**Universidad de La Frontera**
**Facultad de Ingeniería Ciencias y Administración**
**Programa de Doctorado en Ciencias de Recursos Naturales**

# Nitrogen effect on antioxidant capacity, phenolic composition and phenylalanine ammonia-lyase activity of highbush blueberry (*Vaccinium corymbosum L.*)

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**by**

**Erwin Orlando Yañez Mansilla**

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**Nitrogen effect on antioxidant capacity, phenolic composition and phenylalanine ammonia-lyase activity of highbush blueberry (*Vaccinium corymbosum L.*)**

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***"Nunca consideres el estudio como un deber, sino como una oportunidad para penetrar en el maravilloso mundo del saber"***

***Albert Einstein***

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## Summary and outline of this thesis

Scientific evidence suggests an inverse relation between nitrogen (N) concentration and phenolic compounds accumulation in leaf and fruits in some plant species. Some plant species rich in phenolic compounds have not been evaluated in terms of N fertilization and its effects on antioxidant capacity, phenolic compounds accumulation and PAL activity. In this sense, there are not reports related to N concentration specific in leaves that maintain a high antioxidant capacity in blueberry leaves. We hypothesized that there is N concentration threshold that ensures a high phenolic concentration and antioxidant capacity without detrimental effects on plant performance.

In the Chapter 2, a literature review showing an overview about the N effects on plant secondary compounds accumulation is presented. Some considerations are described such as N uptake and its role in higher plants. In addition, Carbon/Nitrogen (C/N) balance theories are described for understanding the fluxes of C and N within the plants in relation to N fertilization. The effects of N fertilization on alkaloids and phenolic compounds are also reviewed.

In Chapter 3, the effects of increasing N doses on the photosynthetic and antioxidant performance of highbush blueberry cultivars (Legacy and Bluegold) under hydroponic conditions at the short-term are evaluated. Accordingly, blueberry cultivars exhibit differential sensitivity to N stress at the short-term. Nitrogen addition decline significantly some antioxidant features and SOD activity is involved in the amelioration of oxidative stress caused by N excess.

In Chapter 4, the influence of increasing N additions on phenolics and antioxidant activity in blueberry cultivars grown at the long-term was assayed. Sensitivity to either N starvation in Legacy or N excess in Bluegold was observed. Despite the differential responses among the cultivars, a threshold of about 15 g N $kg^{-1}$ DW promote high antioxidant activity in blueberry leaves.

In Chapter 5, in soil conditions we tested N uptake and its effect on phenolic concentration and profile in blueberry leaves at increasing N treatments at the long-term. Later on the based on long-term results, a kinetic study was performed where

anthocyanidins profile and PAL activity were evaluated in terms of N uptake at selected N treatments. Long-term results showed that highest N addition induced oxidative damage accompanied by negative effects on $CO_2$ assimilation and antioxidant features for both cultivars. Phenolic acids and flavonols were declined in those plants with 20 g N $kg^{-1}$ DW. Kinetic assay results confirm that blueberry leaves with 15 g N $kg^{-1}$ increased PAL activity, and this response was supported by an increase in ORAC, total phenols and anthocyanins. Conversely, antioxidant capacity and anthocyanins steadily decreased in Bluegold that accumulated about 20 g N $kg^{-1}$. Based on both experiments, the high N dose in both cultivars declined antioxidant parameters at long-term and this behavior was supported at the kinetic assay by a decrease PAL activity.

In Chapter 6, a general discussion and conclusions are presented. In this sense, antioxidant and physiological responses to N concentration are integrated in a model for blueberry cultivars. High N fertilization has detrimental effects on physiological and biochemical traits for blueberry leaves, being Bluegold more sensitive. In general, blueberry maintains great antioxidant attributes growing at low N additions levels. Even though, Legacy and Bluegold showed differential accumulation of phenolic compounds, we found that 15 g N $kg^{-1}$ in blueberry leaves would be adequate N nutrition status for maintain values of antioxidant capacity and phenolic compounds concentration.

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# Chapter 1

## General Introduction

# General introduction

## 1.1 Introduction

Nitrogen (N) is an essential macronutrient for the development and growth of higher plants (Marschner, 1986). Nitrogen is assimilated into organic forms and is a constituent of the photosynthetic apparatus, enzymes and pigments (Lam *et al.,* 1996). However, N depletion or excess triggers negative consequences like senescence, reduced shoot biomass and yellowing of leaves (Smart *et al.,* 1994; Hoque *et al.,* 2012; Britto and Kronzucker, 2013)

Nitrogen stress decreases enzymatic activity and chlorophyll concentration which leads to a detrimental effect in the photosynthetic apparatus (Huang *et al.,* 2004; Witzell and Shevtsova, 2004). Moreover, a decrease of photosynthesis could induce an overproduction of reactive oxygen species (ROS) accompanied by high lipid peroxidation. These facts have been recognized as leading contributors to growth delay in plants (Hachiya *et al.,* 2012; Li *et al.,* 2012). In this sense, plants activate defence mechanisms such as antioxidant enzymes like superoxide dismutase (SOD) like a first defence line for counteract this N stress (Ramalho *et al.,* 1998) and non-enzymatic compounds improve their concentration for avoiding this damage (Diaz *et al.,* 2006). Indeed, N recycling from mature organs to young leaves accompanied by increasing phenolic compounds accumulation is a strategy in N starved plants (Peng *et al.,* 2008; Wolf-Rüdiger *et al.,* 2004; Canton *et al.,* 2005).

Secondary metabolites production is closely related to defence and survival mechanisms in higher plants (Harbone and Willians, 1976). There is a strong relationship between N fertilization and phenolic compounds accumulation in plant tissues (Strissel *et al.,* 2005; Larbat *et al,* 2014). In this sense, a key amino acid phenylalanine (Phe) is a pivotal substrate for the synthesis of proteins and several phenolics, among them cinnamic acids, flavonoids, anthocyanins and tannins. The first step of the phenylpropanoid pathway is deamination of Phe by phenylalanine ammonia-lyase (PAL), which converts Phe to

cinnamic acid as a precursor of phenolic compounds. Ammonium released from the Phe is re-assimilated in the N metabolism. This event occurs mostly in those plants growing in N limited conditions (Da Cunha, 1987; Razal *et al.*, 1996; Singh *et al.*, 1998).

Even though phenolic compounds families are derived from the same precursor (Phe) the variability of phenolics to N fertilization is controversial (Mogren *et al.*, 2006; Kovačik and Klejdus, 2014). In fact, several reports have demonstrated an increase of phenolic compounds and antioxidant capacity in plants grown without or limited N conditions (Steward *et al.*, 2001; Witzell and Shevtsova, 2004; Benard *et al.*, 2009). Otherwise, N addition decreases flavonoids concentration (Awad and Jager, 2002) or anthocyanins (Bongue-Bartelsman and Phillips, 1995).

Excessive N fertilization enhanced the production of biomass in plants, but the quality is often negatively affected due to low concentration of antioxidant compounds. There are some reports about the minimal N doses that are currently recommended applied in several crops and its impact on phenolic compounds production (Stefanelli, 2010; Treuter, 2010). However, there are not evidences in relation to an N concentration in plant tissues that could maintain high levels of phenol compounds and antioxidant capacity as well as diminish the N fertilizers additions to crops.

On the other hand, highbush blueberry (*Vaccinium corymbosum)* has been widely studied due to the large number and variety of phenolic compounds (Cho *et al.*, 2005; Xiaoyong and Luming, 2014). The chemical profile of the fruit and leaves of blueberries is made up mainly of phenolic acids, flavonols and anthocyanins (Sellapan *et al.*, 2002). The chemical structure of the most important phenolics in blueberry has a powerful antiradical scavenger for diminished oxidative damage in plant tissues (Zheng and Wang, 2003). Despite the importance of highbush blueberry as crop with leaves and fruits with high contents in phenolic compounds, physiological and biochemical studies in this species are scarce in relation to N nutrition (Yañez-Mansilla *et al.*, 2015). Under field conditions, N doses commonly applied in blueberry range from 20 to 140 kg $ha^{-1}$ and concentration of N

in the leaf of around 17 and 20 g N $kg^{-1}$ DW have been suggested as an acceptable value for this fruit crop (Hart *et al.,* 2006).

As state above, most of current scientific evidence suggests an inverse relation between N concentration in leaf or fruit and the synthesis of phenolic compounds in some plant species. However, information about the effect of N fertilization on the antioxidant capacity, phenolic composition, PAL activity of highbush blueberry (*Vaccinium corymbosum*) is not available. These facts suggest the need to investigate the relationship between plant N concentration, PAL activity and phenolic composition in species with a high antioxidant capacity like highbush blueberry.

