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"GYPSUM EFFECTS ON PHOTOSYNTHETIC PERFORMANCE, METABOLITES, AND MORPHOLOGICAL FEATURES IN *VACCINIUM CORYMBOSUM* L. CULTIVARS UNDER ALUMINUM TOXICITY IN AN ANDISOL."

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"GYPSUM EFFECTS ON PHOTOSYNTHETIC PERFORMANCE, METABOLITES, AND MORPHOLOGICAL FEATURES IN *VACCINIUM CORYMBOSUM* L. CULTIVARS UNDER ALUMINUM TOXICITY IN AN ANDISOL."

Esta tesis fue realizada bajo la supervisión dela Dra. Marjorie Reyes Díaz, perteneciente al Departamento de Ciencias Químicas y Recursos Naturales de la Universidad de La Frontera y es presentada para su revisión por los miembros de la comisión examinadora.

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Summary of this thesis

Few studies about the relationships between sulfate, a plant nutrient, and aluminum, a toxic ion, are available; hence, the molecular and physiological processes underpinning this interaction are poorly understood. The relatively high concentrations of trivalent toxic aluminum (Al^{3+}) occur in acidic soils, which may hamper root growth, limiting uptake of nutrients, including sulfur (S). Also, in these soils, Al-S interaction is present. On the other side, Al^{3+} may be detoxified by complexation with sulfate in the acid soil solution as well as in the root-cell vacuoles. Therefore, the hypothesis of this Thesis was: *The application of calcium sulfate ($CaSO_4$) on acid soil with toxic Al increases photosynthetic performance, metabolic responses, and modifies leaf anatomy features in highbush blueberry cultivars (V. corymbosum) due to increment of S and Ca in leaves and soil solution.*

This hypothesis was evaluated through the following objectives: (I) To analyze the effect of gypsum application on chemical properties and morphological features on leaves of blueberry cultivars under aid soil and Al toxicity; (II) To evaluate the effect of gypsum application on the photosynthetic performance in blueberry cultivars growing under acid soil and Al toxicity; (III) To evaluate the effect of gypsum application on the total metabolites and metabolic profile in blueberry cultivars growing in acid soil and presence of toxic Al. To answer these goals three studies compiled in chapters were performed: (I) Recent insights into the mechanisms governing plant responses to Al toxicity and its relationship with sulfur nutrition, emphasizing the role of phytohormones, microRNAs, and ion transporters in higher plants (Chapter II); (II) Gypsum amendment improves leaf morphology alterations as well as photochemical and biochemical damages in highbush blueberry under Al-toxicity, being cultivar-dependent. The morphological parameters can be important traits as Al-resistance anatomical markers in highbush blueberry (Chapter III), and metabolite profiling of blueberry cultivars under Al^{3+} toxicity and gypsum application, where gypsum amendment ameliorates the harmful effect of Al^{3+}toxicity, mainly in the Al-sensitive cultivar (Bluegold). In the Al-resistant cultivars (Legacy and Brigitta), two distinct mechanisms (tolerance and exclusion, respectively) to cope with toxic-Al were found. In addition, the results indicated that in roots gamma-aminobutyric acid (GABA) might be related to the responses under toxic Al^{3+} (Chapter IV).

Finally, in Chapter 5 we present the General discussion and concluding remarks of the thesis, concluding that gypsum application decreased Al^{3+} toxicity, mainly in the Al-sensitive cultivar (Bluegold).

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CHAPTER I

General Introduction

1.1 General Introduction

In acid soils (pH<5.5inwater), aluminum(Al) is released into the soil solution in the form of cations[$Al(OH)_2^+$, $AlOH^{2+}$ and Al^{3+}, in order of increasing abundance with decreasing pH],with Al^{3+} being highly toxic to most plants (Rengel 1992; 1996; Mora et al. 2006). Thus, Al toxicity is one of the most important negative factors constraining crop performance in acid soils (Barceló and Poschenrieder 2002; Saracoglu et al. 2009). In South Central Chile, it is frequent to find acid soils derived from volcanic ash (Andisols). These soils are also characterized by the presence of high concentrations of soluble Al cations, particularly (Al^{3+}), which are extremely toxic to many plants (Rengel 1992; 1996; Borie and Rubio 2003; Mora et al. 2004; Mora et al. 2006).Aluminum can be affecting chemical and physiological features as well as metabolic and molecular path ways of plants. It is known, that the first symptom of Al^{3+} injury in plants is root growth reduction (Delhaize et al. 2012; Ryan et al. 2011), due to the Al interaction with the apoplastic side in the cell wall, plasma membrane, and cytoskeleton (Horst et al. 1999). This disturb water and essential nutrients uptake such as calcium (Ca), magnesium (Mg), potassium (K), manganese (Mn), and sulfur (S) (Foy Guo et al. 2003; Mora et al. 2005a; Wangand Shen 2006), because to the competition between Ca^{2+} and Al^{3+} cations (Ryan and Kochian 1993; Kinraide 1998). Also, Al^{3+}can produce negative effects on photosynthetic capacity, mainly due to decreases in photosynthetic pigments, fluorescence parameters of photosystem II (PSII), reduced stomatal conductance (Kochian et al. 2005; Chen et al. 2005). In Citrus, net photosynthesis, non-photochemical quenching (NPQ), photochemical quenching (qP), effective quantum yield of PSII and maximum quantum yield of PSII (Fv/Fm) decreased when subjected to toxic Al (Chen et al. 2005). At cellular levels, toxic Al provoked a high production of reactive oxygen species (ROS), leading, to oxidative damage, in proteins an lipids of cell plasma membranes resulting in lipid peroxidation (LP),,and ultimately cell death (Panda et al. 2009; Huang et al. 2014). For that, the oxidative injury can be measured as LP, as reported in plant species, such as *Pisum sativum* and *V. corymbosum* (Yamamoto et al. 2001; Inostroza-Blancheteau 2011; Reyes-Díaz et al. 2011), where mainly in the Al-sensitive cultivars, increments

in the antioxidant activities in response to Al-stress were found. Nonetheless, plants may resist Al^{3+} by avoidance (Al exclusion) and/or tolerance mechanisms (detoxification of Al inside the cells). The Al exclusion involves the exudation of organic acid from the root, whereas tolerance mechanisms comprise internal Al detoxification by organic acid and enhanced scavenging of free oxygen radicals in cells (Inostroza-Blancheteau et al. 2012). At the molecular levels it is regulated by several genes such as a malate transporter encoded by the gene denominated *TaALMT1* (*Triticum aestivum aluminum-activated malate transporter 1*), *HvAACT1* that encodes a membrane protein HvAACT1, responsible for citrate exudation and a family of multidrug and toxic compound extrusion (MATE) genes, *Hydrangea macrophylla*, plasma membrane Al-transporter 1 (*HmPALT1*) and Vacuolar Al-transporter (*HmVALT*) (Inostroza-Blancheteau et al. 2012; Alarcon-Poblete et al. 2018). These last two transporters belong to the aquaporin family that allowed the Al transport in plants (Alarcón-Poblete et al. 2018). Otherwise, Al affects metabolic processes like synthesis of amino acid and proteins, which is related to different changes in cytoplasmic and membrane proteins. Furthermore, phytotoxic Al can affect primary metabolites, such as amino acid, organic acid and carbohydrate metabolisms in plants (Khan et al. 2000; Lin et al. 2005; Cheng etal. 2010). In this concern, Cárcamo et al. (2019) reported thatamino acid concentration increased significantly in highbush blueberry (*V. corymbosum*) cv. Camellia and decreased in cv. Duke under Al stress. In addition, studies in *Lens culinaris* and *Zea mays* showed that total amino acids and sugars increased in shoots and roots under Al treatments (Azmat et al. 2007; Khan et al. 2008).On the other hand, at the anatomical level it is reported that Al^{3+} reduced the size and thickness of leaf blades due to a decrease in cell size, as well as an increase in the number of stomata from the abaxial epidermis, with a simultaneous reduction of their size in *Capsicum annuum* L. (Konarska 2010). In root tips, microscopic analyses in *V. corymbosum* plants suggested a higher degree of Al-induced morphological injury in Al-sensitive,Bluegold compared to Al-resistant, Brigitta (Inostroza-Blancheteau et al. 2011). In this sense, toxic Al in acid soils is treated by calcareous amendments for it reduction using lime, phosphogypsum and gypsum (Mora et al. 2002; Meriño-Gergichevich et al. 2010; Alarcón-Poblete et al. 2019). It has been demonstrated that gypsum amendments in Andisol pasture,

improves substantially plant growth in soils with high Al concentration (Mora et al. 2002). Positive effects have been reported with this amendment application, which is associated with Al^{3+} precipitation by lime and Al^{3+} complexation by gypsum, decreasing the levels of free toxic Al as a consequence of its toxic effects reduction for plants (van Raij 2008; Tirado-Corbalá et al. 2017). Also, gypsum promotes growth of root system in some plants improving their nutrient availability and water uptake (Ritchey et al. 1995; Al-Oudat et al. 1998;Vyshpolsky et al. 2010; Chi et al. 2012; Batool et al. 2015; Alarcón-Poblete et al. 2019; Mckenna et al. 2019).

In southern Chile, the highbush blueberry (*V. corymbosum*) is an important fruit crop, because to the fruit richness in nutritional and antioxidants properties for human health, together the high market price of its fruits,(Guerrero 2006; Balliga and Katiyar 2006). Therefore, it is highly demanded as healthy food (Prodorutti et al. 2007).This crop is well adapted to acid soils, but it is sensitive to the presence of Al-toxicity, which promotes biochemical and molecular changes (Reyes-Díaz et al. 2010;Inostroza-Blancheteau et al. 2011), decreasing its productivity substantially (Blatt and McRae 1997; Suzuki et al. 1999). This toxicity decreases by calcareous amendments. In fact, Al treatment in *V. corymbosum* plants increase photosynthetic performance by effects of gypsum amendment (Reyes-Díaz et al. 2011). This seems to be related with positive effect of gypsum amendment to reduces the injuries in highbush blueberry under Al-toxicity (Alarcón-Poblete et al. 2019). Despite, the knowledge of positive impact of gypsum on physiological and biochemical properties of plants under toxic Al, the mechanisms involved by this amendment on biological features in plants is scarce. Therefore, the aim of this work was to study the mechanisms displayed by gypsum application at the physiological, morphological and, metabolic levels in highbush blueberry cultivars growing under toxic Al and gypsum amendments.

HYPOTHESIS AND OBJECTIVES OF THE PROJECT.

HYPOTHESIS

The application of calcium sulfate ($CaSO_4$) on acid soil with toxic Al increases photosynthetic performance, metabolic responses, and modifies leaf anatomy features in highbush blueberry cultivars (*V. corymbosum*) due to increment of S and Ca in leaves and soil solution.

OBJECTIVES

General objectives.

To evaluate the effect of gypsum ($CaSO_4$) application on photosynthetic performance, metabolic responses and morphological features in *Vaccinium corymbosum* L. cultivars under aluminum toxicity in acid soil.

Specific objectives.

1.- To analyze the effect of gypsum application on chemical properties and morphological features on leaves of blueberry cultivars under aid soil and Al toxicity.

2.- To evaluate the effect of gypsum application on the photosynthetic performance in blueberry cultivars growing under acid soil and Al toxicity.

3.- To evaluate the effect of gypsum application on the total metabolites and metabolic profile in blueberry cultivars growing in acid soil and presence of toxic Al.

CHAPTER II. Review: "*Molecular regulation of aluminum resistance and sulfur nutrition during root growth"*

REVIEW

Molecular regulation of aluminum resistance and sulfur nutrition during root growth

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CHAPTER II. Review: "Molecular regulation of aluminum resistance and sulfur nutrition during root growth"

Molecular regulation of aluminum resistance and sulfur nutrition during root growth

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CHAPTER II. Review: "Molecular regulation of aluminum resistance and sulfur nutrition during root growth"

Abstract

Main conclusion **Aluminum toxicity and sulfate deprivation both regulate microRNA395 expression, repressing its low-affinity sulfate transporter (*SULTR2;1*) target. Sulfate deprivation also induces the high-affinity sulfate transporter gene (*SULTR1;2*), allowing enhanced sulfate uptake.**

Abstract Few studies about the relationships between sulfate, a plant nutrient, and aluminum, a toxic ion, are available; hence, the molecular and physiological processes underpinning this interaction are poorly understood. The Al-sulfate interaction occurs in acidic soils, whereby relatively high concentrations of trivalent toxic aluminum (Al^{3+}) may hamper root growth, limiting uptake of nutrients, including sulfur (S). On the other side, Al^{3+} may be detoxified by complexation with sulfate in the acid soil solution as well as in the root-cell vacuoles. In this review we focus on recent insights into the mechanisms governing plant responses to Al toxicity and its relationship with sulfur nutrition, emphasizing the role of phytohomones, microRNAs, and ion transporters in higher plants. It is known that Al^{3+} disturbs gene expression and enzymes involved in biosynthesis of S-containing cysteine in root cells. On the other hand, Al^{3+} may induce ethylene (ET) biosynthesis, enhance reactive oxygen species (ROS) production, alter phytohormone transport, trigger root growth inhibition and promote sulfate uptake under S deficiency. MicroRNA395, regulated by both Al toxicity and sulfate deprivation, represses its low-affinity Sulfate Transporter 2;1 (SULTR2;1) target. In addition, sulfate deprivation induces High Affinity Sulfate Transporters (HAST; SULTR1;2), improving sulfate uptake from low-sulfate soil solutions. Identification of new microRNAs and cloning of their target genes is necessary for a better understanding of the role of molecular regulation of plant resistance to Al stress and sulfate deprivation.

Keywords: Al transporters gene regulation ion toxicity microRNA nutrient deficiency S transporters

CHAPTER II. Review: "Molecular regulation of aluminum resistance and sulfur nutrition during root growth"

INTRODUCTION

In acid soils (pH<5.5 in water), aluminum (Al) is released into the soil solution in the form of cations [$Al(OH)_2^+$, $AlOH^{2+}$ and Al^{3+}, in order of increasing abundance with decreasing pH], with Al^{3+} being highly toxic to most plants (Rengel 1992; 1996; Mora et al. 2006). In many soils throughout the world (e.g., Andisols in Chile), Al toxicity is one of the most serious problems limiting plant growth, interfering with physiological and biochemical processes (Tang et al. 2002; Borie and Rubio 2003; Kochian et al. 2005; Wang and Shen 2006; Singh and Chauhan 2011). The first visible symptom of Al^{3+} injury in plants is root growth reduction (measurable within the first 30 minutes; Rengel 1992), which can severely affect uptake efficiency of water and essential nutrients such as calcium (Ca), magnesium (Mg), potassium (K), manganese (Mn) and sulfur (S) (Foy 1984; Guo et al. 2003; Mora et al. 2005a; Wang and Shen 2006).

Under soil acidity conditions, Al is complexed with sulfate, forming $AlSO_4^+$, which is non-toxic to plants (Kinraide and Parker 1987; Alva et al. 1991; Kinraide 1997). This complexation occurs in the outer sphere of Al ion, thus creating a steric shielding effect (Alva et al. 1991), and is regulated by sulfate concentration in the soil solution (Mora et al. 2005b). In addition, $AlSO_4^+$ formation (Mora et al. 1999) may be brought about by formation of basic aluminum sulfates of the type $Al_4(OH)_{10}SO_4\ 5H_2O$ or alumite [$KAl_3(OH)_6(SO_4)_2$] (Bolan et al. 1993). In soils, sulfate decreases Al saturation of the cation exchange complex, improving plant production and yield (Mora et al. 2006). However, strong complexation between Al and sulfate may provoke S deficiency in some soils (Quastel 1965).

Sulfur in plants is a constituent of glutathione (GSH) that operates as a component of glutathione *S*-transferase (GST); this enzyme is crucial for plant defense under biotic and abiotic stresses (Rausch and Wachter 2005). Sulfur deficiency results in oxidative stress that interferes with membrane functioning, enzyme activities, protein synthesis, photosynthesis, and stomatal movement, thereby causing growth retardation.

Several studies reported that microRNAs are involved in Al stress and S nutrition, targeting functions related to root growth (Liang and Yu 2010; He et al. 2014). Likewise, inhibition of root growth provoked by Al toxicity is also mediated via phytohormones like ethylene (Kochian et al. 2005; Doncheva et al. 2005; Ryan et al. 2011). Nonetheless, information about the underlying mechanisms related to Al toxicity and S nutrition is

scarce. Therefore, this review is focused on recent insights into the mechanisms regulating Al toxicity and its relationship with sulfur nutrition, emphasizing the role of phytohomones, microRNAs and ion transporters.

ALUMINUM TOXICITY AND SULFUR ASSIMILATION

The interaction between Al toxicity and sulfate metabolism in plants is complex and poorly understood. Aluminum toxicity may induce programmed cell death (PCD) by inducing accumulation of reactive oxygen species (ROS) in the mitochondrial complexes I and III. Both complexes are damaged by the interaction between Al and the iron-sulfur (Fe-S) cluster proteins (Li and Xing 2010). Although studies about the role of functional proteins in mitochondria are scarce, ROS generation and a loss of mitochondrial transmembrane potential are known to play important roles in the Al-induced caspase-3-like activation and PCD (Li and Xing 2011). Caspases (cysteinyl aspartate-specific proteases) are the important signaling molecules involved in cellular damage and the initiation of PCD (Kumar 2007), and may be involved in the PCD signaling cascades modulated by Al toxicity (Li and Xing 2010).

Sulfate is taken up by plants, reduced and incorporated into bioorganic compounds such as cysteine (Anjun et al. 2015), methionine (Met), vitamins (e.g. biotin and thiamin), Co-A and S-adenosyl methionine (Mazid et al. 2011), and is also involved in many aspects of primary metabolism, including protein synthesis and formation of the low-molecular-weight S-containing defense compounds (Capaldi et al. 2015). For example, Cys is required for glutathione (GSH) biosynthesis that occurs in plastids, mitochondria and the cytosol. Wulff-Zottele et al. (2014) reported that after sulfur fertilization of ryegrass, an increase in Cys concentration was found in roots under Al toxicity. This increase in Cys suggests that Al^{3+} may be involved in up-regulation of genes that encode cysteine synthase in ryegrass roots as it was also demonstrated in the Al^{3+}-resistant rice (*O. sativa*) cultivars (Yang et al. 2007). Glutathione can modify redox balance; an increase in reduced glutathione concentration was found to be associated with enhanced Al resistance in ryegrass (Wulff-Zottele et al. 2014). In addition, a study performed in *Zea mays* under Al stress suggested that the glutathione-*S*-transferase 27.2 (*GST27.2*) gene played a key role in the defense against Al toxicity (Cançado et al. 2005).

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TRANSPORTERS ASSOCIATED WITH ALUMINUM RESISTANCE AND UPTAKE OF ALUMINUM AND SULFATE

Aluminum resistance comprises Al avoidance (exclusion) and Al tolerance (of internalized Al). Aluminum avoidance is based on exudation of organic acid anions such as malate and citrate via anion transporters. In wheat and Arabidopsis, the main mechanism of Al resistance is reliant on Aluminum Activated Malate Transporter 1 (ALMT1) located in the root-cell plasma membrane (Sasaki et al. 2004; Hoekenga et al. 2006; Liu et al. 2009) (see Table 1). Another transporter group associated with Al resistance is the Multidrug and Toxic compound Extrusion (MATE) family (Table 1). Differing in localization and regulation, they are involved in mediating Al-activated citrate exudation in *Zea mays* (Maron et al. 2010), *Sorghum bicolor* (Magalhaes et al. 2007) and *Glycine max* (Wang et al. 2016). Recently, the *TaMATE1B* gene was isolated from wheat (Table 1); it encodes a transporter located in the plasma membrane of root tips, facilitating citrate efflux and enhanced Al resistance (Tovkach et al. 2013). Another transporter related to Al resistance based on organic acid anion exudation in *Hordeum vulgare* is Aluminum-Activated Citrate Transporter 1 (HvAACT1, homologous to ALMT1) expressed in epidermal cells of root tips (Furukawa et al. 2007) (see Table 1). The same transporter has been reported in rice (*O. sativa*) (Yokosho et al. 2009) (see Table 1) and rice bean (*Vigna umbellata*) (Yang et al. 2011) (see Table 1).

Some plants can tolerate relatively high intracellular concentration of Al (Al accumulators). Such plants have the effective Al transporters that facilitate influx of Al from the soil solution into root cells and then into the xylem sap. For example, in the Al-accumulator species *Hydrangea macrophylla,* Plasma membrane Al-Transporter 1 (HmPALT1) and Vacuolar Al-Transporter (HmVALT), both belonging to the aquaporin family, were found (Negishi et al. 2013). It has been pointed out that *HmPALT1* is expressed only in sepals (Negishi et al. 2012), suggesting that other Al transporters should exist for the uptake and long-distance transport of Al. An additional Al transporter, HmPALT2 (a member of the anion permease family) has been localized in the plasma membranes of blue-sepal cells, but *HmPALT2* is also expressed in leaves, stems and roots. It was suggested that HmPALT2 could transport Al into the cytosol of both root and shoot cells (Negishi et al. 2013); however, this transporter is not Al-specific because it also transports iron (Fe), nickel (Ni), copper (Cu), cadmium (Cd), and other elements (Negishi et al. 2013).

Uptake of Al from the external medium into the cytosol can also occur via the Nramp Aluminum Transporter 1 (Nrat1) belonging to the Natural Resistance-Associated Macrophage Protein (Nramp) family; this transporter is expressed in non-epidermal root-tip cells of rice (Xia et al. 2010). Given that a knockout of Nrat1 increases Al sensitivity, it was speculated that Al uptake via Nrat1 was a component of an Al transport pathway from the external medium to the vacuole, where detoxification occurs.

As an essential element, S is taken up and translocated by a set of transport proteins differing in localization and regulation. The genome of *A. thaliana* contains a family of 14 genes encoding putative sulfate transporters (Rouached et al. 2009). This family could be divided into five groups based on the similarity of protein sequences (Hawkesford 2003). The first group includes three sulfate transporters (SULTR1;1 to SULTR1;3) characterized as root-specific and with high affinity for sulfate. The second group comprises two low-affinity transporters involved in uptake (SULTR2;1) and distribution (SULTR2;2) of sulfate (see Table 2). The third group contains five transporters (SULTR3;1 to SULTR3;5) active in the root-to-shoot transport. Group four contains two sulfate transporters located in the thylakoid membranes, and group five comprises SULTR5;1 and SULTR5;2 (recently renamed MOT1) as high-affinity transporters associated with uptake of molybdate from soil (Hawkesford 2003; Baxter et al. 2008; Rouached et al. 2009).

For simplicity, two broad categories of sulfate transporters have been distinguished in Arabidopsis: the Low Affinity Sulfate Transporters (LAST) and High Affinity Sulfate Transporters (HAST) (Rouached et al. 2009). When S is deficient in the rhizosphere, the expression of high-affinity sulfate transporters is induced, and some assimilation enzymes that regulate the cysteine synthase complex are activated (Davidian and Kopriva 2010; Takahashi et al. 2011). In addition, the expression of *HvST1* (*Hordeum vulgare* Sulfate Transporter 1) gene, belonging to the high-affinity sulfate transporter family, in root tissues (Table 2) was inversely correlated with sulfate supply (Smith et al. 1997). The *ShST1 (Stylosanthes hamata* Sulfate Transporter 1) gene encodes a high-affinity Sulfate Transporter (SULTR) in the plasma membrane, permitting uptake of oxyanions (Lindblom et al. 2006) (see Table 2). Other high-affinity sulfate transporters and enzymes involved in sulfate assimilation and reduction have been described (Anjum et al. 2015), including those in *Triticum durum:* high-affinity Sulfate Transporters (TdSultr1.1 and TdSultr1;3), ATP Sulphurylase (TdATPSul1 and TdATPSul2), Adenosine-5'-PhosphoSulfate (APS) reductase (TdAPR), Sulphitereductase (TdSir), *O*-AcetylSerine(Thiol)Lyase (TdOASTL1

and TdOASTL2), and Serine AcetylTransferase (TdSAT1 and TdSAT2) (Ciaffi et al. 2013). *TdSultr1.1* was up-regulated in wheat roots by S deprivation, resulting in increased sulfate uptake capacity, whereas the elevated expression of this gene in shoots was probably an indicator of increased sulfate remobilization and transport in plants (Ciaffi et al. 2013).

