Ca^{2+}] may be expected during early growth following bud-break, but this has not yet been determined in blueberry. Highly localized remobilization of Ca^{2+} from woody stems may support early growth. Limited and localized remobilization via the xylem has been suggested previously as a potential mechanism of Ca^{2+} translocation in plants [162,198]. Alternatively, Ca^{2+} stored in roots may be remobilized to new shoots during early spring growth. Relative contributions of remobilization from the above Ca^{2+} sources to support early growth prior to new soil Ca^{2+} uptake clearly warrants further investigation in blueberry. Evidence for such Ca^{2+} remobilization-based support of early growth has been documented in other fruit species such as apple [162].

Calcium accumulation is frequently observed in plants as Ca-oxalate (CaOx) crystals. These crystals generally accumulate within vacuoles of specialized cells called idioblasts, but can also occur in other regions [199]. Accumulation of CaOx may aid in regulation of Ca^{2+} homeostasis, protection against herbivory, and potentially as a mechanism of elimination of excess Ca^{2+} [198,199]. In many plants, increase in Ca^{2+} availability results in higher CaOx levels. Calcium appears to accumulate to a very large extent as CaOx crystals in leaves and roots of fruit species such as pecan, peach and grape (*Vitis* sp.) [200–202]. It remains to be determined if and where Ca^{2+} accumulates as CaOx in blueberry. Particularly, it would be important to determine if blueberry can accumulate CaOx under conditions of excess Ca^{2+} availability (Figure 2). Blueberries appear to produce oxalic acid (Nambeesan and others, unpublished results). The role of CaOx as a storage form that can be remobilized is debated. Although Ca^{2+} can become bioavailable from CaOx, particularly when in the druse (crystalline aggregation) crystal form, the extent of remobilization is spatially limited [198,203]. Hence, even if CaOx accumulation occurs in blueberry, further investigations will be necessary to determine if it is a source for Ca^{2+} remobilization.

4. Conclusions

Blueberry plants acquire and use multiple sources of N. However, the significance and capacity for use of certain forms such as organic N needs further evaluation in typical cultivation conditions. Blueberry also appears to display N-source preference for the inorganic N-form, NH_4^+. The physiological basis for such preference may be related to its acquisition, translocation and assimilation. Further research is essential to elucidate mechanisms associated with inorganic N-source acquisition, its translocation and assimilation in blueberry. Specifically, identification of NH_4^+ and NO_3^- transporters and their characterization at low and high N availability, and in different parts of the root, is required to better understand the physiology of its acquisition and transport. Furthermore, forms of N translocated in the plant and their assimilation in response to varying N availability need to be determined at various stages of the annual cycle. An initial approach to address this could involve analyses of changes in the root transcriptome in response to different sources of inorganic N, and at varying N availability. An additional area that warrants further research regards N storage and remobilization. Primary organs of storage, forms, and relative proportions of storage N derived from various pools need to be clearly determined in blueberry.

Calcium uptake by plant roots, its transport to the xylem and subsequently to the economically significant organ, fruit, remain to be elucidated in blueberry. Although Ca-related blueberry fruit disorders have not yet been unequivocally identified, it remains likely that Ca^{2+} plays important roles in regulating fruit quality at harvest and during postharvest storage. Hence, it is important to better understand Ca^{2+} entry and distribution in the fruit. In this context, it will be important to determine storage sources and forms that supply Ca^{2+} to the fruit during early development, prior to emergence of significant foliage. Furthermore, roles of fruit transpiration and xylem functionality in determining fruit Ca^{2+} entry and distribution need to be evaluated. Additionally, identification and characterization of various transporters involved in regulation of fruit Ca^{2+} uptake can greatly enhance our understanding of its physiology in the fruit.

Information gained through research in the above areas can aid in understanding N and Ca nutrition in blueberry. Importantly, decisions regarding N and Ca nutrient management in blueberry production can be greatly informed by knowledge gained from such research. This is particularly important to sustain current trends in blueberry production and to ensure its profitability.

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