## 1.2 Hypothesis and research objectives

### 1.2.1 Hypothesis

Due to phenolic compounds metabolism regulation by nitrogen (N) in plant tissues, there is a threshold of N concentration in blueberry leaves that will enhance the content of antioxidant phenolic compounds in highbush blueberry.

### 1.2.2 Research objectives

#### 1.2.2.1 General objective

To evaluate the effect of nitrogen concentration on antioxidant capacity, phenolic composition, and phenylalanine ammonia-lyase (PAL) activity of highbush blueberry (*Vaccinium corymbosum*).

#### 1.2.2.2 Specific objectives

1. To assess antioxidant capacity, profile and concentration of phenolic compounds in leaves under different doses of nitrogen fertilization.
2. To establish the optimal relationship between the nitrogen concentration and phenolic compound concentration in leaves.
3. To evaluate the relationship between N concentration in leaves and activity of phenylalanine ammonia-lyase (PAL) activity that controls the synthesis of phenolic compounds.

# Chapter 2

## Review:
## Secondary metabolism and defense in higher plants in response to nitrogen

# Review: Secondary metabolism and defense in higher plants in response to nitrogen

## Abstract

Nitrogen (N) is a macronutrient for higher plants, basic compounds such as amino acids and proteins and also secondary compounds such as alkaloids are present. In addition, N metabolism influences synthesis and accumulation of phenolic compounds. In this sense, it has been observed that in N could be triggering the production of some amino acids precursors of secondary metabolites such as phenylalanine aromatic amino acid (Phe). Experimental evidence indicates that under N deficiency, some plants improve enzymatic activity and expression of specific genes related with phenolic compounds synthesis. On the other hand, contradictory responses are found in literature, N-fertilization in plants increase phenolic compounds. Nitrogen availability and source could be triggered these responses, but the mechanisms responsible for this response remain unclear. The aim of this review is to show an overview about the N effects on the regulation of the secondary metabolism in higher plants, with emphasis on alkaloid and phenylpropanoid compounds. Some considerations are described such as regulation of enzymatic activity of phenolic compounds as well Carbon/Nitrogen balance under N-treatments. Nonetheless, it is necessary a deepest knowledge about the impact of N nutrition on the plant secondary metabolism. Nitrogen concentration in plant tissues and the synthesis or accumulation of secondary compounds such as alkaloids and phenylpropanoids should be addressed in future researches. Such studies must consider molecular approaches involving key enzymes of secondary metabolic pathways and the enhancement of phenylpropanoids content, without detrimental effects on the primary metabolism and in turn, on the plant growth.

***Keywords:*** Secondary metabolism, Nitrogen, phenylalanine, phenolic compounds.

## 2.1 Introduction

Nitrogen (N) is an integral constituent of higher plants, and it is assimilated in reduced form to be incorporated into amino acids and proteins. Amino acids are stored in the cell vacuole and used for tissue formation and accumulation of secondary metabolites such alkaloids and phenolic compounds (Stitt *et al.*, 2002*;* Petrusa *et al.*, 2013). Plants take up N from soil solution as nitrate ($NO_3^-$) and ammonia ($NH_4^+$), and

their availability depends on physico-chemical and biological properties of soils and environmental factors (Cartes *et al.,* 2009), which in turn govern the N-losses by leaching (Alfaro *et al.*, 2006) and volatilization (Nuñez *et al.,* 2010). In addition, N and Carbon (C) metabolism are interrelated in a complex metabolism network. In this respect, both macronutrients act coordinately regulating the primary and secondary metabolism in higher plants (Urbanczyk-Wochniak and Fernie, 2005). For example, N starvation decreases chlorophyll, amino acids and proteins content (Amtmann and Armengaud, 2009). Furthermore, senescent plants have developed strategies for N mobilization and synthesis of amino acids. This response is accompanied by accumulation of antioxidant compounds such as anthocyanins (Diaz *et al.,* 2006; Zhou *et al.*, 2012). The imbalance in the concentration of N and C in the plant triggers the accumulation of phenolic compounds (Zaghdoud *et al.,* 2015; Goufo *et al.,* 2014), but to date the mechanisms responsible for this response remain unclear. Nevertheless, there is scientific evidence indicating that phenylalanine ammonia-lyase (PAL), the first enzyme acting in the phenolic synthesis pathway, would be regulating the fluxes of some aromatic amino acids precursors of phenolic compounds.

Secondary metabolism is related with N and C and it has been reported that low N concentration in plant tissues have increased the synthesis of phenolic compounds (Stumpf *et al.*, 2015) and some primary metabolites such as sugars (Pompelli *et al.*, 2010). This response has been accompanied by an increase in the gene expression of phenylalanine ammonia-lyase (PAL) enzyme in the phenylpropanoid pathway. However, increased accumulation of phenolics has also been observed in plants as a consequence of N fertilization (Yañez-Mansilla *et al.*, 2015). Therefore, there are conflicting results with respect of N fertilization effect on phenolics accumulation in higher plants.

Likewise, several reports have shown an induction of PAL gene expression as response to C-N imbalances (Lødval *et al*, 2014). Theorical concepts as protein competition model (PCM) and carbon-nutrient balance (CNB) could explain the relationship between C and N metabolisms and their influence on secondary metabolism (Bryant *et al*., 1983; Jones and Hartley 1999).

The amount of aromatic amino acids precursors for alkaloids and phenylpropanoids synthesis is influenced by N concentration in plant tissues, and the effect of N applied as either mineral or organic fertilizers has shown to reduce accumulation of secondary compounds and, in some cases, no variations are seen. The aim of this review is to show an overview about the N effects on the regulation of the secondary metabolism in higher plants, with emphasis on alkaloid and phenolic compounds.

## 2.2. Nitrogen in the soil-plant system

### *2.2.1 Nitrogen cycle and its availability in volcanic soils*

Nitrogen biogeochemical cycle involves oxidation and reduction processes of N forms in the environment. The largest natural N source is found in the atmosphere remains 80% of total components (Follett, 1995). Nitrogen organic forms are present in the soil like proteins and amino acids and other N compounds. In addition, the principal N inorganic forms in the soil are $NH_4^+$, $NO_3^-$ and lesser proportion as $NO_2$.

Anthropogenic activities such as agricultural practices and grazing systems increase N in the ecosystem due to the use of organic matter, crop residues, manures or sludge amendments and N-based fertilizers such as urea. By contrast, N outputs include ammonia volatilization, nitrate leaching and nitrous oxide emissions. Likewise, N transformation processes include biological $N_2$ fixation, mineralization, immobilization, nitrification and denitrification, and they determine the N potentially available to plants in the soil-plant system.

Volcanic ash-derived soils, like Andisols, are naturally acidic (4.5 and 5.5) and they are characterized by low nutrient availability, high phosphorus (P) fixation capacity, pH-dependent cation exchange capacity, low basic cation content and high organic matter (OM), hence high carbon (C) and nitrogen (N) contents (Mora *et al.*, 1999; Escudey *et al,* 2001).

In these soils, the continuous application of N-fertilizers (ammonia) have increased the acidification process because nitrification releases free $H^+$ from ammonia, affecting growth and yield in crop systems (Mora *et al.*, 1999).

On the other hand, N losses by leaching in pastures grown on an Andisol under field conditions were estimate. The application of 300 kg N $ha^{-1}$ added as urea or sodium nitrate the potential N losses were about 90 kg N $ha^{-1}$ during the spring-summer period (Mora *et al.,* 2007). In grazed pastures under heavy-frequent grazing systems in Southern Chile, N leaching was about 58.7 kg N $ha^{-1}$, and ammonia emissions were 10 % greater in intensive grazing systems in comparison with infrequent grazing treatments (Nuñez *et al.,* 2009). Ammonia and nitrous oxide losses are easily produced by volatilization process from the soil. In some countries such as The Netherlands, Australia and New Zealand, N-emissions from grasslands systems ranged between 20 and 50 kg N $ha^{-1}$ (Eckard *et al.,* 2003; Schils *et al.,* 2005).

Likewise, soil organic matter content, temperature and N supply level play a key role in the kinetics of N-mineralization, and in turn in the availability of N in Andisols. $NH_4^+$ and $NO_3^-$ are the main N-species potentially available in soils for plant growth. These species are produced by ammonification and nitrification of soil organic matter, organic amendments and urea-based fertilizers. In this sense, Cartes *et al.* (2009) evaluated the effect of temperature and urea supply on urease activity and N-mineralization in two Andisols of Southern Chile differing in their organic matter content. Nitrogen availability was improved at increasing temperature and urea fertilization rates. Besides, the mineralization was more efficient in sites with high organic matter content. However, agronomical practices, such as crop rotation or no-tillage have proven to affect the labile pool mineralizable-N, and in organic crop systems, at increasing manure supply and crop rotation the pool of mineralizable-N was the highest in comparison with conventional systems (Spargo *et al.,* 2011).