The Sultr3 transporters that belong to the low-affinity family have multiple roles, facilitating sulfate transport to aerial parts, or controlling cysteine levels in seeds and seedlings (Kataoka et al. 2004a). In particular, Sultr3;1 appears to be responsible for sulfate transport into chloroplasts (Gallardo et al. 2014). The low-affinity sulfate transporter AtSultr4;1 has a crucial role in the efflux of sulfate from the vacuole to the cytosol, suggesting a specific function in remobilization of stored vacuolar sulfate (Kataoka et al. 2004b; Zuber et al. 2010; Gallardo et al. 2014). Hence, the SULTR4 transporters maintain the cellular homeostasis of sulfate and optimize sulfate cycling at the cellular level (Takahashi et al. 1999, 2000; Buchner et al. 2004; Kataoka et al. 2004b; Martinoia et al. 2007).

The Al and sulfate transporters implicated in uptake and translocation of these ions in many species and organs are shown in Tables 1 and 2. Based on the studies mentioned above and to better understand the interactions between Al and S, we proposed a possible interactive model in root cells (Figure 1). Under Al toxicity, Al may be sequestered into the vacuole via Aluminum Sensitive 1 (ALS1) transporters (Larsen et al. 2007; Huang et al. 2012) (see Table 1). In contrast, a transporter facilitating sulfate entry into the vacuole has not been characterized yet (Gigolashvili and Kopriva 2014; Kopriva et al. 2015), even though there is no doubt that sulfate does enter the vacuole. In the vacuole, Al may be complexed with organic acid anions (Ma et al. 2001), and we suggest that Al may also form complexes with sulfate ($AlSO_4^+$), similarly to what occurs in the soil solution (Figure 1). Furthermore, polyphenols are also considered as the potential Al-chelating agents due to their high affinity for Al (Barcelo and Poschenrieder 2002; Kochian et al. 2015). Recently, Zhang et al. (2016) reported that oenothein B, as a high-molecular-weight gallate-derived polyphenol, may sequester and precipitate Al in the vacuole. In addition, detoxification of Al and trace elements in the cytosol of non-accumulator plants may be achieved primarily by complexation with S-containing compounds (Na and Salt 2011).

MicroRNAs REGULATED BY ALUMINUM AND SULFATE SUPPLY

CHAPTER II. Review: "Molecular regulation of aluminum resistance and sulfur nutrition during root growth"

The cis-elements that enhance gene expression in plants are 20 to 23 nucleotide-long non-coding RNAs (microRNAs), promoting the cleavage of target mRNAs (He et al. 2014). MicroRNAs are mainly located in the intergenic regions and are involved in the responses to biotic and abiotic stresses in plants (Mallory and Vaucheret 2006), including Cd exposure, Cu starvation, Al stress, and sulfate starvation (Huang et al. 2009; Liang and Yu 2010; Sunkar et al. 2012; Chen et al. 2012); however, information on the role of microRNAs in response to Al toxicity and S deficiency in plants is limited (e.g. Lima et al. 2011; He et al. 2014).

It has been reported that several microRNAs respond to a wide range of abiotic stresses. For example, microRNA319a/b, microRNA319b.2 and microRNA400 respond to several stresses because they possess numerous promoter response elements (RE) located upstream of microRNA genes (Barciszewska-Pacak et al. 2015). MicroRNA319b has at least seven short RE sequences of NGATT (where N=G/A/C/T) that control responses to a variety of environmental stimuli. The ethylene-RE (ERE) and gibberellin-REs (GAREs) often have other cis-elements in the promoter region in response to metal stress (Barciszewska-Pacak et al. 2015).

Some Al-responsive microRNAs involved in the Al-stress-signaling network are: microRNA160, microRNA166, microRNA171, microRNA319, microRNA390, microRNA393, microRNA398, microRNA519, and microRNA529 (Zhou et al. 2008; Yang and Chen 2013; He et al. 2014). Zhou et al. (2008) reported that upon exposure to Al stress, the expression of microRNA171, microRNA319 and microRNA519 was up-regulated (and the expression of microRNA166 and microRNA398 down-regulated) in leaves of *Medicago truncatula*.

Chen et al. (2012) functionally characterized microRNAs under Al toxicity and resistance in *M. truncatula* root tips, identifying 326 known microRNAs and 21 new ones. Among them, 23 microRNAs were responsive to Al toxicity, but, in contrast to the other study on the same species (Zhou et al. 2008), the expression of microRNA166 and microRNA171 was not modified by the presence of Al. These discrepancies between the two studies can be explained by different experimental conditions [Zhou et al. (2008) exposed plants to 50 μM Al^{3+} for 24 h, whereas Chen et al. (2012) used 10 μM Al^{3+} for either 4 or 24 h], suggesting that microRNA166 and microRNA171 responded to relatively high Al^{3+} concentration.

Using quantitative RT-PCR, Chen et al. (2012) found that in *M. truncatula* root tips the expression of microRNA319 rapidly decreased during the first 4 h in response to Al toxicity, but was not responsive to the 24-h Al treatment. Accordingly, the Al-responsive microRNAs were classified into three groups based on their

expression patterns: rapid-response, late-response and sustained-response microRNAs. The majority of Al-responsive microRNAs belonged to the 'rapid-response' category, i.e. they were responsive during the first 4 h (but not after 24 h) of Al treatment.

Low sulfate concentrations in the soil solution activate sulfate transporters, increasing efficiency of sulfate uptake by roots. During sulfate deprivation, sulfur uptake and metabolism in plants are regulated at various levels, with microRNAs being part of this regulatory network (Jones-Rhoades et al. 2006; Lewandowska and Sirko 2008; Huang et al. 2010). A few microRNAs (microRNA319, microRNA403-5p and microRNA2111a-3p) had a 2-fold higher expression in *A. thaliana* under sulfate starvation compared with the control conditions (Barciszewska-Pacak et al. 2015). In addition, differential expression of a range of microRNAs (microRNA156, microRNA160, microRNA164, microRNA167, microRNA168, microRNA394, and microRNA395) was reported in roots, stems and leaves of *Brassica napus* under sulfate-deprived and/or Al toxicity conditions. In particular, microRNA160 was transcriptionally down-regulated, whereas microRNA164b and microRNA394a,b,c were up-regulated in roots and stems of *B. napus* under S deficiency (Huang et al. 2010).

Under Al toxicity, microRNA395 could be up-regulated or down-regulated depending on the species, e.g. as reported in *A. thaliana, O. sativa* and *Nicotiana tabacum* (Liang and Yu 2010; Lima et al. 2011; Gielen et al. 2012). The same microRNA was up-regulated under sulfate starvation in *O. sativa* and *A. thaliana* (Jones-Rhoades and Bartel 2004; Chiou 2007; Liang and Yu 2010) and *B. napus* (Buhtz et al. 2010). In *A. thaliana*, microRNA395 regulated sulfate uptake, transport and assimilation, as well as sulfate concentration in shoots, by targeting three ATP Sulfurylase (APS) genes (*APS1*, *APS3* and *APS4*) that encode the enzymes catalyzing sulfate activation in the sulfur assimilation pathway (Schachtman and Shin 2007; Lewandowska and Sirko 2008). During sulfate deprivation, reciprocal regulation between *SULTR2;1* and *APS* genes through microRNA395 is important for remobilizing sulfate from mature to young leaves in Arabidopsis (Liang and Yu 2010), although the mechanism involved in this regulation is unclear. MicroRNA395 is highly abundant in the phloem parenchyma and is also involved in restricting expression of low-affinity SULTR2;1 (Chiou 2007; Kawashima et al. 2009), limiting internal remobilization of sulfate from leaves during S deficiency. On the other hand, sulfur deprivation induces expression of high-affinity *SULTR1;2*, allowing sulfate uptake and translocation from roots to shoots,

contributing to growth maintenance under S deficiency (Takahashi et al. 2011; Yang and Chen 2013; Paul et al. 2015).

In *O. sativa* roots subjected to Al toxicity, microRNA160 and microRNA168 were up-regulated, while microRNA156 and microRNA395 were down-regulated (Lima et al. 2011). In contrast, microRNA395 was up-regulated in response to sulfate starvation in *A. thaliana* (Jones-Rhoades and Bartel 2004; Liang and Yu 2010). It remains to be tested whether the effects of Al toxicity and S deficiency on regulating microRNA395 expression can be elicited in a single plant species. Given that Al^{3+} is complexed with sulfate in soil (Mora et al. 2005a), it is suggested that the formation of this complex would lower sulfate availability in soil and could provoke S deficiency in plants (Figure 1). Thus, microRNA395, being regulated by both Al toxicity and sulfate deprivation, would repress low-affinity sulfate transporter (*SULTR2;1*) gene (Liang and Yu 2010; Gielen et al. 2012), whereas sulfate deprivation would induce high-affinity sulfate transporter (*SULTR1;2*), improving sulfate uptake from the soil solution containing low S concentration under Al toxicity (Figure 1).

RELATIONSHIP BETWEEN ALUMINUM TOXICITY, SULFATE NUTRITION AND PHYTOHORMONES INVOLVED IN INHIBITION OF ROOT ELONGATION

Roots are the first target of Al toxicity in acid soils, with inhibition of cell growth and elongation being likely mediated via phytohormones (Kochian et al. 2005; Doncheva et al. 2005; Ryan et al. 2011). Ethylene biosynthesis is induced by Al^{3+}, provoking inhibition of root elongation (Stepanova et al. 2005; Sun et al. 2010) due to a decrease in polar auxin transport by Proteinase Inhibitor 2 (PIN2) and Auxin Transporter Protein 1 (AUX1) (Figure 1).

A number of studies have pointed out that microRNAs play a role in Al stress by targeting functions related to root growth (Table 3). He et al. (2014) indicated that microRNA160, microRNA164, microRNA167, and microRNA393 are involved in auxin signaling. MicroRNA393 is also associated with the regulation of leaf development (Si-Ammour et al. 2011) and root growth (Chen et al. 2012) (Table 3). The microRNA393 expression in *M. truncatula* leaves increased in response to several metals, including Cd and mercury (Hg), but it was not affected by the toxic Al exposure (Zhou et al. 2008). In contrast, in *O. sativa* roots, microRNA393b was down-regulated under Al stress (Table 1) (Lima et al. 2011).

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A recent study indicated that microRNA390 is up-regulated in wild soybean (*Glycine soja*) in response to Al (Zeng et al. 2012). This microRNA targets Trans-Acting Short-Interfering 3 (TAS3) that induces degradation of Auxin Response Factors (ARF), resulting in inhibition of lateral root growth (Figure 1, Table 3). In contrast, Mendoza-Soto et al. (2012) reported that the expression of microRNA390 was repressed in roots of *M. truncatula* under Al toxicity, also resulting in the inhibition of lateral root growth. Similarly, in *japonica* rice roots exposed to Al, microRNA393b levels were slightly lower than in the control plants, whereas microRNA160e was up-regulated by the Al treatment (Lima et al. 2011). MicroRNA160e down-regulates ARFs and has an important counter-balancing role through the inverse regulation of microRNA393b in the auxin-signaling pathway in response to Al toxicity (Lima et al. 2011). The authors suggested that up-regulation of microRNA528, microRNA160e and microRNA166k, as well as down-regulation of microRNA393, could contribute to controlling root growth in rice plants exposed to high Al concentration (Table 3).

Wang et al. (2005) and Li et al. (2016) identified ARF10 and ARF16 in *A. thaliana*, which are repressed by up-regulation of microRNA160, as controlling the root-cap cell formation. In *A. thaliana* expressing a microRNA160-resistant form of ARF17, there was an increase in ARF17 mRNA levels, altering the accumulation of auxin-inducible Gretchen Hagen3 (GH3)-like mRNAs that encode auxin-conjugating proteins (Hagen and Guilfoyle 2002). Decreased expression of ARF17 was correlated with developmental defects, including inhibition of root growth, demonstrating the importance of ARF17 regulation directed by microRNA160 (Liu et al. 2007; Li et al. 2016). In addition, ARF17 regulates the GH3-like early auxin response genes (Mallory et al. 2005). Studies performed by Lima et al. (2011) demonstrated an induction of microRNA160 under Al toxicity, regulating ARF and therefore repressing the auxin-responsive gene expression, leading to root growth inhibition (Figure 1).

In *A. thaliana*, over-expression of microRNA160 was associated with cleavage of ARFs that regulate the root-cap development (Turner et al. 2013); in particular, the expression of three *ARF* genes was barely detectable, and root length was reduced (Table 3). On the other hand, the contrasting responses under Al toxicity were found in the regulation of microRNA160 and microRNA390. Up-regulation of microRNA160 in roots of *O. sativa* (Lima et al. 2011) and microRNA390 in roots of wild soybean (*G. soja*) (Zeng et al. 2012) repressed ARFs in both species, triggering root growth inhibition (Figure 1). In contrast, down-regulation of both

microRNAs was associated with (i) repression of the IAA transport and the auxin response genes, (ii) up-regulation of ARF levels, and (iii) root growth inhibition. This may be a cause of the reduced root growth under Al stress in *M. truncatula* found by Chen et al. (2012).

Under optimal conditions, auxin binds to TIR1 through the F-box subunit of the ubiquitin ligase complex named Skp1-Cdc53-F-box protein (SCF), forming the SCF/TIR1 complex that stabilizes the interaction between TIR1 and Aux/IAA heterodimer (Tiwari et al. 2003). This binding triggers the ubiquitination of Aux/IAA by the SCF complex, leading to its degradation by the 26S proteasome (Enders and Strader 2015). This degradation allows activation of ARFs, regulating transcriptional auxin process. In this process, different families of early-response genes are involved, allowing ARFs to activate expression of the auxin-responsive genes (Jungmook et al. 1997; Chapman and Estelle 2009). Under Al toxicity, decreased levels of microRNA393 cause the suppression of TIR1 and increase the cellular availability of the Aux/IAA heterodimer (Figure 1). This increase depresses ARFs through heterodimerization and inhibits the auxin-responsive gene expression, reducing the auxin concentration and consequently decreasing root growth (Figure 1).

Ethylene is related to inhibition of root growth. It is synthesized via the following pathway: methionine is converted to S-Adenosyl Methionine (SAM) by SAM synthetase and then to 1-AminoCyclopropane-1-Carboxylic acid (ACC) by ACC Synthase (ACS) followed by ACC being degraded by ACC oxidase (ACO) to release ethylene (Lin et al. 2009; Sauter et al. 2013). Inhibition of root elongation in *Arabidopsis* under Al toxicity may be associated with Al-induced up-regulation of the *AtACS* and *AtACO* genes (Sun et al. 2010) (see Figure 1). Ethylene production is likely to act as a signal to alter auxin distribution in roots by up-regulation of the *AtPIN2* and *AtAUX1* genes, provoking a negative regulation of AUX1- and PIN2-mediated auxin polar transport, leading to inhibition of root elongation (Sun et al. 2010) (see Figure 1).

It is known that ethylene regulates plant responses to deprivation of various nutrients, such as Fe, P, K, S, and Mg (Garcia et al. 2015), as well as toxicity of heavy metals, such as Cd, Cu and Zn (Maksymiec 2007), and also Al (Sun et al. 2007, 2010). In addition, Al induces an increase in ROS formation (Meriño-Gergichevich et al. 2010; Xie et al. 2015), which may enhance ethylene biosynthesis (Garcia et al. 2015), with ethylene involved in the Al-associated inhibition of root growth and regulation of sulfate uptake (Figure 1). On the other hand, the

formation of Al-sulfate complex in soil solution could cause S deficiency in plants under Al toxicity (Mora et al. 2005a), which may trigger the ethylene-regulated S-deficiency responses.

CONCLUSIONS AND FUTURE PERSPECTIVES

Despite the scarcity of studies on the interaction between Al toxicity and S nutrition in plants under acidic conditions as well as the molecular approaches aimed at elucidating the transcriptional regulation by Al and S, we have suggested the possible mechanisms that regulate the interaction between the two elements.

It is known that Al^{3+} modifies microRNA expression implicated in the auxin responses, triggering root growth inhibition. However, there is little knowledge about the underlying mechanisms by which microRNA393 regulates TIR1 and microRNAs 160 and 390 regulate ARFs in plant cells. In addition, Al^{3+} also induces ethylene biosynthesis and ROS production, interfering with phytohormone transport and consequently inhibiting root growth. Sulfate may ameliorate Al toxicity through the formation of $AlSO_4^+$ complex in the soil solution as well as in the vacuoles, lowering sulfate availability and potentially provoking S deficiency in plants. Both Al toxicity and sulfate deprivation regulate microRNA395 expression, repressing its low-affinity sulfate transporter (*SULTR2;1*) target. In addition, sulfate deprivation induces the high-affinity sulfate transporter (*SULTR1;2*) gene, allowing enhanced sulfate uptake.

The physiological and molecular studies focused on the interaction between Al toxicity and S nutrition are necessary for a better understanding of the mechanisms leading to Al resistance, as an important prerequisite for improving crop growth and food production in acid soils. The identification of candidate microRNAs and cloning of their target genes should provide a further insight into the role of microRNAs and their molecular targets in regulating plant resistance to Al stress and sulfate deprivation. This knowledge will provide a new molecular basis for improving crop Al tolerance via molecular-assisted breeding and genetic engineering.

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Table 1: Localization and function of transporter genes involved in aluminum resistance in higher plants.

Gene name	Organ/tissue; function	Species	Reference
ALMT1	Root cell plasma membrane; malate exudation	*Triticum aestivum,* *Arabidopsis thaliana*	Sasaki et al. (2004); Liu et al. (2014) Hoekenga et al. (2006)
MATE	Plasma membrane of root tip cells; efflux of citrate	*Hordeum vulgare*, *Sorghum bicolor*, *Zea mays,* *Vigna umbellata,* *T. aestivum,* *Oryza sativa,* *Eucalyptus camaldulensis*	Furukawa et al. (2007) Magalhaes et al. (2007) Maron et al. (2010); Yokosho et al. (2011) Yang et al. (2011) Tovkach et al. (2013); Liu et al. (2014) Liu et al. (2014) Sawaki et al. (2013)
AACT1	Epidermal cells of root tips; activated citrate transporter	*H. vulgare,* *O. sativa*	Furukawa et al. (2007) Yokosho et al. (2009)
Nramp (Nrat1)	Plasma membrane of all root tip cells (except epidermal); Al^{3+} uptake	*O. sativa*	Xia et al. (2010); Li et al. (2014)
HmVALT1	Vacuolar and plasma membrane in sepals; transports aluminum into the vacuoles	*Hydrangea macrophylla*	Negishi et al. (2012)
*HmPALT*1	Vacuolar and plasma membrane in sepals only; Al transporter	*H. macrophylla*	Negishi et al. (2012)
HmPALT2	Plasma membrane of cells in sepals, leaves, stems and roots; transports Al from the external environment into the cytosol	*H. macrophylla,* transgenic *Arabidopsis* lines	Negishi et al. (2013)
OsALS1	Tonoplast of root cells; responsible for sequestration of Al into vacuoles	*O. sativa*	Huang et al. (2012); Liu et al. (2014)

4 Table 2: Localization and function of sulfate transporter genes involved in sulfate uptake and translocation in higher plants.

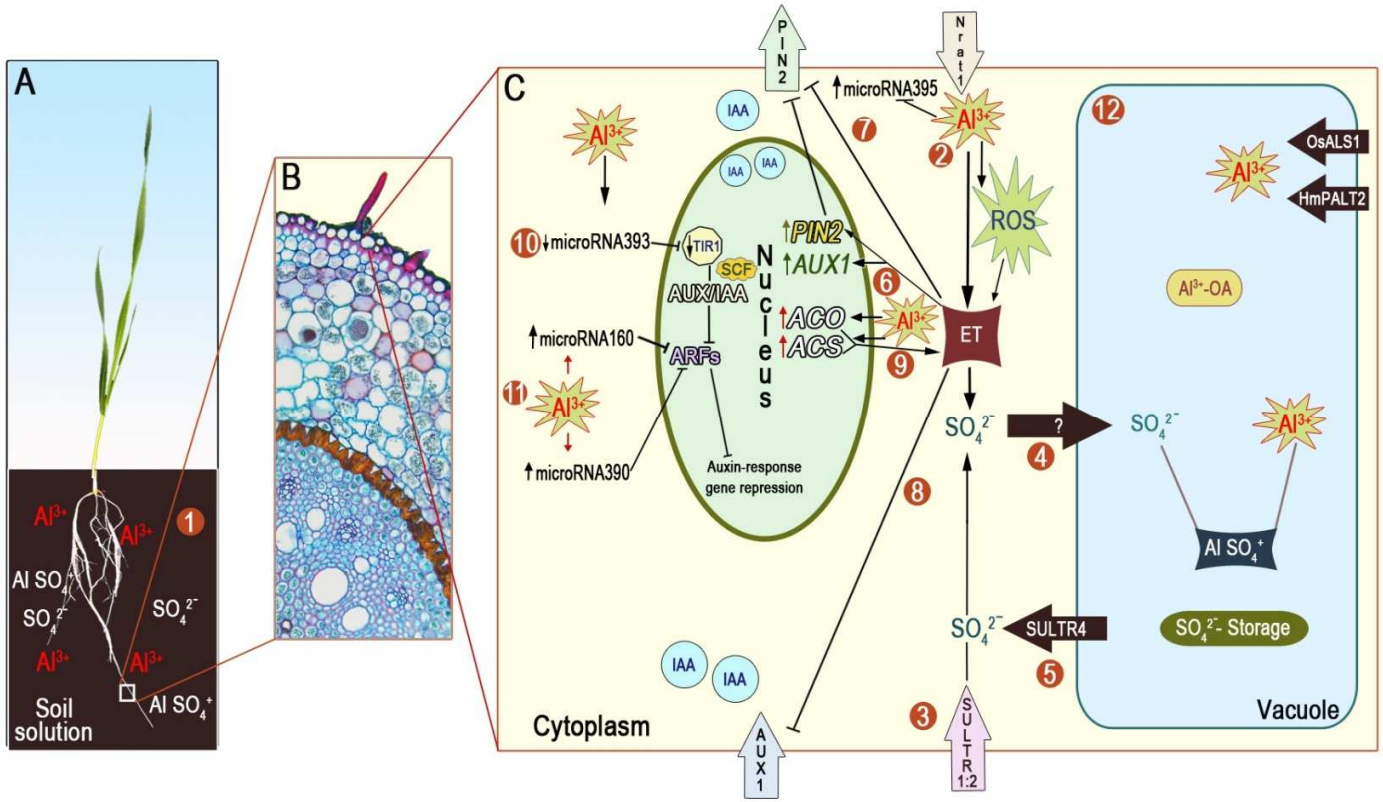
Gene name	Organ/tissue; function	Species	Reference
SULTR1;1	Epidermal and cortical root cells; uptake of sulfate by root hairs	*Brassica oleracea* *Arabidopsis thaliana* *Triticum durum*	Buchner et al. (2004) Maruyama-Nakashita et al. (2004a,b); Yoshimoto et al. (2007); Barberon et al. (2008) Ciaffi et al. (2013)
SULTR1;2	Plasma membrane of root epidermal and cortical cells, pericycle, root apex, and vascular parenchyma cells; absorption of sulfate and internal transport	*A. thaliana* *B. oleracea*	Takahashi et al. (2000); Shibagaki et al. (2002); Hirai et al. (2003); Maruyama-Nakashita et al. (2004a); Yoshimoto et al. (2007); El Kassis et al. (2007); Barberon et al. (2008) Buchner et al. (2004)
SULTR1;3	Phloem cells; transport of sulfate	*A thaliana* *B. oleracea* *T. durum*	Yoshimoto et al. (2003) Buchner et al. (2004) Ciaffi et al. (2013)
SULTR2;1	Vascular tissues, xylem parenchyma and root pericycle cells; sulfate uptake	*A. thaliana*	Liang and Yu (2010)
SULTR2;2	Xylem parenchyma cells; involved in distribution of sulfate from vascular bundles to the palisade cells in leaves. Plays a central role in the regulation of sulfate assimilation	*A. thaliana*	Takahashi et al. (2000)
SULTR3;1	Chloroplasts; responsible for sulfate transport into chloroplasts	*A. thaliana*	Cao et al. (2013)
SULTR3;2	Plasma membrane of leaf and root cells; transport of sulfate	*A. thaliana*	Takahashi et al. (2000)
SULTR3;3	Root, shoot and leaves; transport of sulfate	*A. thaliana* *B. oleracea*	Takahashi et al. (2000) Buchner et al. (2004)
SULTR3;4	Root and leaves; transport of sulfate and connection with abscisic acid (ABA) synthesis	*B. oleracea* *A. thaliana* *Medicago truncatula*	Buchner et al. (2004) Gallardo et al. (2014)
SULTR3;5	Vasculature (xylem parenchyma) and root pericycle cells; root-to-shoot transport of sulfate	*A. thaliana*	Kataoka et al. (2004a)
SULTR4;1	Vacuoles of root xylem parenchyma cells, tonoplast in shoots and chloroplast envelope; efflux of sulfate	*A. thaliana*	Takahashi et al. (1999); Takahashi et al. (2000); Kataoka et al. (2004b); Martinoia et al. (2007)
SULTR4;2	Vacuoles (tonoplast) in shoots and roots; efflux of sulfate	*A. thaliana* *B. oleracea*	Kataoka et al. (2004b) Buchner et al. (2004)
SULTR5;1	Shoots and leaves; unclear whether it is involved in the transport of molybdate or sulfate	*T. aestivum*	Shinmachi et al. (2010)

SHST1	Plasma membrane, primarily in roots; mediates uptake into root cells rather than root-to-shoot translocation	*Stylosanthes hamata*	Smith et al. (1995); Lindblom et al. (2006)
LeST1;1	Root epidermis; uptake of sulfate	*Lycopersicum esculentum*	Abdin et al. (2010)
HVST1	Plasma membrane of root epidermis; uptake of sulfate	*Hordeum vulgare*	Smith et al. (1997)
SLIM1	Root epidermis; sulfate acquisition	*A. thaliana*	Maruyama-Nakashita et al. (2006)
TdATPSul1	Plastids of roots and shoots; genes code for enzymes involved in sulfate assimilation and reduction	*T. durum*	Ciaffi et al. (2013)
TdATPSul2	Plastids of roots and shoots; genes code for enzymes involved in sulfate assimilation and reduction	*T. durum*	Ciaffi et al. (2013)
TdAPR	Plastids of roots and shoots; genes code for enzymes involved in sulfate assimilation and reduction	*T. durum*	Ciaffi et al. (2013)
TdSir	Plastids; genes code for enzymes involved in sulfate assimilation and reduction	*T. durum*	Ciaffi et al. (2013)
TdOASTL1; TdOASTL2	Plastids and cytosol, respectively; genes code for enzymes that catalyze Cys biosynthesis by replacing the activated *o*-acetylserine (OAS) with sulfide	*T. durum*	Lewandowska and Sirko (2008); Ciaffi et al. (2013)
TdSAT1; TdSAT2	Cytosol; encodes a serine acetyltransferase (SAT) isoform and its protein catalyzes the formation of *o*-acetylserine from Ser, also involved in sulfate assimilation and cysteine metabolism in plants	*T. durum*	Ciaffi et al. (2013) Lewandowska and Sirko (2008)

7 Table 3: Identification of Al-responsive microRNAs that influence root development.

microRNA	Function	Species	Reference
gso-miR1509a	Regulates development of root caps and lateral roots. It targets auxin response factors (ARFs), such as ARF10, ARF16 and ARF17 families.	*Glycine soja*	Zeng et al. (2012)
miRNA528	Targets F-box/LRR-repeat (leucine-rich repeat) MAX2 (more axillary branches 2); copper ion binding protein; copper amine oxidase-like; laccase; L-ascorbate oxidase; superoxide dismutase.	*Oryza sativa*	Lima et al. (2011)
miRNA396 miRNA396a	Regulate cell proliferation and lateral root development	*Arabidopsis thaliana* *Medicago truncatula*	Rodríguez et al. (2010) Chen et al. (2012)
miRNA393	Modulates auxin signaling through TIR1 (transport inhibitor response 1) regulation, involved in disease resistance. Regulates root growth	*A. thaliana*	Sunkar and Zhu (2004); Navarro et al. (2006); Si-Ammour et al. (2011); Lima et al. (2011); Chen et al. (2012)
miRNA390	Involved in root development. Regulates ARFs and moderates root growth.	*A. thaliana* *M. truncatula* *G. soja*	Marin et al. (2010) Yoon et al. (2010) Mendoza-Soto et al. (2012); Chen et al. (2012); Zeng et al. (2012)
miRNA319	CCAAT-box binding transcription factor (Glyma18g07890) Targets gene *tcp4*.	*G. max* *A. thaliana* *M. truncatula*	Zeng et al. (2012) Jones-Rhoades et al. (2006); Schommer et al. (2008) Chen et al. (2012); Mendoza-Soto et al. (2012)
gso-miRNA167a-p3	Flowering, leaf development, cold response, drought response, jasmonate signal. TCP family transcription factor (Glyma13g04540).	*G. soja*	Zeng et al. (2012)
PN-miRNA169g MiRNA169c	protein of unknown function Glyma18g03980	*G. max* *O. sativa*	Zeng et al. (2012) Lima et al. (2011)
miRNA166k	Regulates genes related to lateral root formation	*O. sativa*	Lima et al. (2011)

PN-miRNA156f-p3	ARF (Seed germination) and Myb (myeloblastosis) family transcription factor (Glyma13g04030 and Glyma20g11040)	*G. max*	Zeng et al. (2012)
miRNA159	MYB33	*A. thaliana* *M. truncatula*	Alonso-Peral et al. (2010) Reyes and Chua (2007) Chen et al. (2012)
miRNA160	Modulates root-growth-regulating auxin response factors (ARF10, ARF17 and ARF16).	*A. thaliana* *G. max* *M. truncatula* *O. sativa*	Wang et al. (2005); Liu et al. (2007) Turner et al. (2013) Chen et al. (2012) Lima et al. (2011)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TIR1
SCF
AUX/IAA
ARFs
Auxin-response gene repression
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Figure caption:

Figure 1: Model of microRNA action on the auxin (IAA)-related responses under aluminum toxicity and S deficiency. Induction: → Repression: ⊣

On the left, box A shows the complexation of Al^{3+} with sulfate in an acid soil, forming non-toxic $AlSO_4^+$ and potentially causing S deficiency in plants (1). In box B, a partial transverse cut of the root is shown. Box C represents a root cell. Uptake of Al^{3+} from the external medium into the cytosol via Nrat1 transporter may induce ROS formation and ethylene (ET) synthesis (2). Sulfate deficiency induces, and Al^{3+} represses, microRNA395 (2). ET promotes sulfate uptake under S deficiency via the high-affinity sulfate transporter SULTR1;2 (3). Cytosolic sulfate can be sequestered into the vacuole (and can complex Al^{3+} there into non-toxic compounds) even though a corresponding tonoplast transporter has not been identified yet (4). In contrast, vacuolar sulfate can be remobilized into the cytosol via the SULTR4 transporters (5). Al^{3+} and ET upregulate expression of the *AUX1* and *PIN2* genes (6) and negatively regulate the PIN2 (7) and AUX1 protein transporters (8), disrupting IAA transport towards the root tips and altering its concentration there.Al^{3+} enhances the expression of the *ACO* and *ACS* genes, resulting in increased ET production (9). Al^{3+} down-regulates the microRNA393 expression, repressing TIR1 and affecting the binding between TIR1 and AUX/IAA heterodimer through SCF, causing repression of auxin response genes (10). Al^{3+} up-regulates both microRNA160 and microRNA390, repressing ARFs and the auxin-response genes (11). Al^{3+} sequestration into the vacuole through PALT2 and ALS1 transporters contribute to Al tolerance in the Al-hyperaccumulating plant species (12).

CHAPTER III. Research article: "Gypsum application ameliorates morphological and photochemical damages provoked by Al toxicity in *Vaccinium corymbosum L.* cultivars".

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Gypsum application ameliorates morphological and photochemical damages provoked by Al toxicity in *Vaccinium corymbosum* L. cultivars.

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Abstract

BACKGROUND: By acidity, Al^{3+} available form increases, being toxic for plants. Calcium amendments are widely used as an agronomic practice to reduce this effect. OBJECTIVE: To determine the gypsum application effect on leaf morphological and physiological features on three highbush blueberry cultivars growing in acid soil and toxic Al level. METHODS: Legacy and Brigitta, Al-resistant and Bluegold, Al-sensitive were grown in acid soil with 48% Al saturation and three gypsum concentrations (0.7, 1.4, and 2.8 g $CaSO_4kg^{-1}$) for 30 days. Chlorophyll *a* fluorescence measurements, photosynthesis, and photoprotective pigments were analyzed. Samples of leaves and roots were harvested, and Al, Ca, and S concentrations, antioxidant activity (RSA), lipid peroxidation (LP), and leaf anatomy were determined. RESULTS: Gypsum decreased leaves and roots Al concentration in all cultivars. Higher Ca leaves concentration in cultivars was observed compared to roots under gypsum treatment. Aluminum damages were observed in leaf thickness, improving anatomic features in cultivars by gypsum as well as a reduction of LP without changes in RSA. Chlorophyll levels changed differentially according to the cultivar and $CaSO_4$ dose, while antheraxanthin was higher in Legacy with gypsum. The Principal Component Analysis (PCA) scores plot showed that PC1 separated Brigitta from Legacy-Al resistant, while PC2 helped to discriminate Bluegold Al-sensitive cultivar with gypsum treatment from the rest of the samples, including Bluegold without gypsum.

CONCLUSIONS: Gypsum amendment ameliorates leaf morphology alterations as well as photochemical and biochemical damages in highbush blueberry under Al-toxicity, being cultivar-dependent. The morphological parameters can be important features as Al-resistance anatomical markers in highbush blueberry.

Keywords: Acid soils; aluminum; calcium sulfate; highbush blueberry; physiological responses.

Introduction

Acid soils represent around 70% potentially arable soils and 30-40% arable soil in the world, where aluminum (Al) toxicity is the main factor that limit crop production in these soils [1, 2]. In south-central Chile, most crop production is developed in acid soils, which derive from young volcanic ashes (Andisols). The main characteristics of these soils are low pH (from 4.5 to 5.5), high organic matter content, low phosphorus (P) and nitrogen (N) availability as well as low ion exchange, and high water-holding capacities [3, 4]. In these soils, acidification is favored by urea fertilization and heavy rains, especially in winter, which lixiviate the main cations from the soil exchange complex, replacing them by protons (H^+) and acid cations, mainly Al and manganese (Mn) [5]. Under acidic conditions, Al^{3+} available form increases, thus being toxic for plants [6]. The first effect of Al toxicity is root growth reduction, which can severely affect water and nutrient uptake, reducing crop yield [7, 8]. Root growth reduction is due to the Al interaction with the apoplastic side in the cell wall, plasma membrane, and cytoskeleton [9]. Although, it has been reported that Al-toxicity produces disturbances on the peripheral tissues, and roots thickening [10, 11], little information has been reported at shoot levels. Konarska [12] reported that Al exposure reduced the size and thickness of leaf blades due to a decrease in cell size, as well as an increase in the number of stomata from the abaxial epidermis, with a simultaneous reduction

of their size. These effects caused a decrease in photosynthetic pigment levels and photosynthetic parameters. Similarly, a reduction in leaf area and chlorophyll content were found in cotton plants growing under Al-toxicity [13]. Likewise, several reports indicated that Al also affects the photosynthetic performance, reducing the stomatal conductance and electron transport rate [14, 15, 16]. Moreover, Al induces reactive oxygen species (ROS) production, leading to oxidative stress in organelles and eventually provoking cell death [17, 18]. Studies in highbush blueberry have demonstrated that long-term exposure to Al-toxicity increased lipid peroxidation (LP) mainly in Al-sensitive cultivar, augmenting the radical scavenging activity (RSA) in response to Al-stress [11, 14, 15]. In other species such as pea (*Pisum sativum*), lipid peroxidation seems to be an Al-toxicity early symptom [19].

For overcoming Al phytotoxicity, calcium (Ca) amendments have been widely used as an agronomic practice to reduce this effect in acid soils [20, 21, 22]. This strategy is commonly used by farmers for the production of different crop species, being lime and gypsum or phosphogypsum the most common Ca source [23-25]. Among the Ca sources, gypsum has the advantage of ameliorating subsoil acidity with the surface application and thus representing a good nutrients source, such as Ca and S, as well as having higher solubility rates in soil solution [4, 15, 25-27]. Furthermore, it is reported that the strong complex formed between Al and sulfate (SO_4^{2-}) provokes both, decreased Al toxicity in soil solution and decreased mineral nutrients translocation towards the upper part of plants (stem and/or leaves) [4]. Despite a few reports about the effect of gypsum application on soil [27-30], gypsum effects on plant metabolism and physiology are still poorly known.

Vaccinium corymbosum L. (highbush blueberry) is one commercially important berry crop with different cultivars sensitivity to toxic Al levels. Its fruit has exceptional flavor,

nutritional properties, and antioxidant-richness; therefore, it is highly demanded as healthy food [31]. This crop is well adapted to soil acidity [32]; however, its sensitivity to the presence of Al-toxicity decreases its productivity substantially. Therefore, this study aimed to investigate gypsum amendment effects on morpho-anatomical features and physiological performance on three highbush blueberry cultivars growing in acid soil and toxic Al level.

Materials and Methods

Plant material and experimental conditions

One-year-old highbush blueberry plants, from three contrasting cultivars in terms of Al resistance (Legacy and Brigitta as Al-resistant, and Bluegold as Al-sensitive) were used in this study. Plants with uniform size (30 cm high) were obtained from Berries San Luis, Lautaro (38°29 S and 72°23 W), La Araucania Region, Chile. The experiment was performed under controlled greenhouse conditions at 20-25ºC, 16/8 h photoperiod (light/dark, respectively), 80% relative air humidity, and photosynthetic photon flux density (PPFD) of 300 µmol photons m^{-2} s^{-1} as an average, according to [33]. Acid soil (Andisol) from the Gorbea serie [34] was used with pH (in water) 4.7 and Al saturation 48%. Previous to starting the experiment, the gypsum amendment was added to the soil sample, carefully mixed, and then incubated for four weeks, as described by Mora et al. [3]. Thereafter, plants were carefully washed with deionized water and transferred to pots (1 plant per pot) with 2 kg acid soil or incubated soil as follows: i) acid soil with a 48% Al saturation; ii) acid soil + Al + 0.7 g $CaSO_4$ kg^{-1} of soil; iii) acid soil + Al + 1.4g $CaSO_4$ kg^{-1} of soil, and iv) acid soil + Al + 2.8 g $CaSO_4$ kg^{-1} of soil (Table 1). Pots were daily irrigated to maintain soil field capacity during

the experiment (30 days). Each treatment had ten replicates in a completely randomized design.

Soil Chemical properties

The soil chemical properties were determined at the beginning and the end of the experiment (30 days), according to Sadzawka et al. [35] (Table 1). Soil pH was potentiometrically measured in a soil/water solution ratio (1:2.5). Sulfur (S) was extracted with Ca (H_2PO_4) [36] and analyzed by turbidometry [37]. Calcium was extracted with 1 M CH_3COONH_4 at pH 7.0 and analyzed by a simultaneous multi-element atomic absorption spectrophotometer (model UNICAM 969 Atomic absorption Spectrometer, England, UK). Exchangeable Al was extracted with 1 M KCl and analyzed by the same equipment.

Aluminum and calcium concentrations in leaves and roots

At 30 days of the experiment, mature expanded leaves of shoots (from the first to the fourth node) and roots were dried separately at 70ºC in a forced air oven for 48 h. Then, leaves and roots were ashed at 500ºC for 8 h and treated with 2M hydrochloric acid. Aluminum and Ca were quantified using a simultaneous multielement atomic absorption spectrophotometer (Model 969 Atomic Absorption Spectrometer, Unicam, Cambridge, UK) as described in Sadzawka et al. [38].

Sulfur concentration in leaves and roots

For S concentration analysis, plant tissues were collected as mentioned above. Dry leaves and roots were treated with 95% magnesium nitrate ($MgNO_3$ x $6H_2O$) and ashed at 500ºC for 8 h. Ashed samples were digested with 10 mL of 2M HCl for 60 min at 150 ºC before the addition of barium chloride ($BaCl_2$) and Tween-80. The resulting solution was measured in a UV/VIS spectrophotometer (UNICO® 2800 UV/VIS, Spain) at 440 nm, as described by Sawdzaka et al. [38].

Fluorescence of chlorophyll a parameters

Fluorescence of chlorophyll *a* parameters were determined *in vivo* using a portable pulse-amplitude modulated fluorometer (FMS 2; Hansatech Instruments, King's Lynn, UK), to establish the PSII photochemical efficiency, according to Reyes-Díaz et al. [33]. The effective quantum yield (ΦPSII) and electron transport rate (ETR) were calculated as described by Maxwell and Johnson [39].

Photosynthetic and photoprotective pigments quantification

Photosynthetic and photoprotective pigments were extracted with 100% v/v acetone (HPLC grade) and determined, according to García-Plazaola and Becerril [40] by high-performance liquid chromatography (HPLC). The HPLC measurements were performed in an HPLC System Agilent technologies 1200 series, column C-18 Waters spherisorb 5.0 µm ODS1 4.6 x 250 mm. Chlorophyll (Chl) *a*, *b*, α-carotene (α-Ca), violaxanthin (Vx), antheraxanthin

(Ax), neoxanthin (Nx), and Lutein (Lt) were measured using pigment standards purchased from Sigma-Aldrich (Sigma Chemical Co. St. Louis, MO, USA).

Lipid peroxidation measurement

Lipid peroxidation (LP) was determined as an index of oxidative damage in plants and assessed in fresh samples (leaves and roots) by monitoring the thiobarbituric acid reacting substances (TBARS). In order to correct the interference produced by TBARS-sugar complexes, the absorbance was measured at 440, 532, and 600 nm according to a modified method [41]. The LP was expressed as nmol equivalents of malondialdehyde (MDA) concentration per gram of fresh weight (FW) in nmol MDA g^{-1} FW.

Radical scavenging activity quantification

The radical scavenging activity (RSA) of roots and leaves was measured by free radical 1.1-diphenyl-2-picrylhydrazyl (DPPH) method, as described by Chinnici et al. [42]. The absorbance was measured at 515 nm using Trolox as standard. The RSA was expressed as microgram of Trolox equivalent per gram of fresh weight (µg TE g^{-1} FW).

Morpho-anatomical analysis

Leaves were collected as described above and placed in a fixing solution; the central portion of leaves was fixed in formaldehyde, acetic acid, and ethanol (FAA) for 72 h and preserved

in 70% ethanol (v/v). Transverse sections of 10 µm were stained with safranine fast green and mounted in water-glycerol, then visualized by microscopy (Olympus CX31, Tokyo Japan) [43]. Finally, leaf morphology was evaluated using Image J software; the width of cell layers such as adaxial epidermis (ADE), abaxial epidermis (ABE), mesophyll (M), and palisade (P), and leaf thickness were measured.

Experimental design and statistical analyses

The experiment was completely randomized with 3 genotypes x 4 treatments x 5 replicates each. Pots with one plant each were changed every day to minimize positional effects. Measurements of chlorophyll *a* fluorescence parameters were performed at 0, 7, 15 and 30 days after gypsum application, whereas morpho-anatomical, chemical and biochemical quantifications were made at the end of the experiment (30 days). All data passed the normality and the equality of variance after the Kolmogorov-Smirnov test. Data were analyzed with a two-way analysis of variance (ANOVA) (where the factors were cultivars and treatments) for chemical and biochemical analyses and to a three-way ANOVA (where the factors were cultivars, treatments, and time) for photosynthetic parameters. Tukey test was applied to identify means with significant differences at the level of $p \leq 0.05$. Analyses were performed with Sigma Stat 2.0 software (SPSS, Chicago, IL).

The Pearson correlation analysis was carried out by t-test with a significance level of $p \leq 0.05$ to examine the relationships among variables. The Benjamini and Hochberg [44] false discovery rate control was used for correcting the resulting *p*-values, using R script displayed by the Rbio software (www.biometria.ufv.br). To reduce the dimensionality of data

set, and identify the variables that explained a higher proportion of the total variance, which could provide insight into the effects of gypsum application on biochemical and physiological features, a multivariate analysis by Principal Components Analysis (PCA) was used in the Minitab® 17 statistics program (Minitab Inc., Philadelphia).

Results

Soil chemical properties after gypsum treatment

Soil pH increased from 4.7 to 5.0 from the beginning until the end of the experiment in all amendment treatments (Table 1). Aluminum saturation in soil decreased, concomitant with gypsum amendment application in all cultivars, reaching a 6-fold decrease at the highest $CaSO_4$ dose (Table 1). Calcium concentration in soil increased 3-fold in the lowest gypsum dose, 5- and 12-fold in the highest doses in all cultivars (Table 1). Soil sulfur concentration augmented until 4.6-fold with the highest gypsum amendment dose in all cultivars (Table 1).

Aluminum, calcium, and sulfur concentrations in leaves and roots

A significant interaction was observed among organs, cultivars, and treatments for Al concentration ($p \leq 0.001$). The Al concentrations in leaves and roots of all cultivars showed a significant decrease ($p \leq 0.05$) when the amendment was applied (Fig. 1). In acid soil treatment, the roots of the three cultivars presented higher Al concentration than leaves (Fig. 1A, B). The leaves from Bluegold exhibited the lowest Al concentration than the other cultivars under acid soil treatment (Fig. 1A). When the highest dose of the amendment was

applied, Al concentration in leaves decreased. Interestingly, in Brigitta leaves, a stronger reduction in Al levels (55%) was observed, followed by Legacy (27%) and Bluegold (23%) (Fig. 1A). In roots, the three cultivars diminished their Al concentration due to the effect of amendments when compared with non-calcium treated roots (acid soil + Al) (Fig. 1B). Legacy presented the highest Al reduction (around 86%) by the highest doses of amendments, whereas in Brigitta and Bluegold the decrease of Al was lower (62%) at the highest $CaSO_4$ application (Fig. 1B). Significant interactions for Ca concentration were observed between organs *versus* cultivars and cultivar *versus* treatments ($p \leq 0.001$). The Ca concentration in leaves of all cultivars was higher than in roots ($p \leq 0.05$), exhibiting increased levels of Ca when the amendment was applied (Fig. 1C, D). The Ca concentration in Bluegold and Legacy roots did not show any significant change when amendments were added. However, Brigitta roots gradually incremented Ca concentration with treatments ($p \leq 0.05$) regarding Al-treated plants (Fig. 1D).

A significant interaction among organs, cultivars, and treatments was observed in leaves and roots S concentration ($p \leq 0.001$). In general, S concentration in leaves was higher than in roots in all cultivars (Fig. 1E, F). Besides, S concentration was increased in leaves and roots of the three cultivars ($p \leq 0.05$) after the amendment application. An increase of S concentration (30%) was observed in Bluegold leaves with the lowest amendment dose concerning to acid soil without amendment (Fig. 1F). Among cultivars, Brigitta and Bluegold leaves presented higher S concentration than Legacy leaves (Fig. 1E).

Chlorophyll fluorescence parameters

A significant interaction between cultivars and time of measurements was observed for ΦPSII and ETR ($p \leq 0.001$). Legacy slightly decreased ΦPSII and ETR values under acid soil treatment, increasing these parameters with amendment application, being higher (32%) under 1.4 g $CaSO_4$ kg^{-1} of soil treatment (Fig. 2). Brigitta increased ΦPSII and ETR with all amendment application through the time, whereas Bluegold decreased these parameters under acid soil treatment after seven days, reaching the initial values with $CaSO_4$ application (Fig. 2). NPQ did not vary among treatments and times in all cultivars (data not shown).

Photosynthetic and photoprotective pigments

With respect to Chl *a* and Chl *b*, a significant interaction was observed between cultivar and treatments ($p \leq 0.007$ and $p = 0.001$, respectively). In Brigitta and Bluegold, total chlorophyll (Chl *a*+*b*), Chl *a* and Chl *b* concentrations increased with all amendment treatments, being around 43% at the highest $CaSO_4$ treatment respect to the acid soil alone (Fig. 3 A, B and C). Bluegold showed a higher concentration of Chl *a*+*b*, Chl *a,* and *b* in all levels of gypsum application than Brigitta and Legacy (Fig.3A, B and C). Chlorophyll a/b ratio did not change in any cultivar and treatments (Fig. 3D).