Based on the experimental facts mentioned above, appropriate strategies should be developed to improve the efficiency of N fertilizers in the soil plant-system to increase yield and crop quality and reduce N losses towards the environment.

### *2.2.2 Nitrogen role in higher plants.*

It is well known that N is one of the most important inorganic macronutrient in plants, as it is an integral component of amino acids, proteins and nucleic acids (Marschner, 1995). Nitrogen is also an important constituent of photosynthetic apparatus, being a structural part of both the chlorophyll molecule and the ribulose 1,5-bisphosphate carboxilase/oxygenase (Rubisco) enzyme. In the same way, secondary compounds containing N in their structure have been studied by Dewick (2002) and Facchini (2001). One or more atoms of N are present in the chemical structure of alkaloids and other secondary compounds as well as in non-protein amino acids (e.g. L-tryptophan, L-phenylalanine, L-tyrosine and L-ornithin).

In respect to the functions of N in plant nutrition, it has been proven that the maximum photosynthetic capacity is regulated by N concentration in the leaf of higher plants (Field and Mooney, 1986). Cheng and Fuchigami. (2000) observed that total Rubisco activity rose linearly with leaf N increase. Supplemental N has shown to increase the plant height of *Picea asperata* seedlings (Yao and Liu, 2007), whereas in *Swida hemsleyi* proline content increased under enhanced UV-B and N treatment (Yao and Liu, 2009). In *Arabidopsis* plants, it has also been demonstrated that N starvation reduced chlorophyll synthesis and enhanced anthocyanins in the leaves (Wolf-Rüdiger *et al.*, 2004; Giorgi *et al.*, 2009)

Some amino acids, such as L-canavanine, an analogue of arginine, functioning as a storage N-compound in seeds of *Sutherlandia frutescens,* are promoted by N-supply (Colling *et al.,* 2010) as well as essential amino acids in *Camellia sinencis* (Ruan *et al.,* 2010) and tobacco plants (Matt *et al.,* 2002). In maize plants, total chlorophyll, carotenoids, proteins, soluble sugars were raised at increasing N addition levels (Correia *et al.,* 2005), and also *Solanun lycopersicum* leaves exhibited a tendency to accumulate amino acids such as arginine, lysine, phenylalanine, and tryptophan as a consequence of increased N supply (Urbanczyk-Wochniak *et al.,* 2005). Additionally, it has been proposed that N fertilization could be protecting coffee plants against photodamage (Pompelli *et al.,* 2010). In relation to the N source effect on ROS scavenging enzymes (e.g superoxide dismutase), ammonia-grown wheat plants enhanced their antioxidant protection compared with nitrate-grown plants (Polesskaya *et al.,* 2004). On the other hand, ascorbate peroxidase (APX) showed enhanced activity under urea treatments in

comparison with ammonia sulfate treatments in wheat leaves (Russo *et al.*, 2010). Likewise, enzymatic antioxidant enzymes (peroxidase-POD, catalase-CAT and APX) were activated in *Picea asperata* seedlings under ammonia nitrate addition to the growth media (Yao and Liu, 2006). Nevertheless, Dominguez-Valdivia *et al.* (2008) found no clear responses in terms of enzymatic antioxidant activities in *Pisum sativum* and *Spinacia oleracea* under 1.5 and 3.0 mM N as ammonia treatments.

In contrast, N deficiency limits plant growth and crop yield (Lam *et al.*, 1996 and references therein) by altering both the amino acids and the carbon metabolism (Stitt *et al.*, 2002) as described below. Furthermore, it accelerates leaf senescence and yellowing as consequence of chlorophyll breakdown (Feild *et al.* 2001; Hoch *et al.*, 2003). Briefly, a decrease of amino acid contents has been observed, which was followed by an increase in leaf carbohydrates in N-deficient *Nicotiana tabacum* plants (Kovačik *et al.*, 2006) and *Solanun lycopersicum* (Urbanczyk-Wochniak *et al.*, 2005). Fritz *et al.* (2006) evaluated amino acid precursors of some secondary metabolites in wild *N. tabacum* plants. They found that the levels of phenylalanine, tyrosine and tryptophane increased at 12 mM nitrate supply, and this effect was higher at midday. Moreover, ornithine and arginine (nicotine alkaloid substrate) decreased two-fold under 0.2 mM nitrate. In addition, N starvation in *Arabidopsis* appears to activate some strategies to counteract plant stress. Such responses include remobilization of N from old or mature organs and accumulation of anthocyanins (Peng *et al.*, 2008; Diaz *et al.*, 2006).

### *2.2.3 Nitrogen uptake and its assimilation in plants.*

Nitrate ($NO_3^-$) and ammonia ($NH_4^+$) are the largest N sources taken up by the roots of higher plants (Taiz and Zieger, 1998; Stitt, 2002). Nitrogen taken up by roots is firstly reduced to ammonia and then assimilated into organic forms. Reduction of nitrate to ammonia also occurs at leaf and root levels (Glass *et al.*, 2002). Nitrate reduction into nitrite is catalyzed by the enzyme nitrate reductase in the cytosol. Nitrite is then reduced to ammonia by nitrite reductase enzyme in the chloroplast or plastids. In legumes, ammonia is also derived from symbiotic fixation of $N_2$ in root nodules. Ammonia ion is the final form of inorganic N that is assimilated into glutamine and glutamate plant tissues (Lea *et al.*, 1990). They are used for the biosynthesis of the largest N-containing compounds, and for the synthesis of chlorophyll molecule in developing leaves (Forde

and Lea., 2007). Primary N assimilation is controlled by glutamine synthase (GS) and glutamate synthase (GOGAT, glutamate-2-oxoglutarate aminotransferase) isoenzymes. Glutamate synthase transfers the amide-nitrogen of L-glutamine to 2-oxoglutarate, providing two molecules of L-glutamate (for review see Temple *et al,* 1998; Suzuki *et al.,* 2005; Foyer *et al*., 2011). There are other two additional processes for N assimilation by plants: reassimilation of photorespiratory ammonia and N-recycled assimilation (Lam *et al.,* 1996). Photorespiratory process involves the conversion of ribulose bisphosphate into two molecules of phosphoglycolate. Nitrogen-recycled assimilation is a plant strategy that consists of ammonia release from biochemical processes such as protein catabolism.

Secondary N-assimilation is related to amino acid deamination of phenylalanine (Phe) by phenylalanine ammonia-lyase enzyme (PAL), and it seems to be enhanced in N-deprived plant species. Furthermore, it has been reported that glutamine synthase (GS) reassimilates ammonia from organic N during both growth and development (Miflin and Habash, 2002). Secondary N assimilation has been supported by a close relationship between enhanced PAL activity and GS activity in *Camellia sinencis* (Ruan *et al.,* 2010). Although there is increasing evidence indicating that the synthesis of phenolic compounds is induced under N deficiency, there are some controversies in the literature results (see below). For example, Sanchez *et al.* (2000) found that PAL activity and accumulation of total phenolic compounds increased at high N-rates. These facts have been explained by theories that have attempted to provide ecological interpretations of variations of N and Carbon (C) fluxes in the plants. Some theories are Carbon-Nutrient Balance (CNB) (Bryant *et al.,* 1985) and Protein Competition Model (PCM) (Jones and Hartley, 1999). Some considerations are described below.

## 2.3 Relationship between nitrogen assimilation and carbon metabolism in higher plants

### *2.3.1 Carbon-nitrogen fluxes in higher plants*

Nitrogen uptake and assimilation are interlinked with photosynthesis and carbohydrate production. It has been established that during N inorganic assimilation, amounts of fixed C are required to provide the C skeletons that act as acceptors during this process (Hachiya *et al.,* 2007). Higher plants allocate nutrient resources as C and N

for plant defense. Thereby, theory of Carbon-Nutrient Balance (CNB) predicts a variation in the production and allocation of C and nutrients (mainly N) in secondary metabolites (Bryant *et al.*, 1983). This theory suggests that when plants acquire an N excess over the requirements, these resources are used in the secondary metabolite production. Thus, the increase of N-based compounds (alkaloids) is produced when N is acquired excessively and carbon is limited in relation to growth requirements. In contrast, phenolic compounds are produced in plants under N-limited and carbohydrate excess.

Complementary to this theory, Jones and Hartley (1999) proposed the Protein Competition Model (PMC). In this sense, Phe amino acid is precursor to proteins and phenolic compounds synthesis. The PMC indicate that in higher plants under N-deficiency, deamination of phenylalanine amino acid PAL is high for production of phenolic compounds. Otherwise, when protein synthesis is high, deamination of phenylalanine is less for the production of phenolic compound and *vice versa*. Figure 2.1 summarizes the allocation of C and N on primary and secondary metabolites as well as plant responses according to CNB and PMC theories. Thus, $CO_2$ levels in environment and N availability in the soil stimulate a complex network of secondary metabolism in the plant. CNB and PMC show fluxes of N and C and its metabolic products such as carbohydrates, phenolic and N-compounds.