Carotenoids pool was significantly increased in Bluegold (around 2-fold) under gypsum applications compared to the acid soil (Fig. 4A). This increment was lower in Brigitta, while Legacy levels remained unchanged (Fig. 4A). The α-carotene significantly enhanced (2.6-fold) in Bluegold by gypsum application in comparison to acid soil, staying constant in Brigitta and Legacy (Fig. 4B). Lutein concentration showed a significant increase ($p \leq 0.05$) when the amendment was applied in Brigitta and Bluegold cultivars (Fig. 4C). In the

treatment without amendment, the highest Lutein concentration was observed in Legacy, followed by Bluegold, and the lowest in Brigitta (Fig. 4C). The xanthophyll cycle (neoxanthin, violaxanthin, and antheraxanthin) was enhanced in Brigitta and Bluegold after the amendment application, whereas in Legacy, these compounds did not change (Fig. 4D, E, and F). The ratio of antheraxanthin and violaxanthin showed the highest value in Brigitta under acid soil, decreasing with amendment treatments. A similar tendency was observed in Legacy, whereas in Bluegold, this ratio augmented concomitant to the amendment respect to acid soil (Fig. 4G).

Lipid peroxidation and radical scavenging activity

There is significant interaction among cultivars, treatments, and organs of *V. corymbosum* ($p \leq 0.001$). At the end of the experiment, leaves LP values were higher in plants without $CaSO_4$ and with the lowest $CaSO_4$ dose, whereas the highest LP value was found in Legacy (Fig. 5A). In roots, Bluegold showed the highest LP in the treatment without amendment (Fig. 5B).

Regarding the radical scavenging activity (RSA), a significant interaction was observed among cultivars, treatments, and organs ($p = 0.011$). Generally, higher leaves RSA was observed in Bluegold (Fig. 6A). The highest RSA was verified in Legacy roots, followed by Brigitta and Bluegold (Fig. 6B). The RSA was higher, around 2-fold increase, in Bluegold and Brigitta leaves than in roots, whereas in Legacy, this increase was only 1.3-fold (Fig. 6).

Morpho-anatomical analysis

Brigitta leaves thickness, and palisade mesophyll cells were higher than Bluegold and Legacy without amendments ($p \leq 0.005$), whereas an increase in all cultivars was found after gypsum application (Table 2 and Fig. 7). In Brigitta and Bluegold, a significant increase was exhibited (Table 2 and Fig. 7) in the upper epidermis cells (about 26%) with amendments (around 28%) with respect to non-amendment plants. It was also observed that Brigitta palisade mesophyll cells increased by 28% with 2.8 g kg^{-1} $CaSO_4$ and 49% in Legacy with 1.4 g kg^{-1} $CaSO_4$, respect to their values in acid soil. Spongy parenchyma thickness increased with increasing dose of $CaSO_4$, as follows: 45% in Legacy and Brigitta, and 24% in Bluegold (Table 2 and Fig. 7).

Correlation analysis of variables

To reduce the dimensionality of the data set and to identify significant associations, the Pearson correlation was used (Fig. 8). For the Legacy cultivar, some correlations were expected like the positive correlations between palisade parenchyma thickness ($r = 0.97$) and spongy parenchyma thickness ($r = 0.97$) with leaf thickness (Fig. 8A). Others were less intuitive, such as NPQ strongly and positively correlated with sulfur levels in leaves at all times ($r > 0.96$). It is worth mentioning that Ca levels in leaf and root were weakly correlated with other traits (Fig. 8A).

The highest number of significant correlations were found for Brigitta cultivar (70 correlations with a *p*-value below 0.05) (Fig. 8B). The correlation analysis showed that in Brigitta, Al levels in leaf were strongly and negatively correlated with two important pigments involved with the protection of the photosynthetic apparatus, which are α-carotene ($r = -0.99$) and violaxanthin ($r = -0.99$) (Fig. 8B). In Brigitta, the highest correlation was exhibited between epidermis thickness parameters and foliar Ca levels ($r > 0.95$) (Fig. 8B). When we analyzed the Bluegold cultivar, similarly to what was found in Brigitta, photosynthetic pigments such as Chl *a*, Chl *b* and α-carotene have a strong and positive correlation ($r > 0.95$) with most of the xanthophyll cycle pigments analyzed in this study, such as neoxanthin, violaxanthin, and anteraxanthin. On the other hand, only in Bluegold, the foliar lipid peroxidation was negatively correlated with these pigments. Besides, only in the Brigitta cultivar, a remarkably positive correlation was observed between Ca and S foliar levels with root sulfur levels ($r = 0.99$).

Principal component analysis (PCA) ***of the physiological and biochemical measurements from leaves and roots***

In order to investigate the possible common factors that would explain the observed correlations, PCA analysis was performed based on all measured traits from leaves and roots of the three blueberry cultivars (Legacy, Bluegold and Brigitta) (Fig. 9). For this comparison, data obtained for all traits were averaged and normalized. The first two dimensions resumed a higher part of the total variance (66.9%). The first principal component (PC1) and the second principal component (PC2) accounted for 37.7% and 29.2% total variation, respectively (Fig. 9A). The PCA scores plot showed that PC1 separated Brigitta from Legacy Al-resistant, while PC2 helped to discriminate Bluegold Al-sensitive cultivar with gypsum treatment from the rest of the samples, including Bluegold without gypsum (Fig. 9A). Overall, according to the percentage of total variability explained by PC1, the global changes between these two cultivars may be related to changes in the physiological (ETR and NPQ) and epidermis thickness parameters showed by Brigitta in contrast to biochemical changes (neoxanthin, violaxanthin and antheraxanthin) displayed by Legacy (Fig. 9B).

On the other hand, the PC2 clearly separated cultivar Bluegold from all other cultivars separated along PC1. Furthermore, it revealed an interesting ungrouping between acid soil and treatments growing under increasing $CaSO_4$ doses (0.7, 1.4, and 2.8 g $CaSO_4$ kg^{-1} of soil) (Fig. 9A). Thus, this result suggests a great degree of Bluegold sensitivity when exposed to increasing doses of $CaSO_4$. It should also be highlighted that results obtained in the PC2 analysis also revealed that pigments such as Chl *a*, Chl *b* and carotene displayed a major

percentage of the total variability (Fig. 9B). This indicates that the effect of the increasing Ca doses on the photosynthetic pigments was important for Bluegold plants.

Discussion

The $CaSO_4$ effect on interactions between other elements

It is widely known that Al-toxicity in acid soils can be decreased by calcareous amendments applications, such as gypsum ($CaSO_4$) [3, 45-47]. Our results confirm this evidence in the three blueberry cultivars subjected to gypsum amendments, where Al soil saturation, as well as Al-concentrations of leaves and roots, decreased concomitantly with the increase of the amendment application (Table 1, Fig. 1). Studies performed in barley (*Hordeum vulgare*) under Al-toxicity showed that Ca addition reduced Al-toxicity due to Al concentration and lipid peroxidation reduction, which increased antioxidant enzyme activity as well as Ca concentration compared with the Al-treatment alone. Thus, suggesting that Ca supplementation could be related to less Al-uptake in barley plants [48]. This $CaSO_4$ application effectiveness in amelioration of Al-toxicity was also verified in lettuce under field conditions [30].

Furthermore, except for Al-accumulator plants, it has been reported that Al concentrates more in roots than in leaves [12]. In blueberry, Al-accumulation in different organs depended on the cultivar and the treatments. The Al-sensitive cultivar Bluegold accumulated more Al in roots than in leaves with the exception of the highest amendment supply. This high Al-root concentration in this cultivar provoked high oxidative stress as

indicated by the higher LP (Fig. 1 and 5). Similar results have been found in the nutrient solution experiment, where Bluegold accumulated higher Al concentration in roots than in shoots when gypsum amendments were applied [15]. In addition, negative correlations between Al and Ca concentrations were found in Brigitta and Bluegold leaves and roots (r = around -0.6 and -0.84, $p \leq 0.05$, respectively), whereas Legacy leaves showed a lower correlation (r = -0.46; $p \leq 0.05$) and in roots, no statistically significant correlation was found. These results indicated that in Brigitta and Bluegold, but not in Legacy, a decrease of Al^{3+} interacting with Ca^{2+} improved Ca uptake from the gypsum amendments (Fig. 1). High external Ca, in cytosolic root cells, reduces Al^{3+} and favors the Ca uptake. Similar behavior has been proposed for K in *Arabidopsis thaliana* [49].

Based on tissues Ca concentrations, blueberry can be considered as a calcifuge species [29]. They reported that healthy blueberry plants leaves have Ca levels that vary from 3.0 to 8.0 g kg^{-1} DW. Our values are in the range reported, depending on cultivar, being Ca levels higher in Bluegold leaves compared to other cultivars (Fig. 1). In rice (*Oryza sativa*), the application of amendments increased soil pH and reduced Al toxicity, improving growth and development due to the addition of Ca and other nutrients such as S [22]. Similar results have been reported in other crop species [26]. In the current study, blueberry cultivars exhibited negative correlation ($p \leq 0.05$) between Al and S concentration in Brigitta roots and leaves (r = -0.82 and -0.90, respectively) and Bluegold (r = -0.79 and -0.50, respectively), whereas this correlation was present (r = -0.47) only in Legacy leaves. Contrarily, Mora et al. [7] reported that a relationship between S and Al contents in roots was positively correlated (r = 0.683) in ryegrass (*Lolium multiflorum*) plants under Taylor and Foy nutrient solution. In our work, it is remarkable that Bluegold and Brigitta showed a higher S concentration in leaves

as compared to roots. Reyes-Díaz et al. [15] observed a high S concentration in Bluegold leaves subjected to Hoagland nutrient solution with toxic Al, under acidic conditions. In our study, a positive effect of $CaSO_4$ application was observed, increasing Ca and S contents in blueberry leaves and roots. Furthermore, studies performed in wheat and ryegrass indicates that Ca content in leaves increased dramatically according to $CaSO_4$ doses increase [3, 28]. Amendments can increase Ca and SO_4 content in the soil, making it available to plants and reducing the Al toxic-forming Ca-Al complex. Unexpectedly, although Bluegold is an Al-sensitive cultivar, it showed a higher Ca concentration in leaves than in roots, differently than in Legacy and Brigitta, which are more Al-resistant.

Photochemical responses of PSII to $CaSO_4$ and Al levels

The maximum photochemical efficiency of PSII (Fv/Fm) was not affected by any of the Al and $CaSO_4$ treatments (data not shown). Similar results were obtained in blueberry subjected to similar treatments in hydroponic Hoagland nutrient solution [15]. In this study, gypsum application enhanced the ΦPSII in the Al-resistant cultivars (Brigitta and Legacy) compared to the values of toxic Al in acid soil without amendments, whereas in Al-sensitive cultivars (Bluegold), ΦPSII values remained constant or decreased at the end of treatment with toxic Al alone (Fig. 2). This result confirms that this cultivar is more sensitive under Al-stress than the other cultivars, as reported previously [14, 33]. In *Glycine max* varieties, fluorescence parameters (Fv/Fm and ΦPSII) were reduced under toxic Al [50]. To counteract the damage caused by stress factors such as excessive light as well as Al-toxicity, plant tissues possess different photoprotective mechanisms, which include the xanthophyll cycle (XC) [51, 52].

These authors reported that in *Citrus* spp. exposed to Al-toxicity conditions antheraxanthin (A) and zeaxanthin (Z) increased in Al-treated leaves. In Al-resistant Legacy cultivar, a slight conversion of violaxanthin into antheraxanthin by gypsum supply was found, as compared with other cultivars (Fig. 4E and F). In addition, gypsum application increased all photoprotective pigments compared to Al-treatment. Chen et al. [51] reported that under high light and Al-toxicity, the increasing conversion of V to A and Z might help to quench $^1O_2$ augmented in Al-treated leaves due to enhanced closure of PSII in *Citrus*. Nonetheless, in our experiment, we observed a higher increment in lutein pigment by gypsum application, especially in Bluegold compared to the acid soil and other cultivars (Fig. 4). This may be associated with a high PSII sensitivity to the Al-stress of Bluegold, due to the fact that lutein participates in the structural stabilization of light-harvesting antenna proteins, quenching ^{3}Chl states. Besides, lutein has been proposed to be involved in the quenching of ^{1}Chl [53].

A typical symptom of oxidative stress is the increased lipid peroxidation (LP), which is considered a general index of oxidative membrane injury [19, 54]. Another study evidenced that LP is also a manifestation of Al toxicity in highbush blueberry [14]. In our research, the $CaSO_4$ application caused a LP decrease in roots and leaves of the three different cultivars with the exception of Legacy roots (Fig. 5). In Bluegold, this decrease was related to a significant increase of Lutein content and RSA (Fig. 4 and 6). Also, Maxwell and Johnson [39] indicate that high RSA can help minimize thylakoid damages, increasing ETR, which induces an increment of photochemical performance such as shown in our study, where Bluegold increased RSA, maintaining ETR compared to Brigitta and Legacy with Ca supply (Fig. 2 and 6). Gypsum application on Brigitta Al-resistant cultivar enhanced ΦPSII

and ETR under Al treatments, unlike Bluegold Al-sensitive, where this parameter remained constant, decreasing only by Al alone.

Gypsum effects on morphological traits in blueberry leaves under Al toxicity

Aluminum toxic levels in soil promoted alterations in leaves morphological features. In our study, we observed a negative correlation between Al levels in soil and leaf thickness in the Al-tolerant cultivars (Brigitta and Legacy). The highest leaf thickness in Brigitta coincided with an augment of the palisade and spongy parenchyma thickness (Table 2 and Fig. 7). Our results agree with those reported by Konarska [12] with a reduction of morphological characteristics. In addition, studies performed in *Eucalyptus* revealed changes in leaf morphology under Al toxicity, such as thickness reduction of leaf epidermis and palisade layers [55]. Calcium concentration in Al-resistant Brigitta cultivar leaves generally correlated positively and significantly with leaf thickness, in each cellular layer evaluated (Table 2 and Fig. 7). These correlations could be an important trait for Al-resistance observed in Brigitta, and may be used as Al resistance anatomical markers for sensitivity in highbush blueberry cultivars.

Principal component analyses of metabolic, morphologic and physiological traits

Into a broader metabolic, morphological, and physiological context, PCA analysis suggests that Al-sensitive and resistant cultivars display distinct response mechanisms (Fig. 9A). The Al responses presented by the Brigitta cultivar were related to changes in Chlorophyll *a* fluorescence and morphological parameters, such as parenchyma thickness (Fig. 9B). It has

been shown that Al toxic levels induce anatomical changes in plant tissues, reducing cell elongation, and division [56]. In sunflower plants, Al stress increased leaf blade thickness and parenchyma layers [57]. Thus, anatomical changes observed in Brigitta can be important traits of tolerant plants and may be used as anatomical markers of Al tolerance for sensible cultivars such as Bluegold.

On the other hand, in the Legacy cultivar, this tolerance was more associated with a higher Al accumulation in leaves and roots, followed by a clear LP increase for both organs (Fig. 9B). It is well known that plasma membrane is one of the primary Al-stress targets at the cellular level [58] and Al important effects appear on plasma membrane structure and function. Accordingly, high lipid peroxidation was found in all $CaSO_4$ treatments of Legacy cultivar. This effect may be because Legacy displayed more elevated Al accumulation in leaves than the other cultivars, even after the application of $CaSO_4$. As regards the Al-sensitive Bluegold cultivar, PCA revealed a clear separation between Al and calcium treatments (Fig. 9B). These results suggested that Bluegold cultivar has a positive response to $CaSO_4$ applications. Thus, compared to other cultivars, Bluegold might have a differential Al uptake by roots, which are revealed by a strong antagonistic group between Al levels in leaves and roots with calcium treatments (Fig. 9B). Genotypic differences have been characterized in Al uptake and accumulation in other crops like maize [59], wheat [60], and pigeon pea [61].

In conclusion, the gypsum amendment ameliorates leaf morphology alterations as well as photochemical and biochemical damages in highbush blueberry under Al-toxicity. In general, a compensatory effect was observed after the amendment application, increasing all

evaluated parameters, particularly in the Brigitta cultivar with 1.4 g $CaSO_4$ kg^{-1} supply. Gypsum application reverses the strong and negative correlation between Al content in leaves and leaf thickness, recovering the morphology and decreasing mesophyll compaction in leaves of all cultivars. Thus, morphological parameters can be important traits as anatomical markers of Al-resistance for Al-sensitive cultivars such as Bluegold.

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Conflicts of interest

The authors have no conflict of interest to report.

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Table 1. Chemical properties of Gorbea soil series, at the end of the experiment (30 days). Values represent the average of five replicates ± SE (n = 5). Different lower case letters indicate statistically significant differences ($p \leq 0.05$) among treatments within cultivars. Different upper case letters show differences ($p \leq 0.05$) among cultivars within treatments.

				Legacy				Brigitta					Bluegold	
Parameters	Acid soil + Al at the beginning	0	0.7	1.4	2.8	0	0.7	1.4	2.8	0	0.7	1.4	2.8
			doses (g $CaSO_4$ kg soil) + Al				doses (g $CaSO_4$ kg soil) + Al				doses (g $CaSO_4$ kg soil) + Al		
pH (water)	4.7±0.12a	4.8±0.01aA	5±0.03aA	5±0.07aA	5±0.06aA	4.7±0aA	4.9±0.01aA	5±0.06aA	5±0.06aA	4.9±0.01aA	4.9±0.01aA	5±0.03aA	5±0.03aA
Al Sat (%)	48±3.95a	32±0.37bA	23±1.48cA	15±0.59dB	9±0.23eA	34±0.74bA	22±1.18cA	17±0.19dA	8±0.04eA	29±2.41bB	22±1.5cA	17±1.6dA	8±0.01eA
Ca (cmol+/kg)	0.3±0.03a	0.39±0.08aA	1±0.1bA	1.6±0.1cA	3.3±0.06dA	0.32±0.04dA	1.0±0.05cA	1.2±0.14bA	3.7±0.12aA	0.38±0.01dA	1.06±0.13cA	1.7±0.1bA	3.5±0.12aA
S (ppm)	34±1.45a	38±0.29bA	61±4.91cA	82±7dA	136±5eC	40±2.02bA	62±4.33cA	87±4.04dA	158±9.81eA	42±0.29bA	62±4.33cA	70±2.9dA	147±6eB

Table 2 Morphometric leaf parameters of three *V. corymbosum* cultivars under an acid soil with 48% aluminum saturation and different $CaSO_4$ treatments at the end of the experiment (30 days). Values represent the average of five replicates ± SE (n = 5). Different lower case letters indicate statistically significant differences ($p \leq 0.05$) among treatments within cultivars and plant tissue. Different upper case letters show differences ($p \leq 0.05$) among cultivars within treatments and plant tissues.

Treatments	**Cultivars**		
	Legacy	**Brigitta**	**Bluegold**
Leaf thickness (µm)			
Acid soil + Al	10.43 ± 0.66 Bd	14.63 ± 0.01Ac	11.1 ± 0.13Bc
Acid soil + Al+ 0.7 g Ca $SO_4 kg^{-1}$	11.94 ± 0.70 Bc	18.16 ± 0.29 Ab	11.21 ± 0.1 Bc
Acid soil + Al+ 1.4 g Ca SO_4 kg^{-1}	17.33 ± 0.14 Ba	20.21 ± 0.23 Aa	12.86 ± 0.13 Cb
Acid soil + Al + 2.8 g Ca SO_4 kg^{-1}	15.75 ± 0.06 Bb	20.98 ± 0.93 Aa	15.31 ± 0.21 Ba
Upper epidermis thickness (µm)			
Acid soil + Al	1.23 ± 0.01 Aa	1.27 ± 0.06 Ac	1.30 ± 0.01 Ac
Acid soil + Al + 0.7 g Ca $SO_4 kg^{-1}$	1.31 ± 0.04 Aa	1.44 ± 0.04 Abc	1.40 ± 0.04 Abc
Acid soil + Al + 1.4 g Ca SO_4 kg^{-1}	1.30 ± 0.06 Ba	1.48 ± 0.06 ABb	1.55 ± 0.08 Aab
Acid soil + Al + 2.8 g Ca SO_4 kg^{-1}	1.25 ± 0.09 Ba	1.77 ± 0.07 Aa	1.72 ± 0.06 Aa
Lower epidermis thickness (µm)			
Acid soil + Al	0.91 ± 0.07 Aa	0.96 ± 0.03 Ac	0.97 ± 0.01 Aa

Acid soil + Al + 0.7 g Ca $SO_4 kg^{-1}$	1.12 ± 0.02 Aa	1.04 ± 0.04 Abc	1.06 ± 0.01 Aa
Acid soil + Al + 1.4 g Ca SO_4 kg^{-1}	1.09 ± 0.05 Aa	1.27 ± 0.06 Aab	1.01 ± 0.03 Aa
Acid soil + Al + 2.8 g Ca SO_4 kg^{-1}	1.16 ± 0.06 Ba	1.49 ± 0.17 Aa	0.91 ± 0.02 Ca

Palisade parenchyma thickness (µm)

Acid soil + Al	2.42 ± 0.10 Cc	4.96 ± 0.09 Ac	3.37 ± 0.09 Ba
Acid soil + Al + 0.7 g Ca $SO_4 kg^{-1}$	3.26 ± 0.15 Bb	5.57 ± 0.00 Ab	3.37 ± 0.42 Ba
Acid soil + Al + 1.4 g Ca SO_4 kg^{-1}	4.79 ± 0.04 Ba	6.64 ± 0.08 Aa	3.68 ± 0.05 Ca
Acid soil + Al + 2.8 g Ca SO_4 kg^{-1}	3.70 ± 0.20 Bb	6.87 ± 0.17 Aa	3.40 ± 0.12 Ba

Spongy parenchyma thickness (µm)

Acid soil + Al	4.51 ± 0.18 Bb	6.06 ± 0.26 Ad	5.44 ± 0.10 ABc
Acid soil + Al + 0.7 g Ca $SO_4 kg^{-1}$	4.73 ± 0.23 Cb	7.03 ± 0.33 Ac	5.82 ± 0.28 Bc
Acid soil + Al + 1.4 g Ca SO_4 kg^{-1}	9.09 ± 0.34 Aa	9.15 ± 0.28 Ab	6.45 ± 0.20 Bb
Acid soil + Al + 2.8 g Ca SO_4 kg^{-1}	8.19 ± 0.24 Ca	11.01 ± 0.29 Aa	7.16 ± 0.49 Ba

Figure captions

Fig 1 Aluminum, calcium and sulfur concentrations in leaves and roots of three cultivars of *V. corymbosum* subjected to acid soil with 48% aluminum saturation and different $CaSO_4$ treatments for 30 days. A and B: Al concentration in leaves and roots, respectively; C and D: Ca concentration in leaves and roots, respectively and E and F: S concentration in leaves and roots, respectively. Values represent means (n = 5) ± SE. Different lower case letters indicate statistically significant differences ($p \leq 0.05$) among treatments within cultivars and plant tissues. Different upper case letters show differences ($p \leq 0.05$) among cultivars within treatments and plant tissues. Asterisk (*) indicates statistically significant differences between tissues (leaves and roots) for the same cultivar and treatments.

Fig 2 Changes in the effective quantum yield (ΦPSII) and electron transport rate (ETR) of three cultivars of *V. corymbosum* at different times (days) subjected to acid soil with 48% aluminum saturation and different $CaSO_4$ treatments for 30 days. Values represent means (n = 5) ± SE. Asterisk (*) indicates statistically significant differences ($p \leq 0.05$) among treatments within cultivars.

Fig 3 Chlorophyll (Chl) *a* and *b* in *V. corymbosum* L. grown in acid soil with different $CaSO_4$ amendment at 30 days. Acid soil (48% aluminum saturation). A: Chlorophyll *a* + *b*; B: Chlorophyll *a*; C: Chlorophyll *b* and D: Chlorophyll *a*/*b*. Values represent means (n = 5) ± SE. Different lower case letters indicate statistically significant differences ($p \leq 0.05$) among

treatments within cultivars. Different upper case letters show differences ($p \leq 0.05$) among cultivars within treatments.

Fig 4 Photoprotective pigments of highbush blueberry cultivars grown in an acid soil (48% aluminum saturation) under different $CaSO_4$ treatments at 30 days. Values represent means (n = 5) ± SE. A: Total photoprotective pigments; B: α-carotene; C: Lutein; D: Neoxanthin; E: Violaxanthin; F: Anteraxanthin; G: Ant/Viol. Different lower case letters indicate statistically significant differences ($p \leq 0.05$) among treatments within cultivars. Different upper case letters show differences ($p \leq 0.05$) among cultivars within treatments.