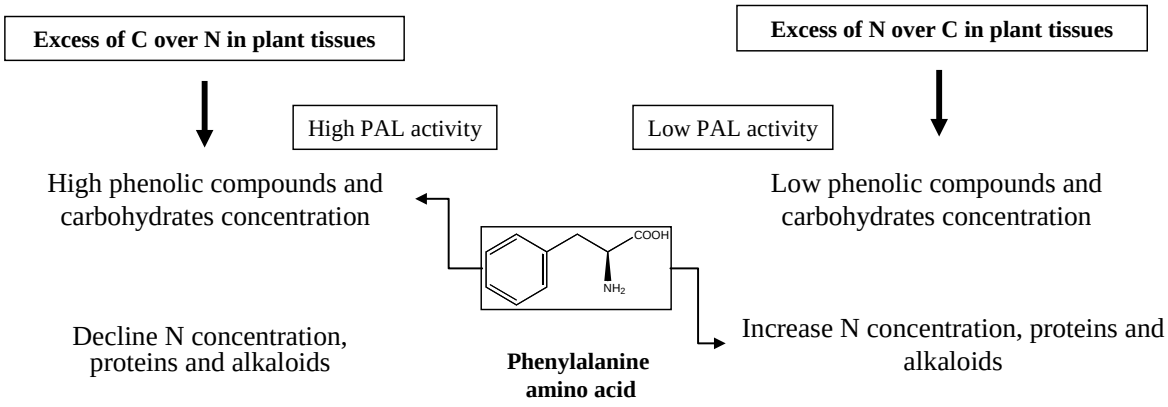


**Figure 2.1.** Allocation of C and N on primary (proteins and carbohydrates) and secondary metabolites (alkaloids and phenolic compounds) in higher plants according to CNB and PMC theories. Phenylalanine (Phe) amino acid is a key structure in higher plants for protein and phenolic compounds accumulation. Adapted from: Bryant *et al.* (1983) and Jones and Hartley (1999).

### *2.3.2 Nutritional Carbon-Nitrogen balance: availability resources for growth or defense?*

It is well known that plant growth and defense depend on the availability of resources such as light and nutrients in the environment. As mentioned above, N and C availability affects plant growth, and their concentration in plant tissues leads the secondary metabolite synthesis. The decrease in C reduces the plant growth, and the excess N is located in amino acids for growth and defense compounds such as alkaloids (see below). Nitrogen deprivation affects differentially C-compounds synthesis. About 40% of the organic matter is destined to the production of phenylalanine and tyrosine. These amino acids are key compounds in the synthesis of proteins, phenolic compounds and aromatic alkaloids. Ammonia released from these amino acids is assimilated into glutamine and glutamate and then re-used in the production of amino acids (Razal *et al.*, 1996). Later on, Coviella *et al.* (2002) determined that cotton plants allocate the N resources in proteins and enzymes when growing under N-starvation and elevated $CO_2$ levels. In such conditions, C-excess resources were destinated for phenolics synthesis.

For example, under N deprivation in *Phaseolus vulgaris* plants, absorbing compounds such as flavonoids increased more than 2 times compared with plants supplied with N. As a result, increased defense to abiotic stress such as UV-B ambient have been observed (Riquelme *et al.*, 2007).

Otherwise, Matros *et al.* (2006) evaluated the effect of two $CO_2$ levels and two N addition doses on the production of secondary compounds in *Nicotiana tabacum* plants. At the highest $CO_2$ supply, and irrespective of the N treatment, chlorogenic acid and some coumarins enhanced its accumulation, and the lowest N dose decreased nicotine content. Thus, *N. tabacum* plants allocated their C-resources on defense compounds against biotic and abiotic stress more that in growth.

Conversely, the concentration of polyphenols and sugars were the lowest under high N supply in *Camellia sinensis* young plants. Moreover, C-flux was shunted into production of amino acids (Ruan *et al.*, 2010). The resources allocation has been more

investigated in terms of the effect of N on alkaloids production. In this sense, alkaloids precursors and alkaloid synthesis appear to be improved by N supply (see below).

## 2.4 Secondary metabolites induction and regulation of key enzymes in response to nitrogen in higher plants

### *2.4.1 Nitrogen compounds: alkaloids*

### *2.4.2 Alkaloids: general characteristics*

Alkaloid classification, synthesis and general characteristics have been reviewed by Dewick, (2002) and Ziegler and Facchini, (2008). Alkaloids are secondary metabolites and they are found mainly in plants. Their chemical structure consists in a heterocycling ring with one or more N-atoms. There are about 12.000 known alkaloid compounds (Facchini, 2001). In general, N is present as primary, secondary and ternary amines in alkaloids. Plant alkaloids have many different biological activities including their defensive role against herbivores, pathogens and other plant species. These biological activities depend on both the protonation of the amine group as well as presence and location of the other functional groups in the structure. Biosynthesis and accumulation of some alkaloids occurs in different tissue types such as epidermis, endodermis, laticifers, idioblasts, pericycle and cortex (Facchini and St. Pierre, 2005).

The general classification of some alkaloids is according to their amino acid precursor. In this regard, piperidine and quinolizidine alkaloids derive from L-lysine. Likewise, pyrrolizidine and tropane alkaloids are derived from L-ornithine. Indole alkaloids derive from L-tryptophan, and imidazole alkaloid from L-histidine (Bernards, 2010). Even though different abiotic stress conditions appear to trigger the synthesis of plant defensive compounds (Dixon and Paiva, 1995) N fertilization and its relation to the synthesis of alkaloids will be reviewed.

### *2.4.3 Alkaloids induction and accumulation*

Higher plants have developed a vast array of defence strategies against adverse environmental conditions. Some of these strategies constitute secondary routes for the production of secondary compounds. Nitrogen supply could increase the concentration of secondary metabolites as alkaloids in plant tissues, and this fact have consequences on plant-herbivores interactions (Chen *et al.*, 2010).

Tables 2.1 summarize the effect of N on the production of alkaloids in higher plants under controlled or field growth conditions. In *Senecio jacobea* plants, the concentration of pyrrolizidine alkaloids (PAs) was examined in nutrient culture experiments. PAs concentration in roots and shoots decreased under high nutrient supply. Nevertheless, jacobine, a typical alkaloid isolated from genus *Jacobea*, was raised in roots at high nutrient levels in solution. In this study, increased accumulation of jacobine suggested important functions in plant defence against herbivores under optimal nutrient rates (Hol *et al.,* 2003). In the same way, the effects of N supply on PAs accumulation in *Senecio* genus were revised by Hol. (2011). Nitrogen fertilization affects the type and concentration of PAs in different plant organs. Hence, N excess could be destined to alkaloid accumulation, and some plant species could be best protected against biotic stress in sites with higher N availability. Nevertheless, the highest PAs concentrations have also been found in *Senecio vulgaris* plants growing in N-poor soils in comparison with vineyard soils. This species showed contrasting phenotypic response in terms of growth and defense in different habitats (Frischknecht *et al.,* 2001). Likewise, galanthamine (an alkaloid from *Narcissus* bulbs) enhanced its content by N applying compared with no N addition (Lubbe *et al.,* 2011). This research also shows that the amount of amino acids and citric acid cycle intermediates increased, but plants did not accumulate galanthamine when applied double N treatment (Lubbe *et al.,* 2011).

Other evidences indicate that increased content of two alkaloids in roots and leaves of *Catharanthus roseus* from 0 to 150 kg $ha^{-1}$ of urea added (Sreevalli *et al.,* 2004). Moreover, *Ilex vomitaria* plants under treatments with ammonium nitrate enhanced caffeine and theobromine alkaloids and these values were directly correlated with the N concentration in leaves ($r = 0.98$ and $r = 0.67$, respectively) (Palumbo *et al,* 2007). Likewise, in *Coffea arabica,* caffeine enhanced its concentration in the phloem exudates at ammonium nitrate added (Gonthier *et al.,* 2011). In *N. tabacum* the levels of amino acids ornithine, arginine and nicotine alkaloids were evaluated at two $KNO_3$ rates (0.2 and 12mM N). At the low N addition level, both amino acids decreased in comparison with the highest N treatment. Furthermore, nicotine experienced a decrease in concentration at 0.2 mM N supplied (Fritz *et al.,* 2006).

Other studies have evaluated the influence of the N source on alkaloids production. In particular, Abdolzadeh *et al.* (2006) compared the effect of ammonia, nitrate and a combination of ammonia and nitrate at different concentrations in hydroponically cultured *Catharanthus roseus* plants. The high contents of amino acids and alkaloids (vincristin and vinblastin) were observed at 11 mM N added as ammonia plus nitrate in comparison with one N-source (ammonia or nitrate). Apparently, nitrate had a greater influence that ammonia on the accumulation of scopolamine and hyoscyamine alkaloids. Similar results were found by Misra and Gupta (2006). In fact, 20 mM N applied as $KNO_3$ increased the total content of alkaloids of plants. According with Yong-Qin *et al.* (2003), different N-sources could generate differential effects in cell cultures of *Taxus yunnanensi* in terms of growth and defence. In particular, this species enhanced diterpenoid alkaloid production (taxol) in ammonium culture media, whereas nitrate promoted cell growth and biomass (Yong-Qin *et al.*, 2003).

Nitrogen treatments and sources enhanced accumulation of N-compounds. A complex secondary metabolism, several amino acids and enzymes are involved in alkaloid synthesis. This fact is not clarified yet, but some alkaloids with specific functions have increased their accumulation under high N-doses applied and would be acting to defend the plant against stress conditions. Nevertheless, are unclear N-source and its relationship with alkaloid synthesis.

### *2.4.4 Phenylpropanoid compounds*

### *2.4.5 Overview*

Phenolic compounds have been extensively reviewed by Harborne, (1976) and Harborne and Williams, (2000). They are secondary metabolites found in leaves, roots, bark, flowers, pollen grains and seeds. The biological activity of phenolic compounds is closely related to the chemical structure of the molecule (double bond conjugation and oxygenation degree). The main functions of phenolic compounds are closely connected with plant defense against different types of stress (e.g cold, wounding, UV-B, herbivory, heavy metals). Some phenolic compounds families are flavonoids, isoflavones, pterocarpans, stilbenes, coumarins, phenolamines, aurones, chalcones, lignans and lignin. These compounds are synthesized principally from phenylalanine aromatic amino acid (Phe) by action of the phenylalanine ammonia-lyase enzyme

(PAL). In general, under N deficiency, phenolic compounds increase its concentration in plant tissues. An example is chlorogenic acid, which is derived from cinnamic acid, probably because it occurs in the early stages of phenylpropanoid pathway and its high powerful antioxidant acts against oxidative stress in N-deprived plants (Zheng and Wang, 2003).

Several kinds of phenolics are accumulated under N-deprivation, including flavonols (Steward *et al.,* 2001), anthocyanins (Bongue-Bartelsman and Phillips, 1995) and phenolic acids (Fritz *et al.*, 2006). Nonetheless, to date, the influence and function of N in the synthesis of phenolic compounds have not been completely elucidated in higher plants (see below).

**Table 2.1.** Some alkaloids that maximize its concentration by N addition in higher plants.

| Specie | Experimental conditions | Alkaloid compound | Tissue | Reference |
|---|---|---|---|---|
| *Hyoscyamus niger* | $CO(NH_2)_2$: 50 mg/L, Hydroponic culture | Scopolamine and hyoscyamine | Leaves and roots | Alaghemand *et al.* (2013) |
| *Coffe arabica* | $NH_4NO_3$ : 23 mM Sandy soil | Caffeine | Leaves | Pompelli *et al.* (2013) |
| *Narcissus pseudonarcissus* | $Ca(NO3)_2$ :110 kg $ha^{-1}$ Soil conditions | Galanthamine | Bulbs | Lubbe *et al.* (2011) |
| *Coffea arabica* | $NO_3NH_4$ : 3 mmol $l^{-1}$ Soil conditions | Caffeine | Seedlings | Gonthier *et al.* (2011) |
| *Ilex vomitoria* | $NO_3NH_4$ : 250 mg Soil conditions | Caffeine, theobromine | Leaves | Palumbo *et al*. (2007) |
| *Catharanthus roseus* | $KNO_3$, $NH_4Cl$: 20 mM Sandy soil | Totals alkaloids | Leaves and roots | Misra and Gupta (2006) |
| *Catharanthus roseus* | $NO_3NH_4$ : 11 mM Soil conditions | Total alkaloids, vincristin and vinblastin | Leaves | Abdolzadeh *et al.* (2006) |
| *Catharanthus roseus* | $CO(NH_2)_2$: 150 kg N $ha^{-1}$ | Vinblastine, vincristine | Leaves and roots | Sreevalli *et al.* (2004) |
| *Datura innoxia* | Nutrient solution: 600 kg N $ha^{-1}$ Field conditions | Hyoscyamine and scopolamine | Leaves and fruits | Al-Humaid. (2003) |

### *2.4.6 Accumulation of phenolic compounds subjected to N*

It is well known that N impacts primary metabolism as well phenylpropanoid metabolism. However, many reports indicate differential responses to N in higher plants. N-deficiency has shown to trigger phenolics synthesis and accumulation in higher plants. In this sense, this response can be a strategic tool to raise the plant defense against abiotic and biotic stress. For example, under N deprivation in *Phaseolus vulgaris* plants, absorbing compounds such as flavonoids increased more than 2 times compared with plants supplied with N. As a result, increased tolerance to abiotic stress such as UV-B ambient has been observed (Riquelme *et al.,* 2007). Possible explanation for increased phenolics production at low N addition levels could be an imbalance of C-N ratio. Specifically, low N supply might be contribute to raise erythose-4 phosphate production in the pentose phosphate pathway, and thus to increase the phenolics contents and PAL activity as suggested by Ibrahim and Jaffar (2011) in a study with *Labisia pumila* herb.

The relationship between N nutrition, secondary metabolism and biotic stress is scarcely documented (Dietrich *et al.,* 2004). In this sense, field experiments with *Vaccinium myrtillus* plants infected by fungus increased the concentration and content of arbutin, chlrorogenic acid and p-coumaric acid under high N-dose (50 kg $ha^{-1}$ $NH_4NO_3$). In the same study, quercetin-3-glucoside and catechin were not affected by N-supply in healthy leaves. This response in *V. myrtillus* was more influenced by environmental conditions rather than N-supply (Witzell and Shevtsova, 2004). In addition, it has been shown that N-supply (0.8 g N per pot applied as $NH_4NO_3$) decreased phenolic content in *Solanum tuberosum* plants (Mittelstraß *et al.,* 2006). However, this species are more likely to be tissues affected by certain fungi that N-fertilization in the synthesis of phenolic compounds.

Table 2 indicates some species of plants and their response to N. Treutter (2010) reviewed the effect of light, temperature, mineral nutrition, water management, grafting and elevated atmospheric $CO_2$ on the improvement of the phenols content in crops. In most of the studies, management with high levels of N decreased the synthesis and accumulation of phenolic compounds in plant tissues. These responses depended on the plant specie, cultivar, time of harvest and N-source. Liu *et al.* (2010) indicated that N

excess (300 mg N $kg^{-1}$ soil) decreased chlorogenic acid and flavonoids, hence antioxidant activity and quality of plants of *Chrysanthemum morifolium.* A classical report by Bongue-Bartelsman and Phillips (1995) with tomato (*Licopersicum esculentum*) skin indicated that two compounds, an anthocyanin (petunidin) and a flavonol (quercetin petunidin-3-O-glucoside) increased in N deficient plants. Awad and Jager (2002) found that at N concentration of 54 mg 100 $g^{-1}$ FW in apple (cv Elstar mutant Elshof) skin, cyanidin-3-galactoside decreased by 40% compared with concentrations of 32 N mg 100 $g^{-1}$ FW. Nevertheless, chlorogenic acid and total flavonoids maintained their concentration irrespective of the N-treatments.

Not always the increase of N fertilization decreases the phenolic compounds and hence antioxidant activity of fruits and leaves. Thus, Mogren *et al.* (2006) did not find variation in the quercetin content in onions (*Allium cepa*) at different doses of N fertilizer (about 40 and 80 kg $ha^{-1}$ calcium nitrate). Similarly, Azaizeh *et al.* (2005) evaluated the inhibition of β-carotene oxidation using medicinal Arabic plant extracts. In this study, the antioxidant activity related with flavonoid content, improved in *Teucrium polium* plants with higher doses of Hoagland solution.

It is well known that organic fertilizers can improve the quality of plants. Compost and vermicompost from different organic sources have been used in lettuce crops (Coria-Cayupan *et al.*, 2009), chicken manure in tomato (Toor and Savage, 2006), peat substrate in blueberry (Ochiam *et al* 2009; 2010), compost in strawberry and blueberry (Whang and Lee, 2003; Montalva *et al.,* 2010) and organic residues from agro-industrial processes in maize (Ertani *et al*., 2011). Organic fertilizers contain large macro and micronutrients, which function as cofactors of several enzymes involved in secondary pathways (Poschenrieder *et al.,* 2008 and references therein). Hence, they have increased the quality of plants by enhancing antioxidant compounds accumulation (Søltoft *et al.,* 2010). Conventional (ammonium sulfate) and organic mix fertilizer (compost, N content 1.5%; blood meal, N content 8%; legume flour, N content 4%) were compared in a study with *Vaccinium corimbosum* plants. Leaf antioxidants phenolic compounds were 70% higher with organic N fertilizer than with conventional N fertilizer. Increasing the quality of the plant by organic fertilizers could be that the optimal N dose resulted in enhanced C fixation, levels of photosynthetic enzymes and

amino acids precursors for phenolic synthesis. In addition, organic fertilizers stimulate N uptake and assimilation and this fact is associated with the synthesis of phenolic compounds by enhanced PAL enzymatic activity (Ertani *et al.*, 2011). Moreover, microbial activity in organically fertilized soils slowly release N other nutrients to be absorbed by plant roots (Montalva *et al.,* 2010). On the other hand, high phosphorus (P) concentration in some organic amendments could be inhibit N uptake and N deficiency in the plant improves antioxidant compounds accumulation (Coria-Cayupan *et al.*, 2009).