Fig 5 Lipid peroxidation measured as malondialdehyde (MDA) concentration in highbush blueberry cultivar leaves (A) and roots (B), grown for 30 days under different treatments in an acid soil (48% aluminum saturation). Values are means ± SE (n = 5). Different lower case letters indicate statistically significant differences ($p \leq 0.05$) among treatments within cultivars. Different upper case letters show differences ($p \leq 0.05$) among cultivars within treatments.

Fig 6 Leaf and root-scavenging capacity, measured as Trolox equivalents (TE), of highbush blueberry cultivars grown in an acid soil (48% aluminum saturation), under different $CaSO_4$ treatments at 30 days. (A): leaves and (B): roots. Values are an average of five replicates ± SE. Different lower case letters indicate statistically significant differences (Tukey's HSD at $P \leq 0.05$) among treatments for the same cultivar. Different upper case letters indicate differences (Tukey's HSD at $P \leq 0.05$) among cultivar and similar treatment. Asterisk (*)

indicates statistically significant differences between tissues (leaves and roots) for the same cultivar and treatments.

Fig 7 Morpho-anatomical features of three highbush blueberry cultivars exposed under acid soil (48% aluminum saturation), and different $CaSO_4$ treatments for 30 days. The image of the transverse section of the leaves shows leaf thickness (LT), upper epidermis thickness (UET), lower epidermis thickness (LET), Palisade parenchyma thickness (PPT), and Spongy parenchyma thickness (SPT). Scale bars represent 5 µm.

Fig 8 Correlation matrices based on Pearson´s correlation coefficients between physiological and biochemical features of leaves and roots as well as leaves morphological traits of blueberry cultivars with contrasting Al resistance (Legacy A, Brigitta B, and Bluegold C), growing under increasing $CaSO_4$ treatments (acid soil + Al, 0.7, 1.4 and 2.8 g $CaSO_4$ kg^{-1} of soil + Al). Physiological parameters analyses were performed at acid soil and Ca-treated plants on different days (0, 7, 15, 30 days). Significant correlation coefficients ($p_{adj} \leq 0.05$) are indicated in bold. Each square represents three biological replicates average, with positive and negative correlations being distinguished by blue and red, respectively. Abbreviations: electron transport rate (ETR); non-photochemical quenching (NPQ); aluminum (Al); calcium (Ca); sulfur (S); radical scavenging activity (RSA); chlorophyll (Chl); upper epidermis thickness (UET); lower epidermis thickness (LET); palisade parenchyma thickness (PPT) and spongy parenchyma thickness (SPT).

Fig 9 Principal component analysis (PCA) representing physiological and biochemical features from leaves and roots as well as leaves morphological traits of three blueberry cultivars with contrasting Ca tolerance (Legacy, Bluegold, and Brigitta), growing under increasing $CaSO_4$ treatments (acid soil + Al, 0.7, 1.4 and 2.8 g $CaSO_4$ kg^{-1} of soil + Al). The analysis of physiological parameters was performed in plants grown in acid soil at 48% aluminum saturation. (A) A score plot of the first component (PC1) against the second component (PC2); it shows the averages of the whole data pool analyzed in the investigated samples. (B) The loadings plot obtained from the resulting distribution of physiological and biochemical data. Numbers in parentheses give the variation percentage explained by the first and the second principal component, respectively. Circle and text colors indicate the cluster assigned using hierarchical clustering. Abbreviations: electron transport rate (ETR); non-photochemical quenching (NPQ); aluminum (Al); calcium (Ca); sulfur (S); radical scavenging activity (RSA); chlorophyll (Chl); upper epidermis thickness (UET); lower epidermis thickness (LET); palisade parenchyma thickness (PPT) and spongy parenchyma thickness (SPT).

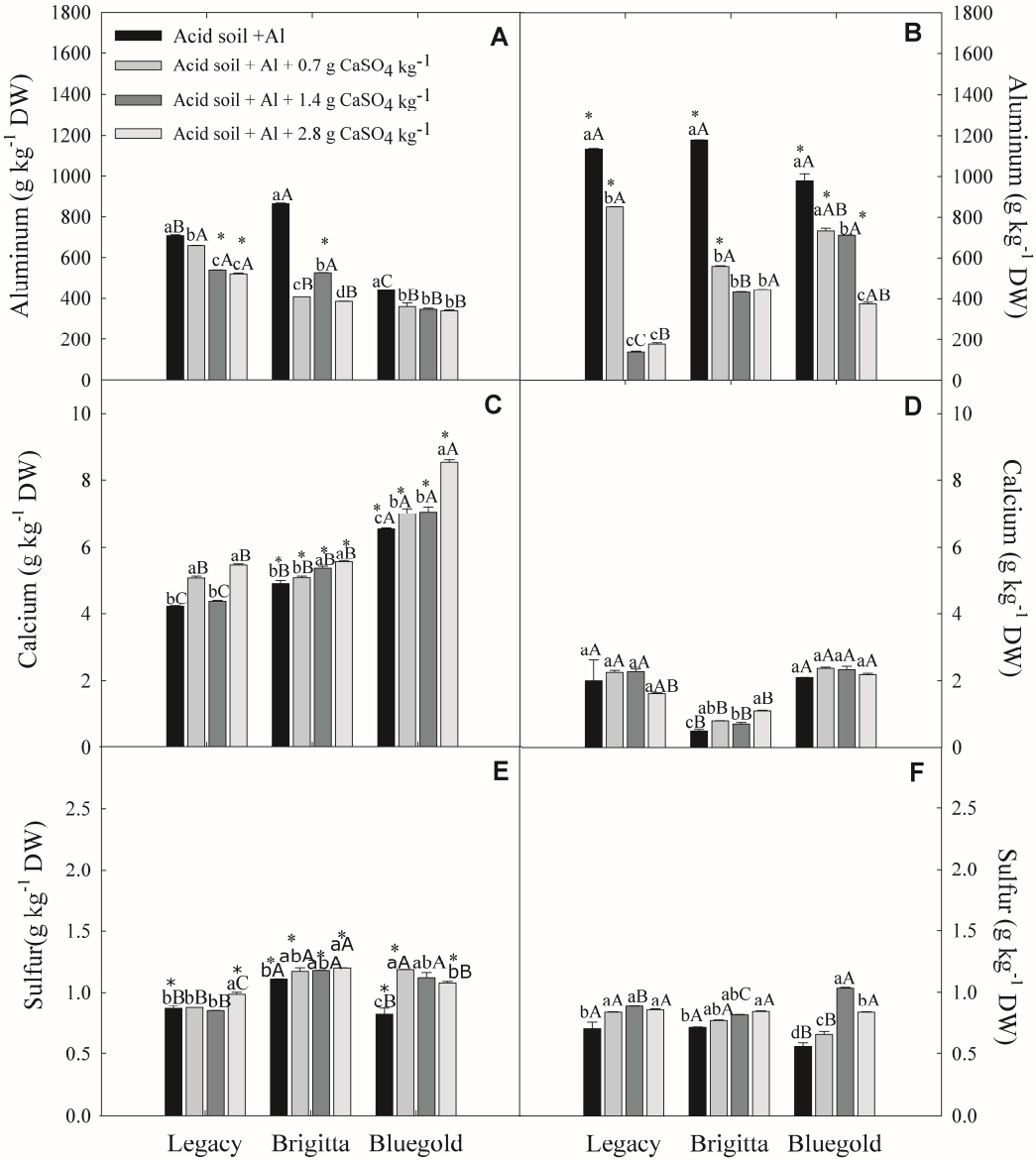
Acid soil +Al
Acid soil + Al + 0.7 g CaSO4 kg-1
Acid soil + Al + 1.4 g CaSO4 kg-1
Acid soil + Al + 2.8 g CaSO4 kg-1
A
B
C
D
E
F
Aluminum (g kg-1 DW)
Calcium (g kg-1 DW)
Sulfur(g kg-1 DW)
Sulfur (g kg-1 DW)
Legacy
Brigitta
Bluegold

Figure 1

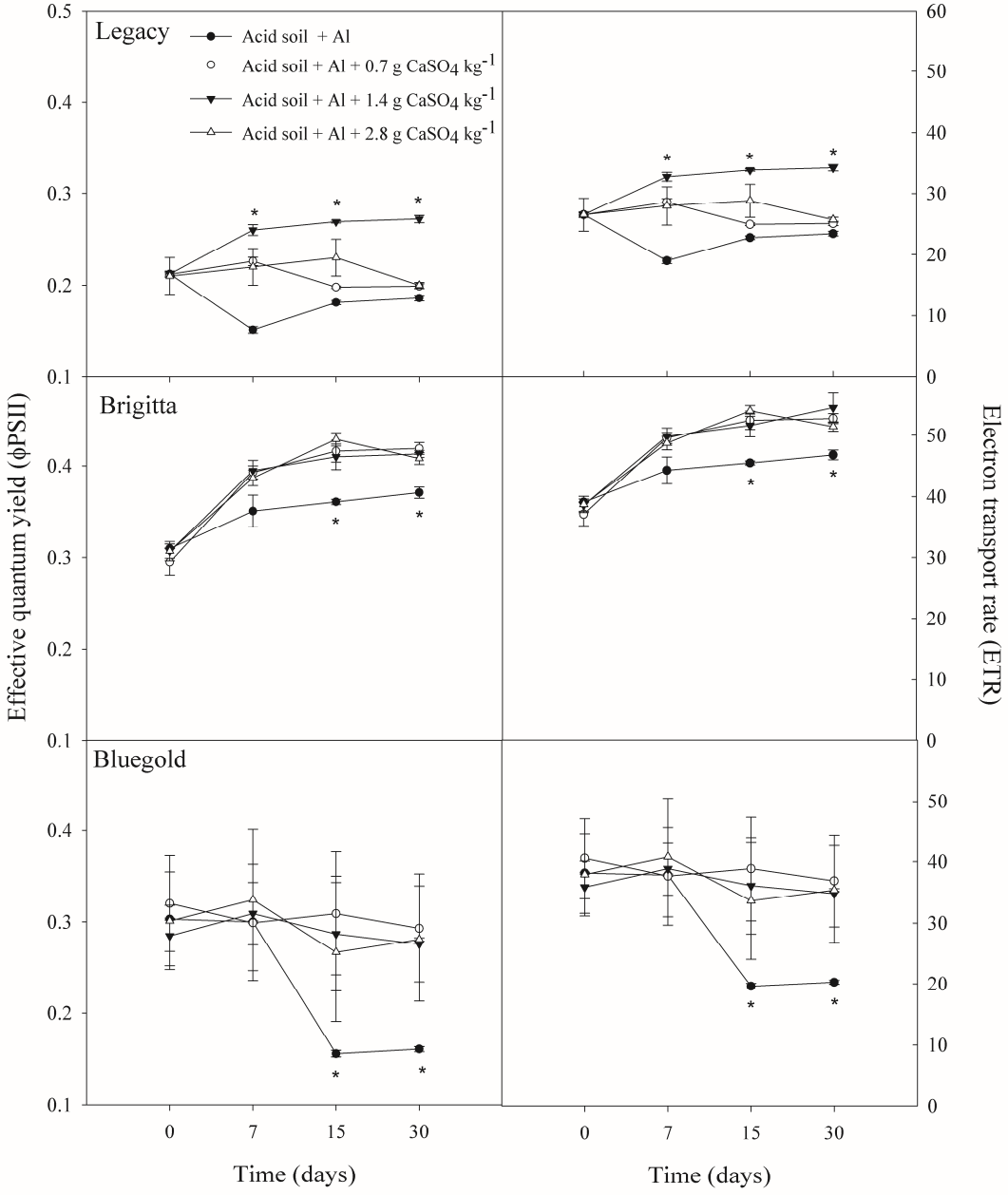
Legacy
Brigitta
Bluegold
Acid soil + Al
Acid soil + Al + 0.7 g CaSO4 kg-1
Acid soil + Al + 1.4 g CaSO4 kg-1
Acid soil + Al + 2.8 g CaSO4 kg-1
Effective quantum yield (ϕPSII)
Electron transport rate (ETR)
Time (days)
Time (days)

Figure 2

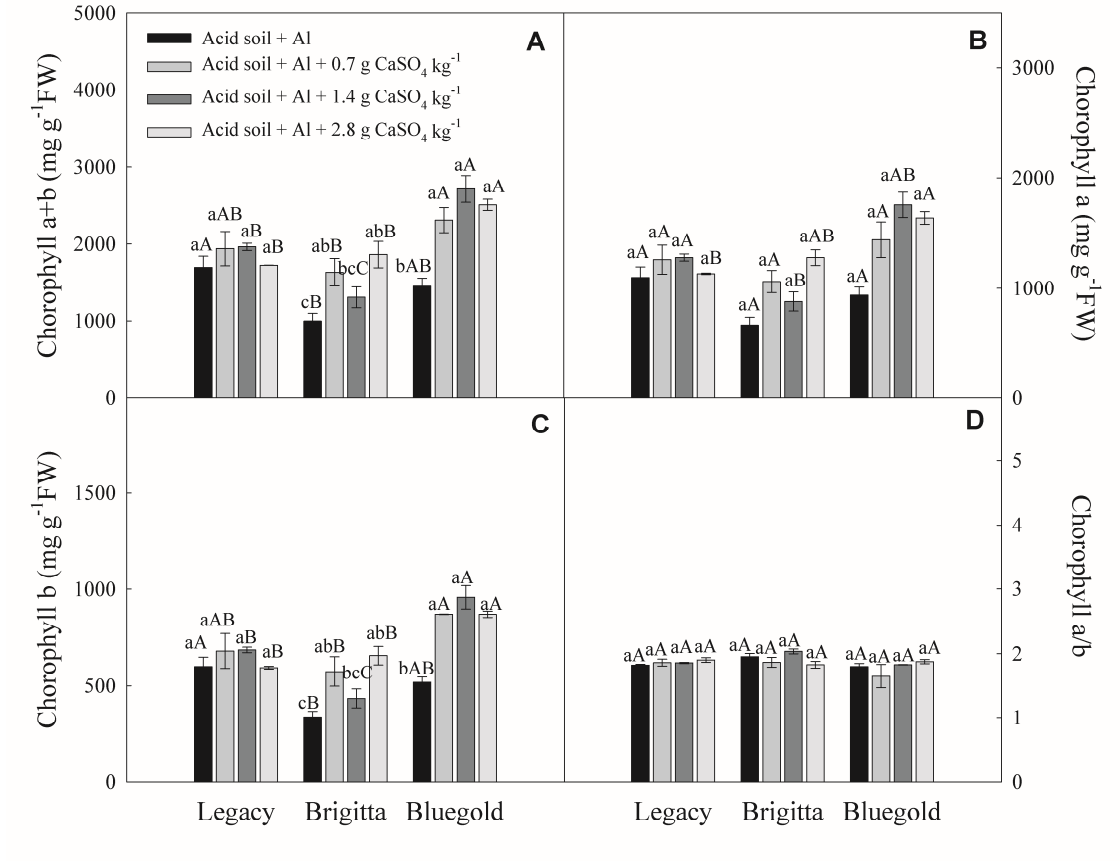
Acid soil + Al
Acid soil + Al + 0.7 g CaSO4 kg-1
Acid soil + Al + 1.4 g CaSO4 kg-1
Acid soil + Al + 2.8 g CaSO4 kg-1
A
B
C
D
Chorophyll a+b (mg g-1FW)
Chorophyll a (mg g-1FW)
Chorophyll b (mg g-1FW)
Chorophyll a/b
Legacy
Brigitta
Bluegold

Figure 3

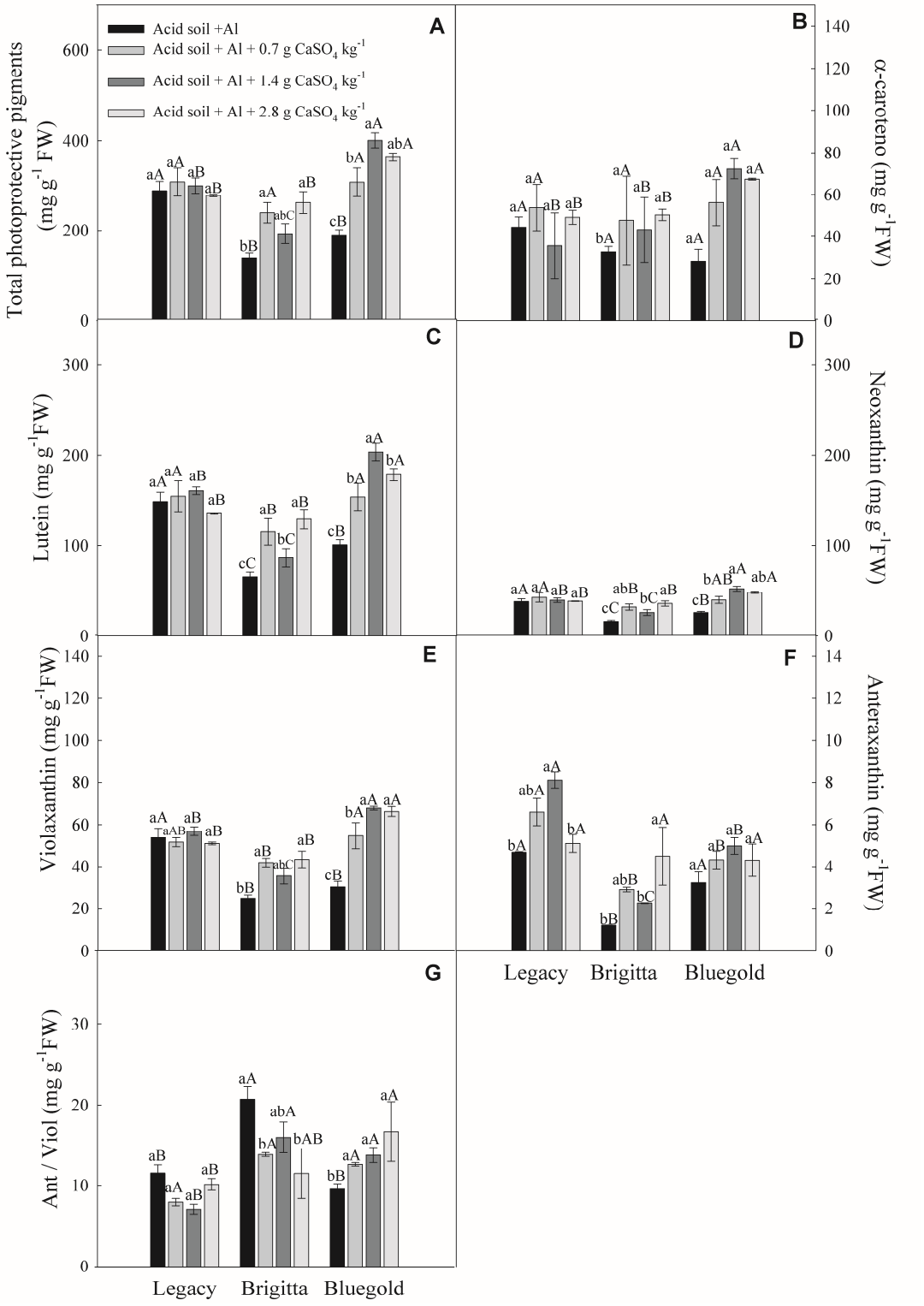
A
B
C
D
E
F
G
Acid soil +Al
Acid soil + Al + 0.7 g CaSO4 kg-1
Acid soil + Al + 1.4 g CaSO4 kg-1
Acid soil + Al + 2.8 g CaSO4 kg-1
Total photoprotective pigments (mg g-1 FW)
α-caroteno (mg g-1FW)
Lutein (mg g-1FW)
Neoxanthin (mg g-1FW)
Violaxanthin (mg g-1FW)
Anteraxanthin (mg g-1FW)
Ant / Viol (mg g-1FW)
Legacy
Brigitta
Bluegold

Figure 4

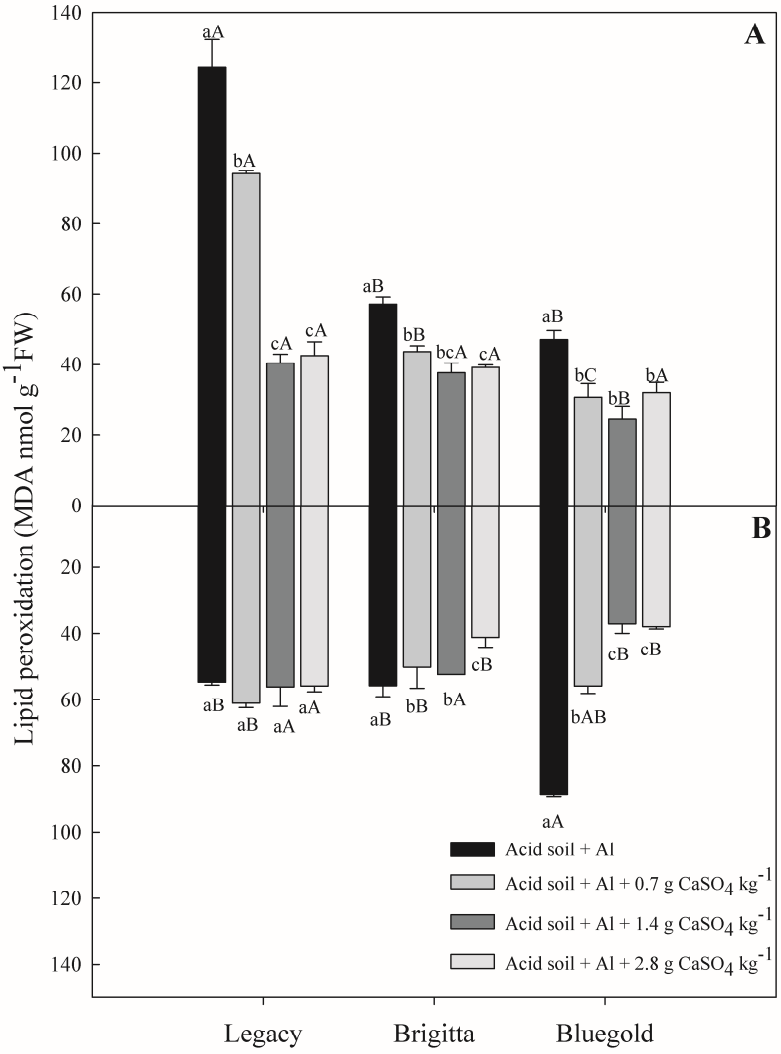
A
B
Lipid peroxidation (MDA nmol g-1FW)
140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
aA
bA
cA
cA
aB
bB
bcA
cA
aB
bC
bB
bA
aB
aB
aA
aB
bB
bA
cB
aA
bAB
cB
cB
Acid soil + Al
Acid soil + Al + 0.7 g CaSO4 kg-1
Acid soil + Al + 1.4 g CaSO4 kg-1
Acid soil + Al + 2.8 g CaSO4 kg-1
Legacy
Brigitta
Bluegold

Figure 5

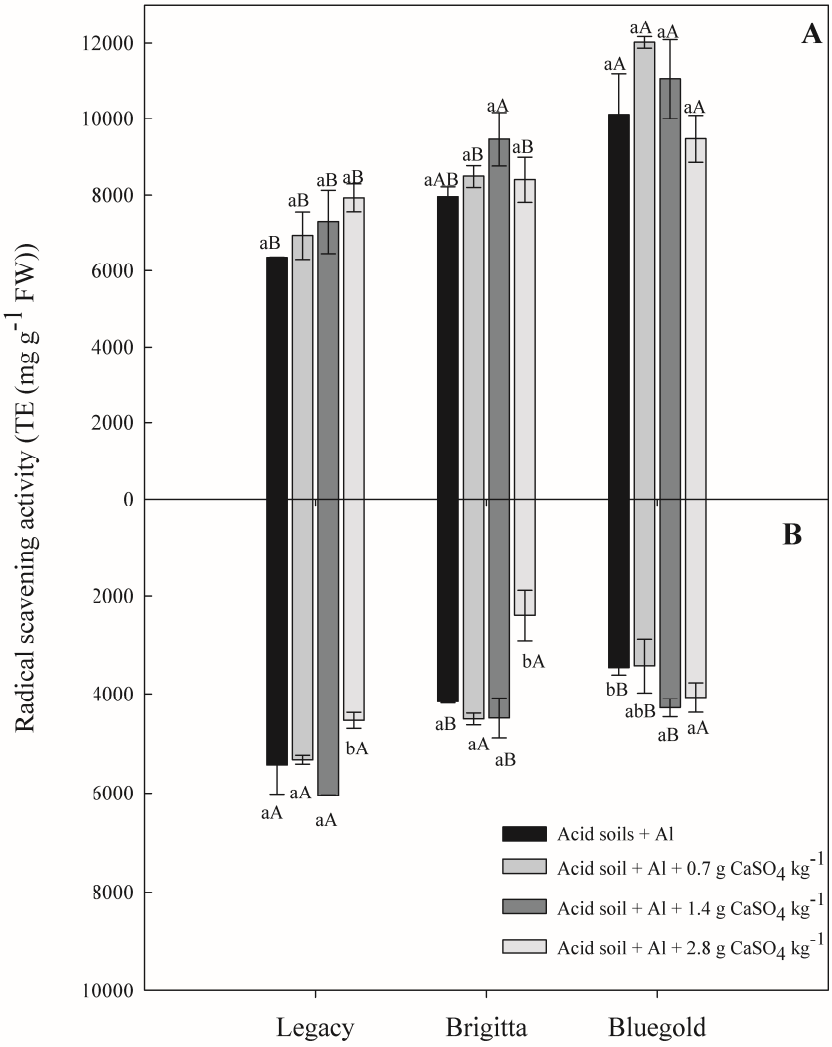
A
B
Radical scavening activity (TE (mg g-1 FW))
12000
10000
8000
6000
4000
2000
0
2000
4000
6000
8000
10000
Legacy
Brigitta
Bluegold
Acid soils + Al
Acid soil + Al + 0.7 g CaSO4 kg-1
Acid soil + Al + 1.4 g CaSO4 kg-1
Acid soil + Al + 2.8 g CaSO4 kg-1

Figure 6

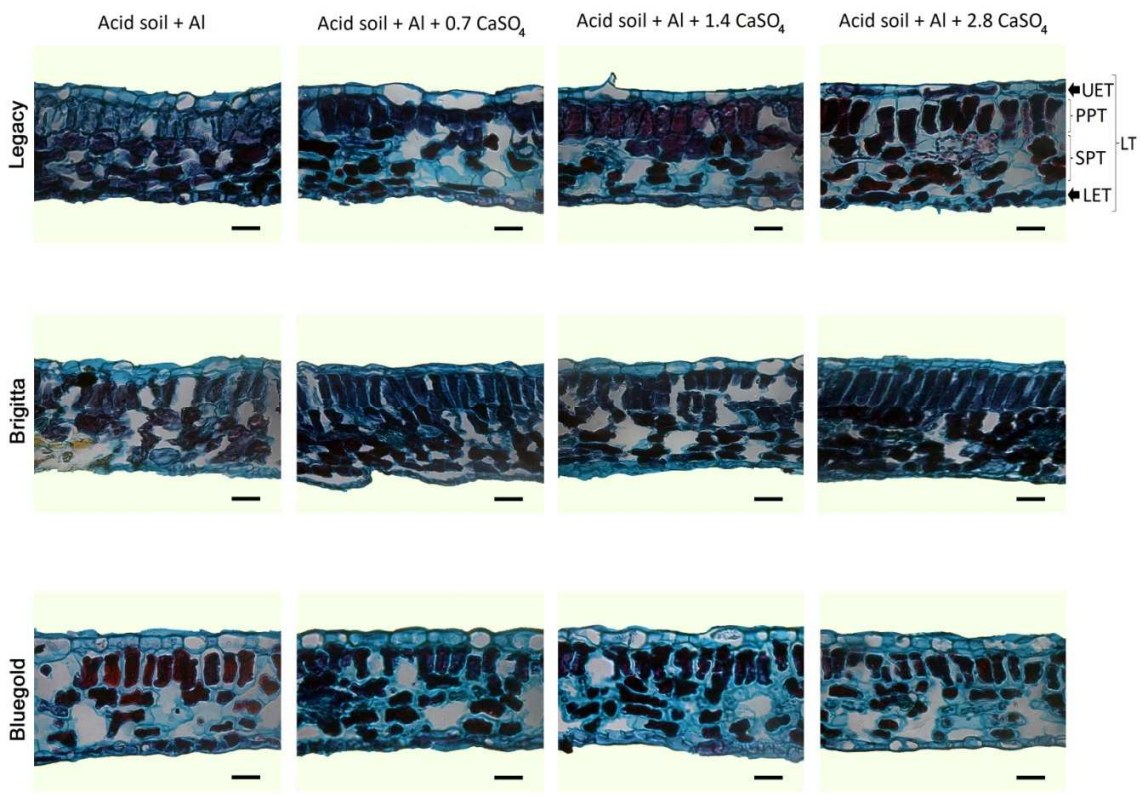
Acid soil + Al
Acid soil + Al + 0.7 CaSO4
Acid soil + Al + 1.4 CaSO4
Acid soil + Al + 2.8 CaSO4
Legacy
Brigitta
Bluegold
UET
PPT
SPT
LET
LT

Figure 7

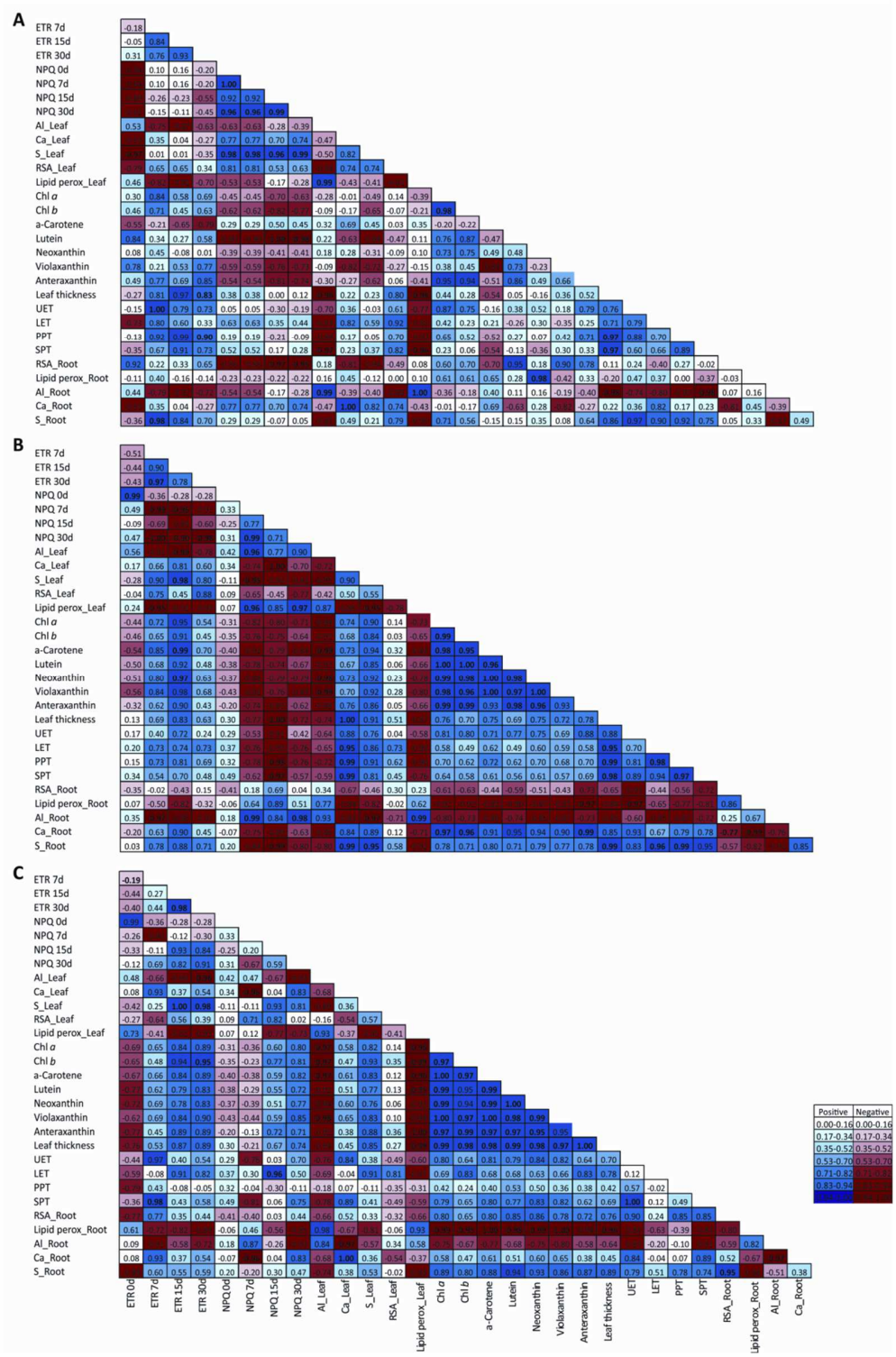
A
B
C
ETR 7d
ETR 15d
ETR 30d
NPQ 0d
NPQ 7d
NPQ 15d
NPQ 30d
Al_Leaf
Ca_Leaf
S_Leaf
RSA_Leaf
Lipid perox_Leaf
Chl a
Chl b
a-Carotene
Lutein
Neoxanthin
Violaxanthin
Anteraxanthin
Leaf thickness
UET
LET
PPT
SPT
RSA_Root
Lipid perox_Root
Al_Root
Ca_Root
S_Root
Positive
Negative
0.00-0.16
0.17-0.34
0.35-0.52
0.53-0.70
0.71-0.82
0.83-0.94

Figure 8

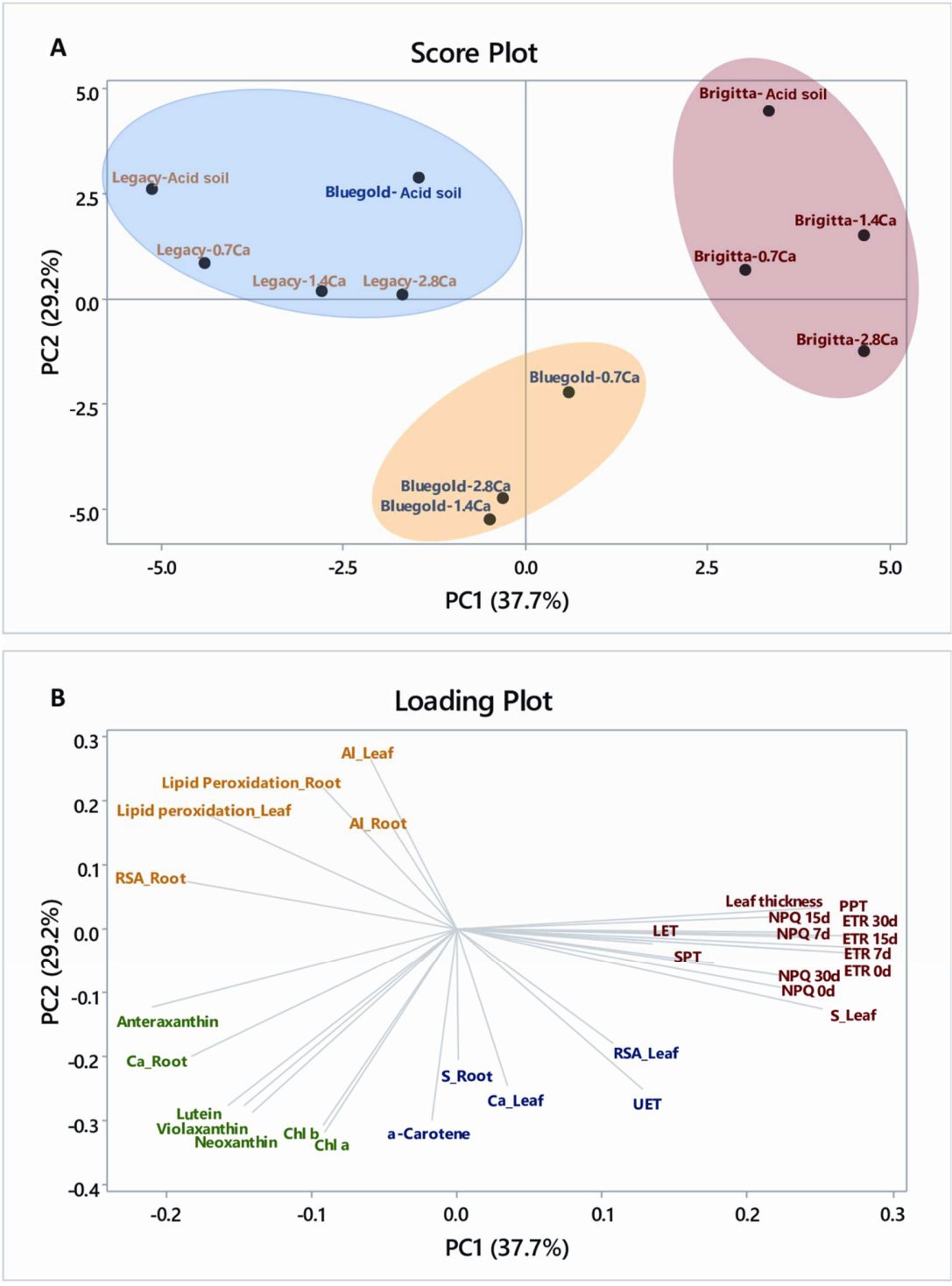
A
Score Plot
Brigitta- Acid soil
Legacy-Acid soil
Bluegold- Acid soil
Brigitta-1.4Ca
Legacy-0.7Ca
Brigitta-0.7Ca
Legacy-1.4Ca
Legacy-2.8Ca
Brigitta-2.8Ca
Bluegold-0.7Ca
Bluegold-2.8Ca
Bluegold-1.4Ca
PC2 (29.2%)
PC1 (37.7%)
B
Loading Plot
Al_Leaf
Lipid Peroxidation_Root
Lipid peroxidation_Leaf
Al_Root
RSA_Root
Leaf thickness
PPT
NPQ 15d
ETR 30d
LET
NPQ 7d
ETR 15d
SPT
ETR 7d
NPQ 30d
ETR 0d
NPQ 0d
S_Leaf
Anteraxanthin
Ca_Root
RSA_Leaf
S_Root
Ca_Leaf
UET
Lutein
Violaxanthin
Neoxanthin
Chl b
Chl a
a-Carotene
PC2 (29.2%)
PC1 (37.7%)

Figure 9

CHAPTER IV. Research article: *"Metabolic responses of Vaccinium corymbosum L. cultivars to Al toxicity and gypsum amendment"*.

Metabolic responses of *Vaccinium corymbosum* L. cultivars to Al^{3+} toxicity and gypsum amendment.

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Metabolic responses of *Vaccinium corymbosum* L. cultivars to Al^{3+} toxicity and gypsum amendment

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ABSTRACT

Highbush blueberry (*Vaccinium corymbosum* L.) is an important crop well adapted to acid soils, but sensitive to Al^{3+} toxicity. Gypsum amendments are frequently used to reduce Al^{3+} toxicity in *V. corymbosum*. However, little is known about the physiological and metabolic responses to gypsum application in plants growing in the presence of Al^{3+} toxicity. Thus, we evaluated the mechanisms displayed by gypsum application at the metabolite levels in *V. corymbosum* cultivars growing under Al^{3+} toxicity. We characterized three cultivars (Brigitta, Legacy, and Bluegold) with different response mechanisms to Al^{3+} toxicity. Furthermore, four treatments were applied: (i) Acid substrate without Al (Control), (ii) Acid substrate + 1.4 g $CaSO_4$ kg^{-1}, (iii) Acid substrate + 0.9 g $AlCl_3$ kg^{-1} and (iv) Acid substrate + 0.9 g $AlCl_3$ kg^{-1} + 1.4 g $CaSO_4$ kg^{-1}. After ten days of treatment exposition, leaves, and roots were harvested for metabolite profiling analyses. Starch and amino acid concentrations in leaves and roots decreased in all cultivars growing under toxic Al^{3+} levels. However, gypsum amendment reduced Al concentration in leaves and roots, as well as increased Ca concentrations in leaves, and recovered amino acid and starch levels. In addition, metabolite profiling and multivariate analyses indicated that in roots, gamma-aminobutyric acid (GABA) might be a metabolite related to Al^{3+} toxicity. Taken together that gypsum amendment ameliorates the Al^{3+} toxicity, mainly in the cultivar Al-sensitive, Bluegold. The two Al-resistant cultivars (Legacy and Brigitta) showed distinct Al mechanisms (tolerance and exclusion, respectively).

Keywords: Aluminum; highbush blueberry; primary metabolism; photosynthesis

1. Introduction

Acid soils (pH $\leq$ 5.5) cover from 30 to 50% of the arable soils in the world, are characterized by the presence of high aluminum (Al^{3+}) concentrations, which is one of the main negative factors constraining crop yields (Barcelo and Poschenrieder, 2002; Kochian et al., 2005; Saracoglu et al., 2009; Pontigo et al., 2017; Teng et al., 2018). The toxic Al^{3+} becomes available to plants in acid soil solution, negatively affecting physiological, biochemical and molecular processes (Borie and Rubio, 1999; Mossor-Pietraszewska, 2001; Du et al., 2009; Imadi et al., 2016; Zhang et al., 2019). It is known that root growth inhibition is the first symptom of Al^{3+} toxicity in plants, which affects uptake and translocation of water and nutrients such as calcium (Ca), potassium (K), sulfur (S), and magnesium (Mg), altering plant metabolism (Kinraide, 1998; Mora et al., 2005; Ryan et al., 2011; Delhaize et al., 2012; Silva et al., 2019). However, Al toxicity also provokes damages on the upper part of plants, reducing chlorophyll concentrations, photochemical efficiency of PSII, changes chlorophyll *a*/*b* ratio, and inhibiting the chloroplast electron transport chain, reducing the photosynthetic performance (Moustakas et al., 1995; Azmat and Hasan, 2008; Alarcón-Poblete et al., 2019; Santos et al., 2019). Indeed, previous studies in our group have shown that Al^{3+} toxicity strongly decreases the effective quantum yield of PSII (ΦPSII) in *V. corymbosum*, reducing CO_2 assimilation (Reyes-Díaz et al., 2010; Alarcón-Poblete et al., 2019; Cárcamo et al., 2019). Therefore, studies about metabolic responses under Al^{3+} toxicity in leaves and roots of plants are necessary. In fact, Al^{3+} toxicity can affect primary metabolic pathways such as amino acid, organic acid and carbohydrate metabolisms in leaves and roots of plants (Khan et al., 2000; Lin et al., 2005; Cheng et al., 2010; Cárcamo et al., 2019). Cárcamo et al.

(2019) reported that amino acid concentration increased significantly in *V. corymbosum* cv. Camellia and decreased in cv. Duke subjected to Al^{3+} toxicity. Likewise, total amino acids and sugars increased in shoots and roots of *Lens culinaris* and *Zea mays* under Al^{3+} toxicity (Khan et al., 2000; Azmat et al., 2007). Contrarily, Ismail (2005) reported that length growth of shoots and roots decreased as well as amino acids and sugar in *Daucus carota* and *Raphanus sativus* plants subjected to Al^{3+} toxicity; although, proline was increased in both species. Therefore, the responses on primary metabolite accumulation to Al^{3+} toxicity are species, genotype, and organ dependent. In this context, Yan et al. (2019) showed that 17 amino acids and 8 sugars in *Poncirus trifoliate* plants were accumulated under Al-stress, mainly histidine, cycloleucine, asparagine, raffinose, and trehalose, compared to control plants.

Recently, different studies reported that a non-protein amino acid, known as gamma-aminobutyric acid (GABA), is accumulated in plant tissues subjected to Al^{3+} toxicity (Ramesh et al., 2015; Ramesh et al., 2018; Seifikalhor et al., 2019). This effect has been associated with the modulation of adventitious root formation and growth in Poplar and also with enzymatic antioxidant system stimulation and modulation of Al transporters; thus, playing an important role in abiotic stress tolerance (Shelp et al., 2012; Seifikalhor et al., 2019; Xie et al., 2020).

The Al^{3+} toxicity in acid soils is usually decreased by calcareous amendments, such as lime, phosphogypsum, and gypsum (Mora et al., 2002; Meriño-Gergichevich et al., 2010; Alarcón-Poblete et al., 2019). It has been demonstrated that gypsum amendments in Andisol pasture, with toxic Al^{3+} levels, improves substantially plant growth (Mora et al., 2002). The positive effects of amendment application are usually related to Al^{3+} precipitation by lime and Al^{3+} complexation

by gypsum, decreasing the levels of free Al^{3+} and thus reducing its toxic effects in plants (Meriño-Gergichevich et al., 2010; Tirado-Corbalá et al., 2017). In addition, gypsum promotes the growth of root system, improving nutrient availability and water uptake to plants (Ritchey et al., 1995; Al-Oudat et al., 1998; Vyshpolsky et al., 2010; Batool et al., 2015; Alarcón-Poblete et al., 2019).

In southern Chile, the highbush blueberry (*V. corymbosum*) is an important fruit crop, due to the high market price of its fruits, which are rich in nutritional properties for human health, such as antioxidants (Guerrero, 2006; Baliga and Katiyar, 2006; Guerrero et al., 2010). This crop is well adapted to acid soils, but it is sensitive to the presence of Al^{3+}, which promotes biochemical and molecular changes, decreasing its productivity (Blatt and McRae, 1997; Suzuki et al., 1999; Reyes-Díaz et al., 2010, Inostroza-Blancheteau et al., 2011; Ulloa-Inostroza et al., 2019). Thus, the gypsum amendment has been proposed as important crop management to decrease the Al^{3+} toxicity for fruit crop species. In fact, Al^{3+} treatment reduces the photosynthetic performance of *V. corymbosum* plants, which is clearly restored by the gypsum amendment (Reyes-Díaz et al., 2011; Alarcón-Poblete et al. 2019). This effect seems to be associated with the positive effect of gypsum amendment on leaf morphology, as well as photochemical and biochemical damages in highbush blueberry under Al-toxicity (Alarcón-Poblete et al., 2019). Despite the positive effects of gypsum on the physiology of plants under toxic levels of Al^{3+}, little is known about the effect of gypsum application on primary metabolic pathways in leaves and roots tissues. Therefore, the aim of this work was to investigate the mechanisms displayed by gypsum application at the metabolite levels in *V. corymbosum* cultivars growing under Al^{3+} toxicity.

2. Material and Methods

2.1 Plant material and growth conditions

One-year-old highbush blueberry (*V. corymbosum*) plants from cultivars Legacy, Brigitta, and Bluegold, with contrasting levels of resistance to Al^{3+} toxicity (Reyes-Díaz et al., 2009; 2011), were used in this study. Plants were provided by Berries San Luis, Lautaro (38°29 S and 72°23 W), Araucania Region, Chile. The experiment was carried out under greenhouse conditions at the Universidad de La Frontera (Temuco, Chile). Growth conditions were exactly the same described in our previous studies (Reyes-Diaz et al., 2009; 2011; Alarcón-Poblete et al., 2019) with a temperature of 25/20 ºC (day/night), 16/8-h (light/dark) photoperiod, 80% of relative air humidity and an average of 300 µmol photons m^{-2} s^{-1} photosynthetic photon flux density (PPFD).

Before starting the experiment, plants were washed with deionized water and transferred to 2 liters pot (1 plant per pot) containing peat, vermiculite, and acid bark, in proportion 50:30:20 and watered with Hoagland solution (Hoagland and Arnon, 1959) at pH=4.5. Afterwards the following treatments were established: 1) Control (acid substrate without Al, pH 4.5; 2) Ca, 1.4 g $CaSO_4$ kg^{-1} of acid substrate (Ca); 3) Al, 0.9 g $AlCl_3$ kg^{-1} of acid substrate (Al); and 4) Ca + Al, 1.4 g $CaSO_4$ + 0.9 g $AlCl_3$ kg^{-1} of acid substrate (Ca + Al). The plants were maintained under these conditions for 10 days, and physiological measurements were evaluated in leaves from five individual plants per treatment at 0, 5, and 10 days of the experiment. At the end of the experiment (10 days under treatment), leaves and roots samples were collected in the middle of the light period, snap-frozen in liquid nitrogen, and stored at -80ºC until chemical and biochemical analyses.

2.2 Chemical analysis

Aluminum and calcium concentrations in leaves and roots

At the end of the experiment, mature expanded leaves from the first to the fourth node of shoots and roots were dried separately at 70ºC in a forced air oven for 48 h. Samples were ashed at 500ºC for 8 h and then treated with 2M hydrochloric acid. Al and Ca were quantified using a simultaneous multielement atomic absorption spectrophotometer (Model 969 Atomic Absorption Spectrometer, Unicam, Cambridge, UK), as described in Sadzawka et al. (2007).