Few studies have evaluated the impact of N concentration in plant tissues (root, leaf or fruits) on phenolics production. Skupien (2006) determined N content and total phenols in fruits of four cultivars of *Vaccinium corymbosum*. Whereas cv. Bluecrop contained the highest concentrations of phenols at 3.5 g N $kg^{-1}$ FW, cv. Spartan with 4.2 g N $kg^{-1}$ FW contained the lowest concentration of phenolics (Table 2.2). This fact confirms that some species at lower N concentration, allocate C-resources to the formation of secondary compounds. In this case, higher N content in fruits of cv. Spartan had not relationship with totals phenols synthesis. The evidence above suggests further research to elucidate the relationship among N-fertilization, synthesis and accumulation of phenolic and N-compounds and enzymes involved in their biosynthesis (Kovacik *et al.,* 2007; Ruan *et al.,* 2010 and references therein). Nevertheless, the activation or inhibition of the synthesis of individual phenolic compounds may be also related other stress conditions that could not be explained by carbon-nitrogen balance (Keski-Saari *et al.,* 2005).

**Table 2.2.** Phenolic compounds that maximize its concentration by N in higher plants.

| Specie | Experimental conditions | Phenolic compounds | Tissue | Reference |
|---|---|---|---|---|
| *Triticum aestivum* | $NH_4NO_3$ : 0.25-2.0 g N pot Soil conditions | Total phenols | Leaves | Stumpf et al. (2015) |
| *Vitis vinifera* | $CO(NH_2)_2$: 0.9-1.5 kg N $ha^{-1}$ Soil conditions | Anthocyanidins | Fruits | Portu et al. (2015) |
| *Cichorium intybus* | Nutrient solution: mineral/organic N Soil condition | Chorogenic acid | Leaves | Sinkovič et al. (2015) |
| *Cecropia peltata* | $NO_3$: 0.2 mM Hydroponic condition | Chlorogenic acid | Leaves | Mora-Izquierdo et al. (2011) |
| *Chrysanthemun morifolium* | $NH_4SO_4$: 0.1, 0.3,0.5 g $kg^{-1}$ Soil pot | Chlorogenic acid | Leaves | Lui et al. (2010) |
| *Solanun lycopersicum* | Hoagland Nutrient solution | Chlrogenic acid and rutin | Leaves | Løvdal et al. (2010) |
| *Vaccinium myrtillus* | $NH_4NO_3$: 12.5-50 kg $ha^{-1}$ Soil conditions | Delphinidin, malvidin, cyanidin, petunidin | Fruit | Åkerström et al. (2009) |
| *Achillea collina* | $Ca(NO_3)_2$: 0.1-1 mM Hydroponic condition | Chlorogenic and caffeic acid | Leaves | Giorgi et al. (2009) |

| | | | | |
|---|---|---|---|---|
| *Matricaria chamomilla* | Hoagland solution with or without N | caffeic, chlorogenic, o- and p-coumaric and ferulic acids | Leaves | Kovačik et al. (2007) |
| *Nicotania tabacum* | $KNO_3$: 0.2 mM Hydroponic conditions | Chlorogenic acid, rutin | Leaves | Fritz et al .(2006) |
| *Vaccinium myrtillus* | $NH_4NO_3$: 0, 12.5, 50 kg $ha^{-1}$ Soil conditions | Chlorogenic acid, arbutin, p-coumaric acid | Leaves | Witzell and Shevtsova, (2004) |
| *Vitis vinifera* | $NH_4NO_3$: 1.4, 3.6, 7.2 mM Sandy Soil | Malvidin- delphinidin-petunidin-3-glucoside | Fruit | Hilbert et al. (2003) |
| *Malus domestica* | $CO(NH_2)_2$: 35-210 kg $ha^{-1}$ Soil conditions | Cyanidin-3-galactoside | Fruit | Awad and Jager, (2002) |
| *Solanum lycopersicum* | $NH_4NO_3$: 0-60 mM Hydroponic condition | Quercetin, kaempferol | Leaves | Stewart et al. (2001) |
| *Solanum lycopersicum* | $NH_4NO_3$ Hydroponic condition | Quercetin-3-O-ß-glucoside | Leaves | Bongue-Bartelsman and Phillips (1995) |

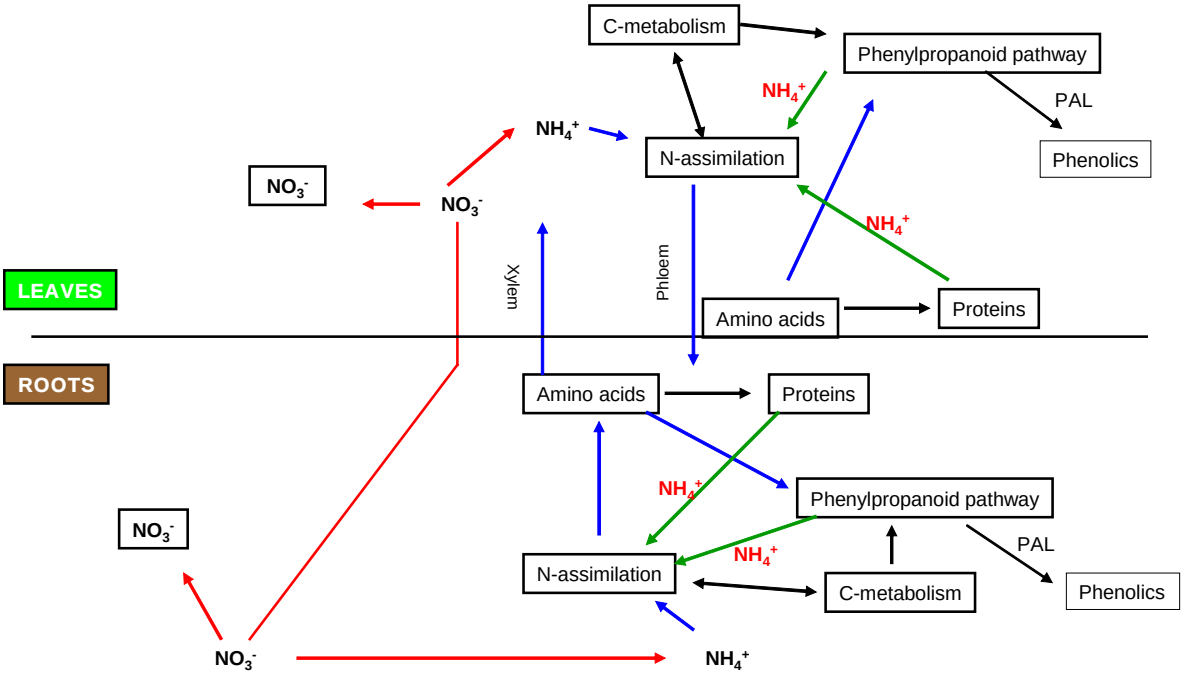


**Figure 2.2.** A model proposal for N-recycled metabolism in roots and leaves of N-starved plants via phenylpropanoid pathway. Red arrows indicate nitrate reduction to ammonia. Blue arrows indicate ammonia assimilation into organic compounds (amino acids, proteins and phenylpropanoid pathway). Green arrows indicate ammonia released from amino acids from phenylpropanoid pathway and protein catabolism. Black arrows indicate interlink between N-assimilation, C-metabolism, phenylpropanoid pathway and phenolics synthesis. Furthemore N-recycled is reassimilated by NADH-GOGAT/Fd-GOGAT enzymes. Adapted from Suzuki, (2010).

PAL activity can be used indicator the effect of N addition on phenolic synthesis in plant tissues. In this sense, positive correlation has been observed between PAL activity and total phenolics at increasing N-supply in *Phaseolus vulgaris* . In this study, secondary metabolites accumulation contributed to the improvement in biomass production (Sanchez *et al.*, 2000). In a hydroponic system with *Cecropia peltata* plants, Mora-Izquierdo *et al.* (2011) suggested that PAL enzyme could be activated by nitrate deficiency. In addition, leaves suffered chlorosis at lowest nitrate addition level (0.2 mM) and this fact may be due to the accumulation to chlorogenic acid and isoorientin. Thus, in N-starved plants, PAL enzyme activity appears to act by recycling N for the

production of amino acids and shunting the C-skeletons into phenylpropanoid compounds synthesis.