Sulfur concentration in leaves and roots

For sulfur (S) concentration analysis, plant tissues were collected as mentioned above, dried and treated with 95% magnesium nitrate ($MgNO_3$ x $6H_2O$), and ashed at 500ºC for 8 h. Ashed samples were digested for 60 min in 10 mL of 2M HCl at 150ºC before the addition of barium chloride ($BaCl_2$) and Tween-80. The resulting solution was measured in a UV/VIS spectrophotometer (UNICO® 2800 UV/VIS, Spain) at 440 nm, as described by Sawdzaka et al. (2007).

2.3 Physiological and metabolic analyses

Chlorophyll fluorescence parameters of PSII

In vivo chlorophyll *a* fluorescence parameters were determined using a portable pulse-amplitude modulated fluorimeter (FMS 2; Hansatech Instruments, King's Lynn, UK) to determine the photochemical efficiency of PSII according to Reyes-Díaz et al. (2009). The maximum quantum yield (Fv/Fm), effective quantum yield (ΦPSII), electron transport rate (ETR) and non-photochemical quenching (NPQ) were calculated as described by Maxwell and Johnson (2000).

2.4 Total metabolites analysis

The extraction of total metabolites was performed after grinding leaf and root samples in liquid nitrogen, followed by the immediate addition of the appropriate extraction buffer exactly as previously described (Gibon et al., 2004). Total protein and starch (in the insoluble fraction) and amino acids (in the soluble fraction) were quantified, according to Cross et al. (2006). In addition, Chlorophyll *a* and *b* contents were determined as detailed in (de Oliveira-Silva et al., 2018).

2.5 Metabolite profiling analysis

A total of 20 mg of lyophilized material was extracted and derivatized following the protocol described by Lisec et al. (2006). The metabolites were determined in a Gas-chromatography-TruTof-mass-spectrometry (GC-MS) system, according to Lisec et al. (2006). Chromatograms were exported from ChromaTof software (version 3.25) and processed in the program R. Peak detection, retention time alignment, and library matching were performed using the Target Search from Bioconductor package in R software (Cuadros-Inostroza et al., 2009). Each analyte peak was normalized to the peak of the internal standard (ribitol) and by the dry weight of each sample, following the recommended reporting format for metabolic profiling (Fernie et al., 2011).

2.6 Experimental design and statistical analyses

The experimental design was completely randomized, with five replicates per each treatment. For the determination of interactions among physiological and biochemical parameters, a two-way ANOVA was used, with the exception of fluorescence parameters, where three-way ANOVA was performed. A Tukey test

was used to identify those values with significant differences. All statistical determinations were carried out by five replicates, using Sigma Stat software (version 2.0; SPSS, Chicago). Differences between the values were considered significant at $P \leq 0.05$.

Multivariate analysis by Principal Components Analysis (PCA) was performed in the Minitab® 17 statistics program (Minitab Inc., Philadelphia, USA), in order to provide insight into the effects of gypsum application on biochemical and physiological features. The PCA permits to reduce the dimensionality of the data set, and identify the variables that explained a higher proportion of the total variance. Furthermore, a mapping of leaf and root metabolites in representative pathways was represented as a heatmap using Microsoft PowerPoint (Microsoft Corporation, Seattle, USA). For this purpose, each data set was transformed into Log_2, and the values were normalized to the average response calculated for the control treatment.

3. Results

Al, Ca, and S in leaves and roots of highbush blueberry cultivars

Al, Ca, and S concentrations were determined in leaves and roots of *V. corymbosum* cultivars subjected to different treatments (Fig. 1). As expected, the Al treatment leads to higher accumulation of Al in leaves and roots of all cultivars compared to the other treatments. In all analyzed cultivars, Al concentration was higher in roots than in leaves. In roots, Bluegold and Legacy accumulated higher amounts of Al than Brigitta cultivar (Fig. 1B), whereas the Al-sensitive cultivar Bluegold accumulated more Al concentration in leaves compared to Brigitta and Legacy considered as Al-resistant cultivars (Fig. 1A). Gypsum treatment leads to

a reduced Al level in both tissues of all cultivars with respect to Al treatment. Interestingly, the cultivars exhibited different Al concentration in the Ca + Al treatment, with the highest reduction (about 78%) in the sensitive cultivar Bluegold, in comparison with Al alone treatment in roots (Fig. 1B). In leaves, Al levels were reduced, reaching near to control levels when the gypsum amendment was applied in all cultivars (Fig. 1A).

The Ca concentrations in leaves were significantly reduced in the Al treatments compared with their controls in all cultivars (Fig.1C). Meanwhile, gypsum application increased Ca concentration in leaves of Brigitta and Legacy, respect to their controls, with the highest Ca concentration in Brigitta (Fig. 1C), whereas in roots, Ca concentration did not change under all treatments and cultivars (Fig. 1D). Furthermore, we evaluated S levels in both organs in *V. corymbosum* cultivars. As expected, gypsum application increased S levels in leaves of Brigitta (35%), Legacy (28%), and Bluegold (28%) compared to the control (Fig. 1E). Surprisingly, Al-sensitive cultivar Bluegold decreased S levels in roots of all treatments compared to the control (Fig. 1F).

Fluorescence parameters of the PSII and chlorophyll pigments

Fluorescence emission was measured *in vivo* at the beginning of experiment 0, 5, and 10 days (Supplementary Table 1). The maximum photochemical efficiency of PSII (Fv/Fm) did not vary among the three cultivars and among treatments. This parameter varied in the range of 0.81 to 0.85, indicating healthy plant leaves (Maxwell and Johnson 2000). Furthermore, the ΦPSII, ETR, and NPQ values were not altered among cultivars, treatments and time points. In agreement, the levels of chlorophyll *a* and *b* did not change significantly under all treatments, and

cultivars (Supplementary Fig. 1). Despite that, the total Chlorophyll levels (Chl *a*+*b*) in Brigitta increased significantly in all treatments with respect to the control (Supplementary Fig. 1C). Meanwhile, Chl *a*+*b* levels were not altered in Legacy and Bluegold. The Chl *a*/*b* ratio of Brigitta strongly decreased in leaves from plants under gypsum amendment (46%) and Al (36%) treatments compared to the control treatment, while this ratio remained constant in the other cultivars (Supplementary Fig. 1D).

Metabolites levels in leaves and roots of highbush blueberry cultivars

Metabolite analyses revealed different responses in protein, amino acids, and starch levels in leaves and roots of blueberry cultivars under different gypsum treatments (Fig. 2). In Brigitta, the protein levels were drastically reduced in leaves of plants subjected to all treatments compared to control (Fig. 2A). In Legacy, protein levels in leaves were reduced only when Ca + Al treatment was applied. Intriguingly, the protein levels increased significantly in the roots of Legacy plants in the presence of Ca (Fig. 2B). Meanwhile, the roots of Brigitta and Bluegold did not change in protein levels among the treatments. The Al treatment decreased by about 35% of the total free amino acid levels in leaves (Fig. 2C) and about 45% in roots compared to the control treatment in all cultivars (Fig. 2D). Interestingly, the amino acid levels were recovered at the level of controls when applied gypsum amendment and gypsum in combination with Al in Brigitta and Legacy leaves (Fig. 2C). In roots, the levels of the free amino acid were increased in Legacy (30%) and Bluegold (3-fold) under Ca + Al treatment compared to Al treatment, whereas in Brigitta roots, any difference was observed (Fig. 2C and D).

On the other hand, Al treatment leads to a strong decrease in starch levels in Bluegold (4-fold) followed by Legacy (40%) leaves, compared with their controls (Fig. 2E). Meanwhile, a strong reduction in starch levels was observed for Bluegold (2.5-fold) and Legacy (3-fold) roots growing under Al treatment, as compared with their control treatments (Fig. 2F), whereas gypsum amendment recovered the starch levels increasing up to 3.5-fold in Legacy followed by Bluegold (2.5-fold) (Fig. 2F).

Metabolite profile in leaves and roots of highbush blueberry cultivars

Regarding the levels of other groups of metabolites, such as main sugars, organic acids, and amino acids, we also analyzed the changes in leaves and roots of *V. corymbosum* cultivars under the tested conditions (Fig. 3 and 4). In general, the presence of Ca or Al displayed several and similar patterns of changes in the levels of these metabolites in leaves and roots of the three cultivars. However, the observed responses were clearly organ and cultivar dependent. Thus, glucose only decreased in leaves and roots of Brigitta, while in Bluegold, it only decreased in leaves under both Ca and Al treatments. Meanwhile, Legacy increased glucose levels in roots under Al treatment (Fig. 3 and 4). Sucrose levels in leaves and roots did not show any significant difference between treatments in all cultivars. Maltose, a starch degradation product, exhibited a mild reduction in leaves of Brigitta cultivar with a significant increase in roots under Al treatment.

Several changes were observed in leaves and roots in the abundance of the tricarboxylic acid (TCA) cycle intermediates following Al or Ca application (Fig. 3 and 4, respectively). Malate and succinate decreased in leaves and roots of the Brigitta cultivar. Citrate decreased in roots of Legacy under Ca application,

whereas in leaves of Bluegold, citrate increased under Al treatment. Fumarate levels were significantly increased in leaves of Legacy and Bluegold cultivars, without changes in roots under Al and Ca treatments. Malate levels, an alternative fumarate precursor, showed similar changes observed for fumarate with the exception of a significant decrease in roots. Glycerate and citramalate levels were reduced in Brigitta leaves under Al and Ca treatments, and glycerate was reduced in Bluegold roots. The presence of Al in the substrate also displayed changes in dehydroascorbate levels, which was increased in leaves of Brigitta and Bluegold; meanwhile, it decreased significantly in the roots of Legacy and Bluegold cultivars.

Concerning to amino acids, glutamine levels increased in Brigitta leaves under Al; meanwhile, it decreased in Legacy leaves. The serine levels were significantly reduced in Brigitta leaves and Legacy roots of plants growing under Al and Ca treatments. Alanine levels exhibited a clear tendency of increase in leaves of Legacy and Bluegold but was reduced in Brigitta following Al and Ca treatment. Interestingly, the levels of gamma-aminobutyric acid (GABA) were significantly increased in leaves of Legacy under Al treatment, being higher in roots of the resistant cultivar (Legacy) than in the sensible (Bluegold) ones, where GABA significantly decreased (Fig. 3 and 4).

Principal Component Analysis

Principal component analysis (PCA) plot of metabolite profile from leaves and roots indicated in the first component clear differences among Al-resistant cultivars Brigitta, Legacy, and the sensitive cultivar Bluegold (Fig. 4A). Distributions along the first and second components are shown in the same

figure. The first and second components accounted for 29.4% and 16.1% of the total variation, respectively. Furthermore, when we analyzed the second component, a separation between treatments (Al, Ca, Al + Ca, and Control) and a gradient of responses, represented by dotted arrows in Fig. 4A is observed. In Legacy and Bluegold cultivars, Al + Ca treatment is closer to control treatment than in Brigitta.

Interestingly, the separation of cultivars showed that the first cluster composed by Brigitta and Legacy was influenced by changes of the most leaves metabolites observed, and the second cluster composed by Bluegold was influenced by changes of the most roots metabolites observed (Fig. 4B). On the other hand, this separation observed in the first component was characterized by changes in amino acid levels, especially GABA, histidine, valine, isoleucine, and glutamate (Fig. 4B), thus suggesting that these metabolites may have a central role in *V. corymbosum* responses to toxic levels of Al^{3+}.

4. Discussion

It is well known that in acid soils, Al^{3+} toxicity negatively affects physiological and biochemical processes, reducing plant growth and crop yield (Reyes-Diaz et al., 2011; Imadi et al., 2016; Zhang et al., 2019). In previous studies, we classified Brigitta, and Legacy as Al-resistant cultivars, and Bluegold as Al-sensitive cultivar, according to their physiological and biochemical features under Al^{3+} toxicity (Reyes-Díaz et al., 2009; 2011; Alarcón-Poblete et al., 2019). In this study, we analyzed the metabolite responses in leaves and roots of Legacy, Brigitta and Bluegold cultivars subjected to Al^{3+} toxicity and gypsum amendment in the acid substrate. Although it is known that roots are the main target of Al

toxicity, several studies have indicated that toxic Al also provokes damages in the upper part of plants (Reyes-Díaz et al., 2009; 2011; Yang et al., 2015; Banhos et al., 2016; Guo et al., 2018; Alarcón-Poblete et al., 2019; Cárcamo et al., 2019). As we expected, in our study, the Al concentration increased in leaves and roots of all *V. corymbosum* cultivars subjected to Al^{3+} toxicity, being higher in leaves of the Al-sensitive Bluegold cultivar; meanwhile, Ca concentrations in leaves were significantly reduced in the same treatment, suggesting that Al can be translocated from root to shoot, inhibiting Ca transport by blocking the Ca^{2+} channels previously reported (Ryan and Kochian 1993; Plieth, 2005). Therefore, Ca-Al relation is strongly associated with Al^{3+} toxicity. Our findings are in agreement with previous results published by our group (Reyes-Díaz et al., 2011), where Brigitta and Legacy accumulated lower Al concentration in leaves compared to Bluegold cultivar under Al^{3+} toxicity treatment and decreasing Al concentration under gypsum application. In this sense, the gypsum amendment is used in order to cope with Al^{3+} toxicity in plants (Mora et al., 2006; Tirado-Corbalá et al., 2017). Gypsum amendment has been demonstrated that improves substantially plant growth by Al^{3+} complexation, decreasing the levels of free Al^{3+} and thus reducing its toxic effects in plants (Meriño-Gergichevich et al., 2010; Tirado-Corbalá et al., 2017). Thus, Alarcón-Poblete et al. (2019) showed that $CaSO_4$ application reduced the presence of free Al^{3+} in the substrate, and consistently decreased the Al levels in both leaves and roots of *V. corymbosum* cultivars, which was observed in our study. According to some studies, gypsum is also an important source of Ca^{2+} and S, which can improve mineral concentration such as nitrogen, phosphorus, calcium, and sulfur in plant organs (Bolan et al., 1993; Caires et al., 2006; Tuna et al., 2007). Calcium plays a key

role in physiological and biochemical processes, including a structural role in cell walls and cell membranes, and as a secondary messenger in signal transduction pathway (White and Brodley, 2003; Riveras et al., 2015; Thor 2019). In our study, Ca concentration significantly increased in Brigitta and Legacy leaves under $CaSO_4$ treatment. $CaSO_4$ amendment also increased S levels in leaves of all *V. corymbosum* cultivars. Similar results were verified in *Lolium perenne* subjected to Al^{3+} toxicity (Mora et al., 2002). Reyes-Díaz et al. (2011) showed a significant and positive correlation (r=0.6) between S concentration of leaves and CO_2 assimilation in *V. corymbosum* cultivars subjected to Al^{3+} toxicity. According to Mobin (2010), S deficiency triggers stomatal closure decreasing CO_2 assimilation in plants. Therefore, gypsum amendment can be used to ameliorate Al^{3+} toxicity, and recovers Ca and S uptake, improving CO_2 assimilation in *V. corymbosum* cultivars.

Aluminum toxicity has also been reported to affects synthesis of protein, amino acids, sugars, and organic acids (Azmat et al., 2007; Cheng et al., 2010; Yan et al., 2019). Our results indicated a decrease in total amino acids under Al treatment with respect to the control plants in all *V. corymbosum* cultivars. Our findings are in agreement with Ismail (2005), where total amino acids and starch were reduced in roots and leaves of *Daucus carota* and *Raphanus sativus* subjects to Al^{3+} toxicity. As reported by Zhao and Shen (2018), Al^{3+} toxicity may inhibit nitrogen uptake and its assimilation, decreasing amino acid and protein levels in plants. In contrast, $CaSO_4$ amendment recovered amino acid and protein levels in our *V. corymbosum* cultivars subjected to Al^{3+} toxicity. We also observed that the gypsum amendment significantly increased valine levels in roots of all *V. corymbosum* cultivars subjected to Al toxicity. However, the function of some

amino acids, such as valine, is not fully understood under abiotic stress conditions (Hildrebrandt, 2018). On the other hand, carbohydrates are among the most abundant root components, and it is plausible to suppose that changes in their quantities and rates would be influenced by Al^{3+} toxicity (Hoshino et al., 2000). Aluminum toxicity has been shown to decrease photosynthetic performance in several species such as *Citrus sinensis, Citrus grandis, Eucalyptus grandis*, and *V. corymbosum* (Reyes-Díaz et al., 2011; Yang et al., 2015; Banhos et al., 2016; Guo et al., 2018; Alarcón-Poblete et al., 2019). Indeed, Reyes-Díaz et al. (2011) observed that CO_2 assimilation was reduced in *V. corymbosum* cultivars subjected to Al^{3+} toxicity, being Bluegold the most affected cultivar. This result coincided with our findings, which was observed lower starch and sugar levels in plants subjected to Al treatment. However, our results showed that the $CaSO_4$ amendment recovered starch levels in all *V. corymbosum* cultivars under Al^{3+} toxicity.

Plants increase the synthesis of certain organic acids in plant tissues in order to cope with Al^{3+} toxicity (Ma et al., 2001; Nunes-Nesi et al., 2014; Yan et al., 2019). In our study, leaves and roots exhibited distinct organic acid profiles among cultivars indicating different activity of TCA cycle enzymes following by Al treatment. In this regard, Legacy roots accumulated a higher amount of Al (2-fold) compared to Brigitta, exhibiting a higher increase of organic acid in Legacy to tolerate Al toxicity. These results suggest distinct Al responses mechanisms used by the two Al-resistant cultivars, exclusion, and tolerance mechanisms to Brigitta and Legacy, respectively. On the other hand, we observed different patterns of Al accumulation between roots and shoots, being Bluegold, who showed the highest Al concentration in shoots compared to the other cultivars, indicating a

major translocation of Al from roots to shoots (Fig. 1). Thus, differences among cultivars were also observed by Cárcamo et al. (2019), where the lowest Al concentration was presented in Camellia roots, followed by Duke, Star, Brigitta, and Cargo.

The PCA scores plot revealed that based on metabolite data from root and leaf organs a clear separation in the first component of the Brigitta and Legacy cultivars from the Al-sensitive cultivar Bluegold (Fig. 4A), where the most changes of metabolites in leaves were observed by the first cluster and the most changes in roots was observed in the second cluster (Fig. 4B). These results confirm our suggestion that the Al-sensitive and Al-resistant cultivars display distinct response mechanisms to Al toxicity. This can be clearly observed in leaves and roots, where GABA concentrations were lower in the sensitive cultivar than in the resistant cultivar Legacy. It has been reported that in seedlings of *Triticum aestivum* GABA levels in roots were significantly higher in the Al-resistant compared with the Al-sensitive cultivar, which also negatively regulates Aluminum-activated Malate Transporter (ALMT) proteins, decreasing organic acids efflux (Ramesh et al., 2015). This result suggested that GABA negatively regulates ALMT, inhibiting the efflux of malate. In this sense, our findings demonstrated that the Al-resistant cultivar Legacy also increases GABA, which could regulate malate efflux in roots, permitting that this organic acid chelates Al in roots of this cultivar. However, studies showing the GABA involvement in Al^{3+} toxicity are still limited, and further studies may uncover novel insights into the role of this amino acid in plants against metal toxicity (Seifikalhor et al., 2019). Taken together, these findings suggest that GABA might be involved in metabolite reprogramming of resistance mechanism responses to Al^{3+} toxicity.

5. Conclusion

Our results indicate that the gypsum amendment ameliorates the Al^{3+} toxicity effects at the metabolite levels in *V. corymbosum* cultivars, mainly in the Al-sensitive Bluegold. On the other hand, our findings demonstrated distinct Al response mechanisms used by the two Al-resistant cultivars, exclusion, and tolerance mechanisms to Brigitta and Legacy, respectively. Noteworthy that the number of metabolites displayed significant changes even after the $CaSO_4$ amendment was higher in roots than in leaves, demonstrating distinct metabolic patterns between tissues. In addition, GABA seems to be related to the metabolic responses displayed by Al-resistant cultivar Legacy due to its increase in both leaves and roots. Thus, these apparent complex metabolic reprogramming displayed by different tissues and cultivars of *V. corymbosum* provided us new knowledge for understanding the changes that occur during Al^{3+} toxicity, and that leads to a considerable decrease in crop yield. In order to understand this new association between GABA and Al^{3+} toxicity responses in highbush blueberry, further investigations of metabolite levels at different time points in plants under Al^{3+} toxicity, as well as transcript profiling at the same time points provide scope for future research work.

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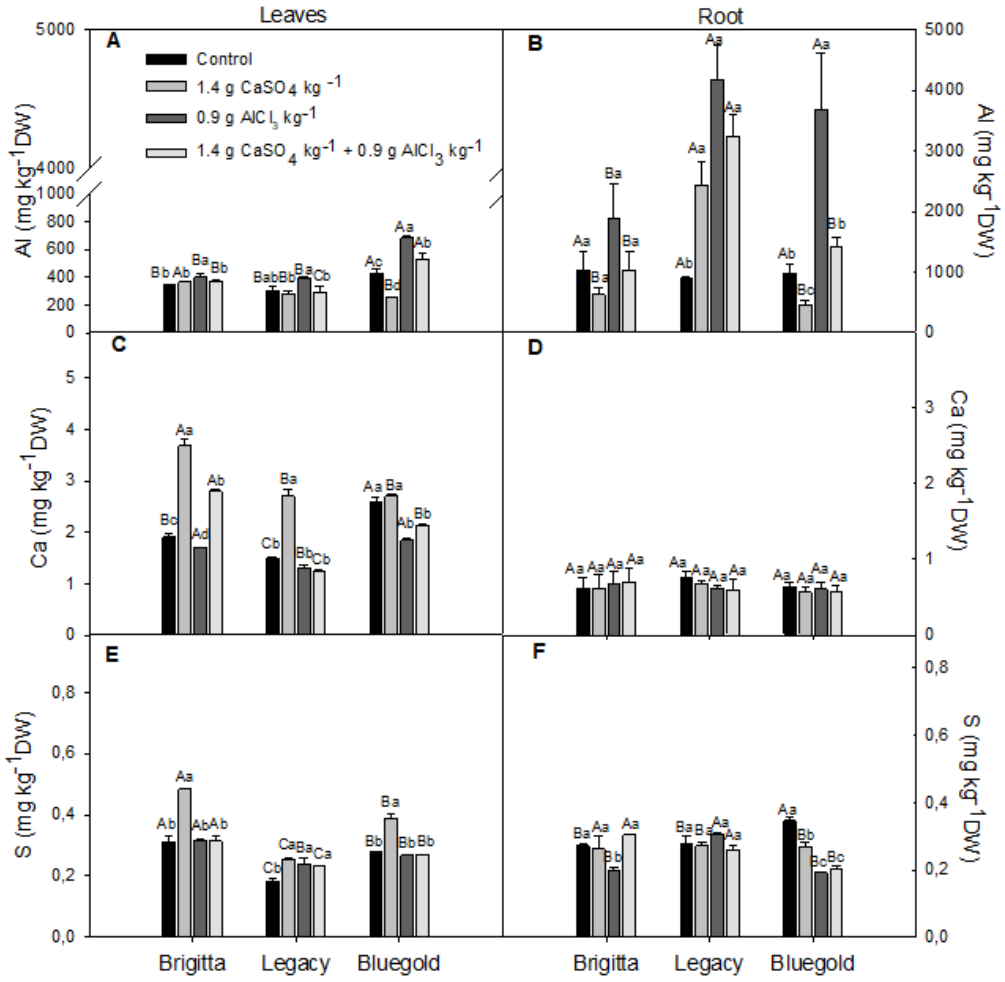


Fig. 1. The abundance of Al, Ca, and S in leaves and roots of three cultivars of highbush blueberry with contrasting Al tolerance grown in peat substrate (control), with Ca or Al alone, or in combination. Values represent the average of three replicates ± SE. Different lower-case letters indicate statistically significant differences (P≤0.05) between treatments for the same cultivar. Different upper-case letters show differences (P≤0.05) between cultivars for the same treatments.

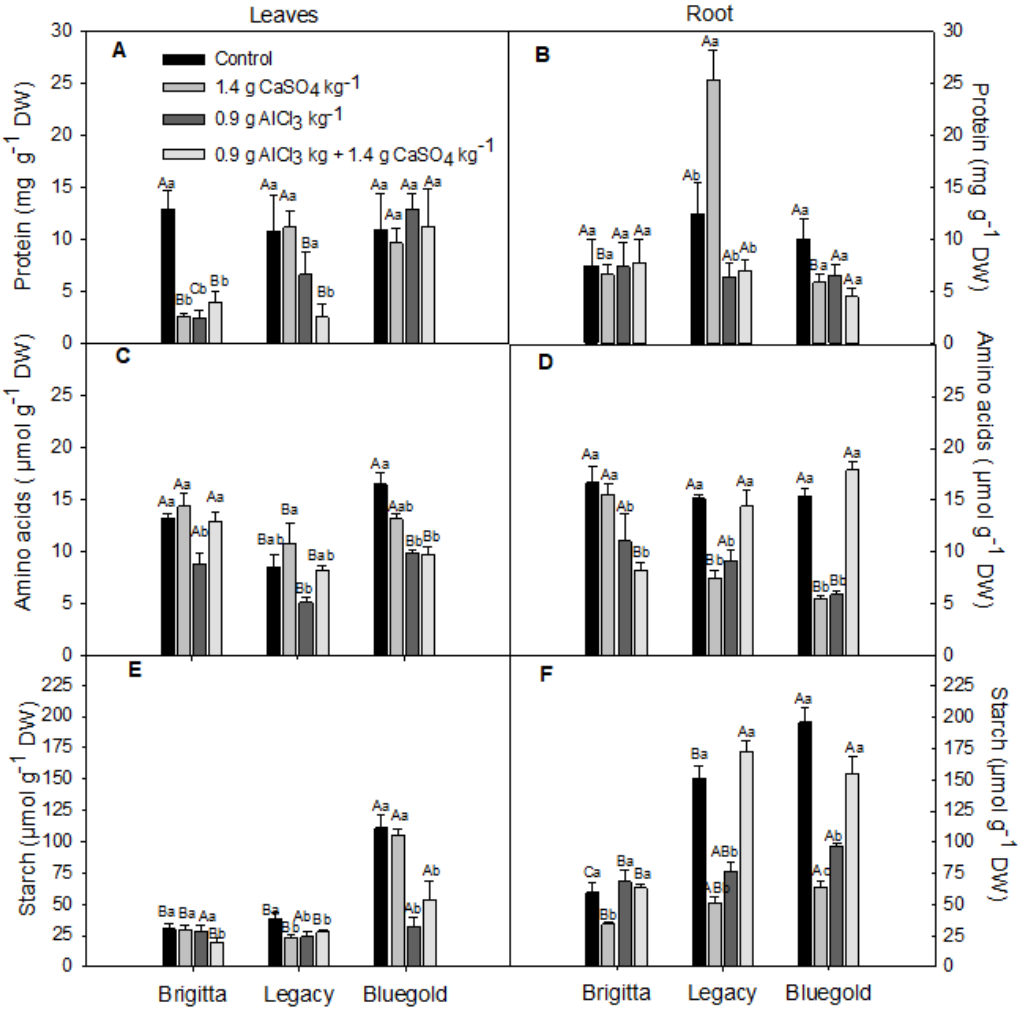


Fig. 2. Protein, amino acid, and starch concentrations in leaves and roots of three cultivars of highbush blueberry with contrasting Al tolerance (Al-resistant Brigitta, and Legacy, and Al-sensitive Bluegold), grown in peat substrate (control), with Ca or Al alone, or in combination. Values represent the average of three replicates ± SE. Different lower-case letters indicate statistically significant differences (P≤0.05) between treatments for the same cultivar. Different upper-case letters show differences (P≤0.05) between cultivars for the same treatments.

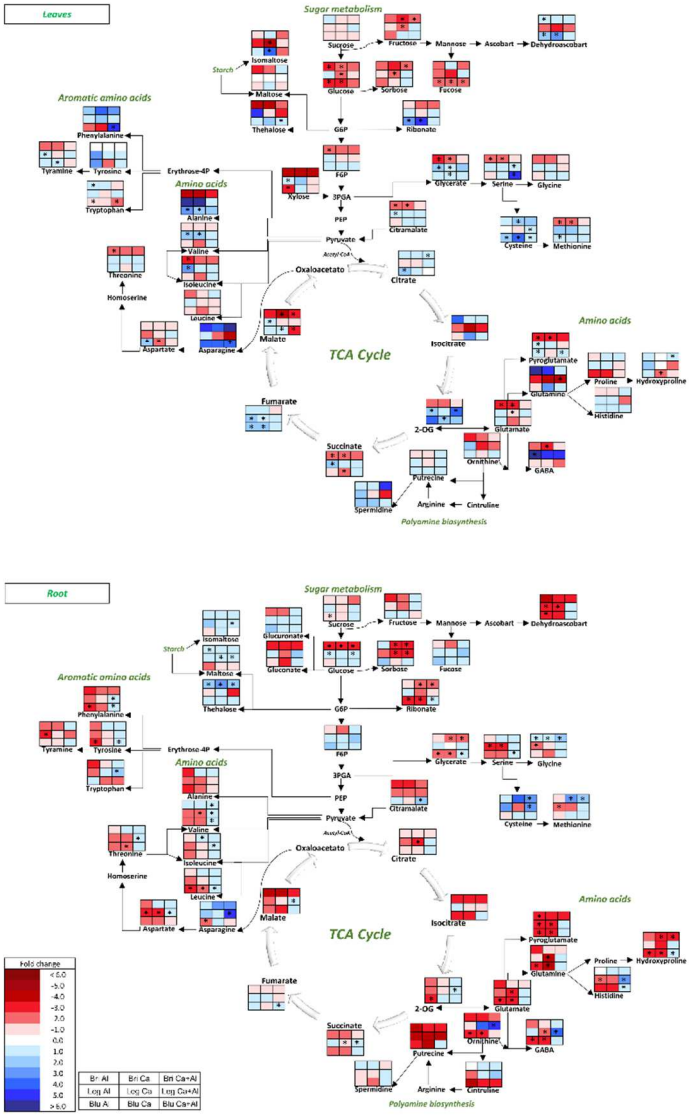


Fig. 3. Change in metabolites of the major primary metabolic pathways in leaves (A) and roots (B) of three blueberry cultivars with contrasting Al tolerance (Legacy, Bluegold and Brigitta), growing in peat substrate with Ca or Al alone, or in combination, for ten days (see explication legend in the box in the upper part to the right side of figure). Values represent the average of three replicates ± SE. The differences in the high degree of the color are deeper, and differences in the lower levels of the color shallower. Metabolites in black numbers indicate significant differences with respect to control.

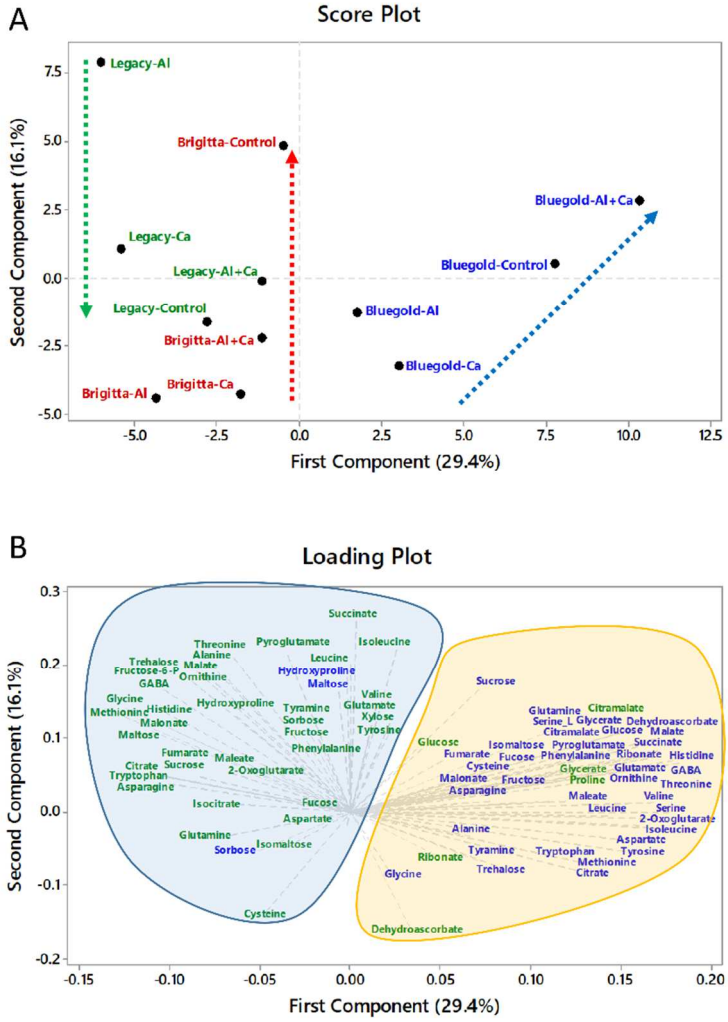
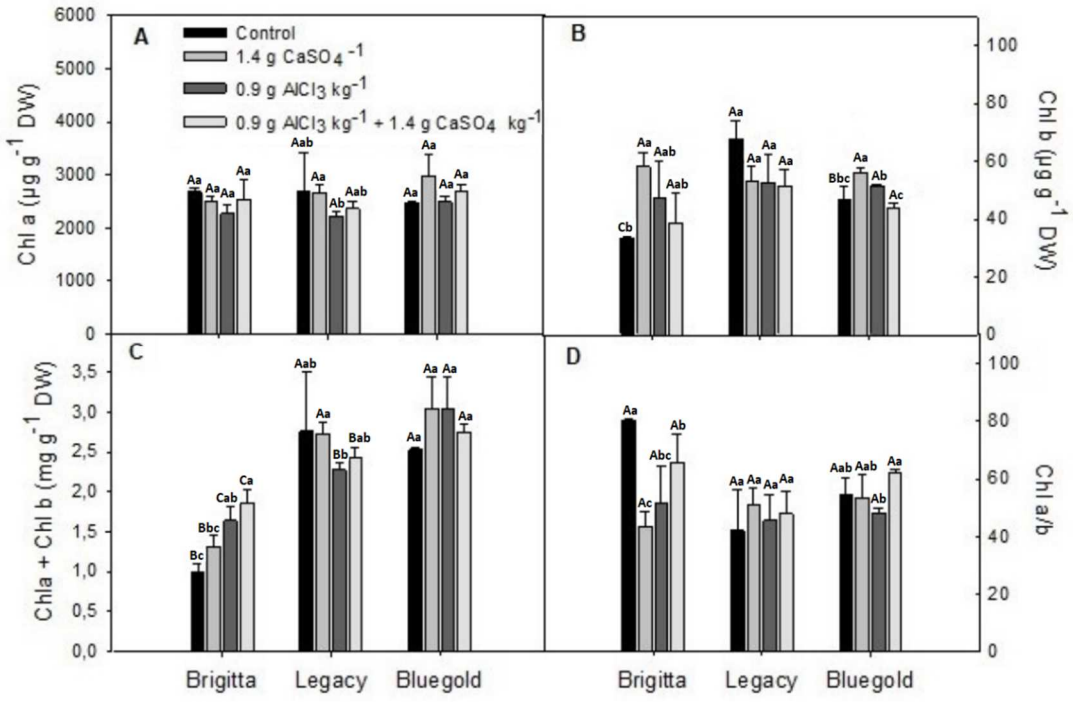


Fig. 4. Principal component analysis (PCA) based on metabolite profiles of the leaves and roots of Legacy, Brigitta, and Bluegold cultivars. The analysis was performed on the correlation matrix of least-square means of accessions averaged. Numbers in parentheses give the percent variation explained by the first and the second principal component. Figure A shows the scores and figure B the loadings plot obtained for the resulting distribution of treatments and metabolites data, respectively. Arrows dotted in figure A indicate the distance between treatments within each cultivar. Colors circles in figure B represents the cluster formed by Pearson distance and text colors indicate the metabolites from leaves and roots, in which leaves are represented in green and roots in blue.

Supplementary Table 1. Effective quantum yield (ΦPSII) and electron transport rate (ETR) of three cultivars of *V. corymbosum* at different times (days) subjected to and acid peat at 4.5 pH and different Al and $CaSO_4$ treatments. Values represent the average of three replicates (n = 3) ± SE.

Treatments	**Control**			**1.4 g $CaSO_4$ kg^{-1}**			**0.9 g $AlCl_3$ kg^{-1}**			**1.4 g $CaSO_4$ kg^{-1} + 0.9 g $AlCl_3$ kg^{-1}**		
Time (days)	**0**	**5**	**10**	**0**	**5**	**10**	**0**	**5**	**10**	**0**	**5**	**10**
Fluorescence Parameters Brigitta												
Fv/Fm	0.83±0.001	0.85±0.008	0.84±0.001	0.84±0.007	0.84±0.006	0.84±0.005	0.83±0.00	0.84±0.004	0.83±0.002	0.84±0.002	0.85±0.001	0.84±0.003
ΦPSII	0.14±0.002	0.15±0.019	0.14±0.005	0.14±0.022	0.15±0.01	0.16±0.01	0.14±0.002	0.13±0.02	0.15±0.01	0.13±0.01	0.13±0.003	0.15±0.013
ETR	17±0.194	16.5±1.19	18±0.74	17.76±2.76	19.28±2.25	24.7±2.84	17.7±0.21	18.41±0.62	0.15±0.01	17.5±2.3	17.3±0.4	17.7±0.45
NPQ	2.7±0.06	2.54±0.209	2.16±0.35	2.69±0.07	2.71±0.23	2.03±0.2	2.61±0.06	2.55±0.17	1.91±0.079	2.52±0.25	3.10±0.22	2.24±0.08
						Legacy						
Fv/Fm	0.83±0.003	0.85±0.006	0.83±0.01	0.85±0.01	0.83±0.001	0.83±0.004	0.81±0.006	0.83±0.002	0.83±0.006	0.83±0.006	0.83±0.005	0.83±0.005
ΦPSII	0.14±0.001	0.13±0.008	0.13±0.008	0.14±0.001	0.15±0.01	0.16±0.01	0.13±0.00	0.13±0.01	0.15±0.004	0.14±0.009	0.13±0.01	0.16±0.01
ETR	17.7±0.2	16.8±1.6	18.5±3.1	17.7±0.21	17.5±2.0	21.39±1.33	17.5±0.04	18.5±2	19.24±3.71	17.1±0.4	15.6±2.3	16.8±1.51
NPQ	2.76±0.4	2.86±0.3	2.96±0.17	2.89±0.32	2.51±0.04	2.69±0.11	2.66±0.15	2.19±0.13	1.92±0.02	2.82±0.17	2.27±0.24	2.17±0.29
						Bluegold						
Fv/Fm	0.84±0.003	0.84±0.008	0.83±0.005	0.85±0.004	0.82±0.01	0.85±0.004	0.84±0.001	0.83±0.002	0.82±0.008	0.83±0.03	0.84±0.006	0.83±0.00
ΦPSII	0.13±0.0003	0.13±0.01	0.14±0.013	0.14±0.006	0.14±0.003	0.14±0.003	0.14±0.001	0.11±0.009	0.14±0.04	0.14±0.007	0.139±0.01	0.17±0.001
ETR	17.5±0.04	16.4±0.59	19.8±1.7	17.7±0.86	15.8±0.3	17±0.5	17.8±0.3	13±0.16	17±0.98	17.07±0.37	15.20±0.99	18±0.38
NPQ	2.79±0.11	2.55±0.16	2.65±0.43	2.86±0.22	2.55±0.10	2.84±0.75	2.12±0.03	2.20±0.08	2.23±0.138	2.82±0.12	2.51±0.11	2.52±0.27



Supplementary Fig. 1. Chlorophyll content in leaves of three cultivars of highbush blueberry with contrasting Al tolerance (Al-resistant Brigitta, and Legacy and Al-sensitive Bluegold), grown in peat substrate (control), with Ca or Al alone, or in combination. Values represent the average of three replicates ± SE. Different lower-case letters indicate statistically significant differences (P≤0.05) between treatments for the same cultivar. Different upper-case letters show differences (P≤0.05) between cultivars for the same treatments.

Chapter V. *“General discussion and concluding remarks”*

5. General discussion

It is widely known that Al-toxicity in acid soils can be decreased by calcareous amendments applications, such as gypsum ($CaSO_4$) (Alarcón et al. 2018). Our results confirm this evidence in the studied blueberry cultivars subjected to gypsum amendments, where Al soil saturation, as well as Al-concentrations of leaves and roots, decreased concomitantly with the increase of the amendment application (Table 1, Fig. 1). Studies performed in barley (*Hordeum vulgare*) under Al-toxicity showed that Ca addition reduced Al-toxicity due to Al concentration and lipid peroxidation reduction, which increased antioxidant enzyme activity as well as Ca concentration compared with the Al-treatment alone, suggesting that Ca supplementation could be related to less Al-uptake in barley plants (Guo et al. 2006). The $CaSO_4$ application effectiveness in amelioration of Al-toxicity was also verified in lettuce under field conditions (Chutichudetet al. 2009).

Table 1. Morphological and biochemical features of highbush blueberry under Al toxicity with or without amendment.

	Acid soil	
Parameters	**With amendment**	**Without amendment**
Al concentration leaves	decreased	increased
Al concentration roots	decreased	increased
Leaf thickness	improving anatomic features	damages
LP	reduction	Increased
Photoprotective pigments	increased	decreased
Leaves morphological features.	ameliorate leaf morphology alteration	morphology alterations

Aluminum toxicity has been shown to decrease photosynthetic performance in several species such as *V. corymbosum*, *Citrus sinensis, Citrus grandis,* and *Eucalyptus grandis* (Reyes-Díaz et al. 2011; Yang et al. 2015; Banhos et al. 2016; Guo et al. 2018; Alarcón-Poblete et al. 2019).

Furthermore, except for Al-accumulator plants, it has been reported that Al concentrates more in roots than in leaves (Konarska 2010). In blueberry, Al-accumulation in different organs depended on the cultivar and the treatments. The Al-sensitive cultivar Bluegold accumulated more Al in roots than in leaves with the exception of the highest amendment supply. This high Al-root concentration in this cultivar provoked high oxidative stress as indicated by the higher LP (Fig. 1 and 5). Similar results have been found in the nutrient solution experiment, where Bluegold accumulated higher Al concentration in roots than in shoots when gypsum amendments were applied (Reyes-Díaz et al. 2010). In addition, negative correlations between Al and Ca concentrations were found in Brigitta and Bluegold leaves and roots (r = around -0.6 and -0.84, $p \leq 0.05$, respectively), whereas Legacy leaves showed a lower correlation (r = -0.46; $p \leq 0.05$) and in roots, no statistically significant correlation was found. These results indicated that in Brigitta and Bluegold, but not in Legacy, a decrease of Al^{3+} interacting with Ca^{2+} improved Ca uptake from the gypsum amendments (Fig. 1). High external Ca, in cytosolic root cells, reduces Al^{3+} and favors the Ca uptake. Similar behavior has been proposed for K in *Arabidopsis thaliana* (Babourina et al. 2005).

Based on tissues Ca concentrations, blueberry can be considered as a calcifuge species (Hanson and Berkheimer 2004). They reported that healthy blueberry plants leaves have Ca levels that vary from 3.0 to 8.0 g kg^{-1} DW. Our values are in the range reported, depending on cultivar, being Ca levels higher in Bluegold leaves compared to other cultivars (Fig. 1). In rice (*Oryza sativa*), the application of amendments increased soil pH

and reduced Al toxicity, improving growth and development due to the addition of Ca and other nutrients such as S (Elisa et al. 2016). Similar results have been reported in other crop species (Rahman et al. 2018).

Table 2. Al and Ca concentrations, RSA and metabolites in leaves and roots of highbush blueberry under acid soil.

Parameters	Leaves	root
Al concentration	less	higher
Ca concentration	Higher	less
RSA	Higher leaves	Less
Number of metabolites that displayed significant	less	higher in roots
Starch	less	higher

The Al concentration increased in all *V. corymbosum* cultivars subjected to Al^{3+} toxicity in leaves and roots, being higher in the Al-sensitive Bluegold cultivar in leaves; mean while, Ca concentrations in leaves were significantly reduced in the same treatment, suggesting that Al can be translocated from root to shoot, inhibiting Ca transport by blocking the Ca^{2+} channels previously reported (Ryan and Kochian 1993); Plieth, 2005). Therefore, Ca-Al relation is strongly associated under Al^{3+} toxicity. The Al concentration is higher in root than in leaves, it can be attributed to the fact that phospholipids, in the cell membrane in the roots, are one of the cellular targets of aluminum, causing changes in potential and inducing its depolarization, affect the Ca^{2+} homeostasis, which could lead to less aluminum translocation.

According to some studies, gypsum also is an important source of Ca^{2+} and S, which can improve mineral concentration such as nitrogen, phosphorus, calcium, and sulfur in plant organs (Bolan et al., 1993; Caires et al., 2006; Tuna et al., 2007).

Auminum toxicity also has been reported to affect synthesis of protein, amino acids, sugars, and organic acids (Azmat et al., 2007; Cheng et al., 2010; Yan et al., 2019). Our

results indicated a decrease in total amino acids under Al treatment with respect to the control plants in all *V. corymbosum* cultivars.

Plants increase the synthesis of certain organic acids in order to cope with Al^{3+} toxicity (Ma et al., 2001; Nunes-Nesi et al., 2014; Yan et al., 2019). In our study, leaves and roots exhibited distinct organic acid profiles among cultivars indicating different activity of TCA cycle enzymes following by Al treatment. In this regard, Legacy roots accumulated higher amount of Al (2-fold) compared to Brigitta, exhibiting higher increase of organic acid in Legacy to tolerate Al toxicity. These results suggest distinct Al responses mechanisms used by the two Al-resistant cultivars, excluding and tolerance mechanisms to Brigitta and Legacy, respectively.

The PCA scores plot revealed that, based on metabolite data from root and leaf organs a clear separation in the first component of the cultivars Brigitta and Legacy from the sensitive cultivar Bluegold (Fig. 4A), confirming our suggestion that the Al-sensitive and Al-resistant cultivars display distinct responses mechanisms to Al toxicity. This can be clearly observed in roots, where GABA concentrations were lower in the sensitive cultivar than in the resistant cultivar Legacy. It has been reported that in seedlings of *Triticum aestivum* lines GABA levels in roots were significantly higher in the Al-resistant line compared with the Al-sensitive line (Ramesh et al., 2015). This result suggested that GABA negatively regulates Aluminum-activated Malate Transporter (ALMT), inhibiting the efflux of malate, provoking TCA cycle modifications as a consequence of Al-stress. In this sense, our findings demonstrated that the Al-resistant cultivar Legacy also increase GABA, which could regulate malate efflux in roots, permitting that this organic acid chelates Al in roots of this cultivar. However, studies showing the GABA involvement in Al^{3+} toxicity are still limited and further studies may uncover novel insights into role of this amino acid in plants against metal toxicity (Seifikalhor et al., 2019). Taken together

these findings suggest that GABA might be involved in metabolite reprogramming of resistance mechanism responses to Al^{3+} toxicity.

6. Concluding remarks

Gypsum amendment ameliorates the Al^{3+} toxicity effects on morphological, photochemical and biochemical features as well as the metabolite levels in highbush blueberry, mainly in the Al-sensitive Bluegold. A compensatory effect was observed after the amendment application, increasing all levels indicated before. Gypsum application reverses the strong and negative correlation between Al content in leaves and leaf thickness, recovering the morphology and decreasing mesophyll compaction in leaves of all cultivars. On the other hand, our findings also demonstrated distinct Al responses mechanisms used by the two Al-resistant cultivars, exclusion, and tolerance mechanisms to Brigitta and Legacy, respectively. Noteworthy that the number of metabolites that displayed significant changes even after the $CaSO_4$ amendment was higher in roots than in leaves, demonstrating distinct metabolic patterns between tissues. In addition, GABA seems to be related to the metabolic responses displayed by Al-resistant cultivar Legacy due to its increase in both leaves and roots. Furthermore, morphological parameters can be also important traits as anatomical markers of Al-resistance for Al-sensitive cultivars such as Bluegold. Thus, these apparent complex metabolic reprogramming displayed by different tissues and cultivars of *V. corymbosum* provided us new knowledge for understanding the changes that occur during Al^{3+} toxicity, and that leads to a considerable decrease in crop yield. In order to understand this new association between GABA and Al^{3+} toxicity responses in highbush blueberry, further investigations of metabolite levels at different time points in plants under Al^{3+}toxicity, as well as transcript profiling at the same time points provide scope for future research work.