The gene expressions of PAL, chalcone synthase (CHS) as well as of other enzymes from the initial stages of phenylpropanoid metabolism have showed variations in response to N-fertilization. At 2 mM $KNO_3$ and 1 mM $NH_4NO_3$ added several secondary metabolites are repressed as well, levels of transcripts and enzyme activity in the phenylpropanoid pathway (Wolf-Rüdiger *et al.,* 2004). The constitutive genes that control the PAL activity have been identified in various species such as rice, parsley, tomato, potato and *Arabidopsis*. Four isoforms (PAL1, PAL2, PAL3 and PAL4) were described to be affected by N supply in *Arabidopsis* plants (Rohde *et al*., 2004). PAL1 and PAL2 transcripts accumulation increased in plants grown in Hoagland solution without N. This fact was highly correlated with both the PAL enzyme activity and the production of flavonoids, kaempferols, quercetins and anthocyanins in *Arabidopsis* plants (Olsen *et al*., 2008). In tomato plants, some structural genes were expressed under environmental conditions such light, temperature and N-depletion. PAL5, CHS2, F3H and FLS raised its expression at low N-treatment. These genes encode the synthesis of anthocyanins, quercetin, kaempferol and caffeoyl derivates (Løvdal *et al.*, 2010). Moreover, *Nicotania tabacum* plants growing in N-starved conditions increased their levels of some phenylpropanoids and lignin precursors in the stems. This fact was proven by enhanced levels of PAL1 and 4CL transcripts (Fritz *et al.,* 2006).

Thus, it is necessary to deep in the knowledge about the N content of roots, leaves or fruits that would induce the production of phenolic compound, and therefore could that enhance the antioxidant activity and plant defense against stress conditions.

### *2.5 Concluding remarks and future perspectives*

The experimental facts are unclear in relation to the influence of N nutrition on secondary metabolism in higher plants. It is generally postulated that N deficiency enhances the synthesis of phenolic compounds. These metabolites are accumulated in tissues to improve plant defense. Conversely, N sufficiency has been usually related with raised contents of amino acids precursors of alkaloids and alkaloids compounds. Considering the benefits of secondary metabolites compounds for human health, more studies are necessary to improve N fertilization management practices, especially in

crops of agronomical interest like berries due to their antioxidant richness. To date the N management in agricultural systems has been mainly focused in the improvement of crop yields and primary metabolism in higher plants as well as in the reduction of the negative impact of N losses by leaching and volatilization in the environment. Nevertheless, at the moment, it is necessary a deepest knowledge about the impact of N nutrition on the plant secondary metabolism. Moreover, the understanding of the relation between N concentration in plant tissues and the synthesis or accumulation of secondary compounds such as alkaloids and phenylpropanoids should be addressed in future researches. Such studies must consider molecular approaches involving key enzymes of secondary metabolic pathways and the enhancement of phenylpropanoids content, without detrimental effects on the primary metabolism and in turn, on the plant growth.

# Chapter 3

# Photosynthetic and antioxidant performance are differentially affected by nitrogen supply in highbush blueberry cultivars at the short-term

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# Photosynthetic and antioxidant performance are differentially affected by nitrogen supply in highbush blueberry cultivars at the short-term

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## Abstract

Nitrogen (N) is an essential nutrient for photosynthesis and could influence phenolic compounds synthesis in higher plants. The effect of different N supply (0 to 38 mM) on the photosynthetic and antioxidant performance of highbush blueberry cultivars (Legacy and Bluegold) grown in nutrient solution at the short-term was studied. In both cultivars, N concentration of leaves slightly increased in response to N supply, with Bluegold showing frequently higher N concentrations than Legacy. Photosynthesis was reduced in Bluegold at the highest N dose, whereas in Legacy a decrease of $CO_2$ assimilation occurred under N starvation. This decrease in photosynthesis was accompanied by enhanced lipid peroxidation but only in Bluegold. In both cultivars SOD was activated at increasing N supply. Legacy also showed increased SOD activity aimed to counteract oxidative stress at the greater N addition level. Radical scavenging activity was not affected due to N supply. However, total phenols and anthocyanins steadily declined in leaves of Legacy, and flavonoids were significantly raised in roots of both cultivars at increasing N treatments. Thus, our findings indicate that blueberry cultivars exhibit differential sensitivity to N stress at the short-term, and SOD appear to be more involved than phenolic compounds in the amelioration of N-induced oxidative stress. Further studies are required to confirm the sensitivity to either N starvation in Legacy or N excess in Bluegold at the long-term.

***Key words:*** Nitrogen, oxidative damage, phenolic compounds, superoxide dismutase. *Vaccinium corymbosum*

## 3.1. Introduction

Nitrogen (N) is well recognized as an essential element for plant growth and also regulates the synthesis of secondary metabolites such as phenolic compounds (Ruan *et al.,* 2010; Åkerström *et al.,* 2009). In this sense, it has been observed dissimilar responses to N supply by enhancing, diminishing or non influencing phenolics accumulation in plants (Lødval *et al.,* 2010; Fritz *et al.,* 2006; Mogren *et al.,* 2006).

It has been hypothesized that high N levels in plant tissues conduct to the formation of more amino acids and proteins for growth in relation to defense compounds (Bryant *et al.,* 1983) like secondary metabolites. Conversely, phenolics rather than aminoacid compounds could be increased under N starvation. In fact, the accumulation of phenolics was raised by N depletion at the short-term in *Arabidopsis* (Olsen *et al.,* 2008). Moreover, chlorogenic acid concentration was enhanced by two-fold in N non-treated chamomile plants compared with those subjected to N nutrition (Kováčik *et al.,* 2007).

On the other hand, the lack of N could trigger reactive oxygen species (ROS) accumulation and the concomitant oxidative damage due to alterations in photosynthetic functionality (Huang *et al.,* 2004; Pompelli *et al.,* 2010). Nevertheless, the activity of antioxidant enzymes such as SOD (Ramalho *et al.,* 1998; Logan *et al.,* 1999) and phenolic compounds as anthocyanins (Diaz *et al.,* 2006) could counteract oxidative stress under N starvation.

Since 1985, highbush blueberry (*Vaccinium corymbosum* L.) is positioned as an important crop cultivated in Chile (Lyrene and Muñoz, 1997) due to its high fruit and leaves antioxidant compounds (Ribera *et al.,* 2010; Ehlenfeldt and Prior 2001, respectively). Nonetheless, information concerning the physiology of this crop behind these beneficial effects is still poor. Moreover, there is scarce information about the N requirements of this crop, and to our knowledge only there are

few reports showing that blueberry can be sensitive to N excess, decreasing plant growth (Hanson and Retamales, 1992; Bañados *et al.,* 2012). However, to date there is also a lack of studies showing the relation between N nutrition and antioxidant behavior for blueberry. The objective of this work was to study the effect of different N supply on the photosynthetic and antioxidant performance of highbush blueberry cultivars grown in nutrient solution at the short term.

## 3.2 Materials and methods

### *3.2.1 Plant material and growth conditions*

Two-years-old highbush blueberry plants of two cultivars (Legacy and Bluegold) were grown in nutrient solution during 4 days under greenhouse conditions: temperature 25/20°C (day/night), photoperiod 16/8 h (light/dark) and 70% relative humidity. Plants were conditioned in plastic boxes filled with 10 L of modified Hoagland solution during 72 hours (Hoagland and Arnon, 1950). Then, plants were transferred to containers with Hoagland solution without N for 72 hours. Thereafter, plants were subjected to different $NH_4NO_3$ concentrations in the culture media (0, 9, 18, 28 or 38 mM N). Hydroponic solutions were continuously aerated and the pH of the solutions was adjusted daily to 4.8 using 0.1 M HCl. During the time-course of the experiment *in vivo* $CO_2$ assimilation measurements were recorded. At the end of the experiment, completely expanded leaves from the second node and roots were collected. Fresh samples were stored at -20 or -80ºC for biochemical analyses; subsamples were dried for N concentration analyses.

### *3.2.2 Measurements of $CO_2$ assimilation*

Assimilation of $CO_2$ was measured between 9 and 10 a.m. in intact leaves belonging to the second node using a portable photosynthesis system (LI-6400, LI-COR Bioscience, Inc., Lincoln, Nebraska, US & Canada) as described by Reyes-Díaz *et al.* (2011).

### *3.2.3 Plant nitrogen concentration*

Nitrogen concentration in leaves and roots was determined by the Kjeldahl method (Sadzawka *et al.*, 2004).

### *3.2.4 Lipid peroxidation and radical scavenging activity (RSA)*

Thiobarbituric acid reactive substances (TBARS) were spectrophotometrically assayed in leaves and roots as an index of oxidative stress, according to the modified method of Du and Bramlage. (1992). For the radical scavenging activity (RSA) the free radical 2.1-diphenyl-1-picrylhydrazyl (DPPH) scavenging method was assayed in leaves and roots at 515 nm, using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as standard (Chinnici *et al.*, 2004).

### *3.2.5 Quantification of phenolic compounds*

Total phenols were determined at 765 nm by the Folin-Ciocalteu method, using chlorogenic acid as the standard (Slinkard and Singleton, 1977). Total flavonoids were analyzed by the aluminum chloride colorimetric assay, using rutin as the standard (Cheng and Breen, 1991). Total anthocyanins were spectrophotometrically measured at 530 and 657 nm by the method described by Chang *et al.* (2002).

### *3.2.6 Superoxide dismutase activity (SOD)*

The activity of SOD (EC. 1.15.1.1) was analyzed in leaves and roots by measuring the photochemical inhibition of nitroblue tetrazolium (NBT) at 560 nm (Giannopolitis and Ries, 1977). One SOD unit was defined as the amount of enzyme corresponding to 50% inhibition of NBT reduction (Donahue *et al.*, 1997). The enzyme activity was expressed on protein basis, and protein concentration was measured at 595 nm by the Bradford (1976) method.

### *3.2.7 Data analyses*

Chemical and biochemical data were analyzed by two-way ANOVA after the normality and homoscedasticity tests. Tukey's test was used to evaluate differences between means ($P \leq 0.05$). In addition, Pearson correlation was used to assess the relationships between two response variables. Analyses were performed with Sigma Stat software v. 2.0 (SPSS, Chicago, IL, USA).

## 3.3 Results

For both cultivars, differential responses were observed in $CO_2$ assimilation as a result of variable N supplies (Figure 3.1). When no N was applied, a decrease of about 28% in

photosynthesis was found in Legacy respect to the plants grown at increasing N supply, which did not varied among them. Conversely, in Bluegold there was no effect of N supply on $CO_2$ assimilation, except at the highest N dose, decreasing by about 60% ($p \leq 0.05$).

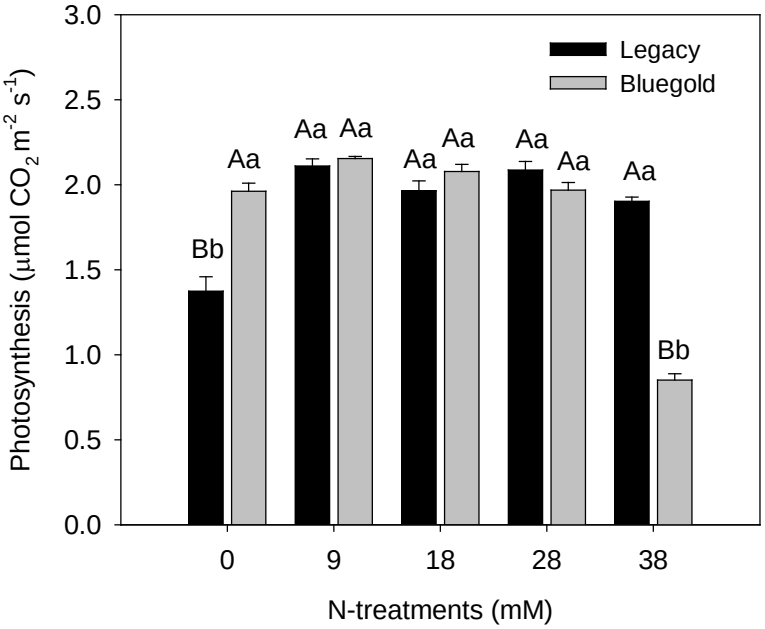


**Figure 3.1.** Photosynthesis rates of two highbush blueberry cultivars grown under different N treatments at the short-term. The values represent the average of three replicates ± SE. Different lowercase letters indicate statistically significant differences among N treatments for the same cultivar. Different uppercase letters indicate statistically significant differences between cultivars for the same N treatment ($p \leq 0.05$).

No statistically significant interaction between cultivars and N treatments for N concentration was found neither for leaves ($p = 0.055$) nor for roots ($p = 0.327$). In Bluegold, an increase in leaf N concentration was observed in treatments with 9 mM N and higher compared to the 0 N treatment, whereas in Legacy leaf N concentration was steady in treatments with 0 to 28 mM N, and increased only at 38 mM N (Table 3.1). Thus, N concentration in leaves was frequently higher ($p \leq 0.05$) in Bluegold than in Legacy. Both cultivars had similar root N concentrations across N treatments (Table 3.1).

Nitrogen applications did not significantly influence lipid peroxidation in leaves and roots of Bluegold at N application levels up to 28 mM, but it increased by about 10% (leaves) and 34% (roots) at the highest N supply (Table 3.1). In Legacy leaves oxidative damage decreased by 20% at doses above 18 mM N, whereas in roots it did not vary significantly due to the N treatments (Table 3.1).

A statistically significant interaction between cultivars and N treatments for SOD activity was found for leaves ($p \leq 0.001$) and roots ($p = 0.008$). The activity of SOD was higher in Legacy than Bluegold leaves at each N treatment ($p \leq 0.05$), except at the greatest N supply (Table 3.1). In addition, SOD was activated under either N starvation or N addition levels above 18 mM in Legacy leaves. Nonetheless, in Bluegold leaves SOD activity rose progressively as the N supply increased, being around 95% higher at 38 mM N in comparison with plants grown without N. In Legacy and Bluegold roots, a significant increase of SOD activity was detected at N supply up to 28 mM N.

In terms of RSA, leaves showed a higher antioxidant activity than roots in both cultivars, and in general no significant differences were found due to N treatments or between cultivars (Table 3.1). In leaves, there was significant interaction between N treatments and cultivars for total phenols ($p \leq 0.001$), flavonoids ($p \leq 0.001$) and anthocyanins ($p = 0.006$), whereas for roots, a statistically significant interaction was only found for total phenols ($p = 0.024$). In both cultivars, more phenols were accumulated in leaves than in roots (Figure 3.2a, b). Total phenols in leaves did not statistically vary in Legacy cultured at up to 18 mM N, but then decreased at 28 and 38 mM N, resulting in approximately 3-fold difference between 0 and 38 mM N treatments. In contrast, Bluegold leaves showed no difference in phenolics concentration. In general, Legacy accumulated more phenols than Bluegold in roots (Figure 3.2b). No difference in phenols concentration was observed in Bluegold roots under increasing N supply, but a decrease of about 23% occurred at 38 mM N compared with 0 mM N in Legacy roots.

**Table 3.1.** Chemical and biochemical properties of leaves and roots of highbush blueberry cultivars grown under different N treatments at the short-term

| N treatments (mM) | Cultivars | N concentration (g kg$^{-1}$ DW) | | Lipid peroxidation (nmol MDA g$^{-1}$ FW) | | SOD activity (U mg$^{-1}$ protein) | | Radical scavening capacity (mg TE g$^{-1}$ FW) | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Leaves* | *Roots* | *Leaves* | *Roots* | *Leaves* | *Roots* | *Leaves* | *Roots* |
| 0 | Legacy | 14.2±0.9 **Ab** | 11.3±0.7 **Aa** | 129±7 **Aa** | 56.5±1.2 **Aa** | 229±6 **Ab** | 69±12 **Ac** | 11.1±1.4 **Aa** | 2.8±0.3 **Aa** |
| 9 | | 13.1±0.1 **Bb** | 13.1±0.6 **Aa** | 119±3 **Aa** | 58.2±1.1 **Aa** | 204±7 **Ac** | 93±2 **Abc** | 10.6±1.3 **Aa** | 3.0±0.3 **Aa** |
| 18 | | 13.6±0.5 **Bb** | 13.1±1.0 **Aa** | 124±12 **Aa** | 56.2±3.5 **Aa** | 222±18 **Abc** | 140±13 **Aa** | 9.7±1.3 **Aa** | 2.4±0.3 **Aa** |
| 28 | | 14.1±0.7 **Bb** | 12.8±0.7 **Aa** | 96±1 **Bb** | 53.5±2.2 **Aa** | 251±9 **Aa** | 164±10 **Aa** | 8.9±0.6 **Aa** | 2.6±0.2 **Aa** |
| 38 | | 16.2±0.8 **Aa** | 12.6±0.02 **Aa** | 91±4 **Bb** | 53.2±5.0 **Aa** | 251±2 **Aa** | 113±2 **Ab** | 10.6±0.7 **Aa** | 2.5±0.5 **Aa** |
| 0 | Bluegold | 14.9±0.6 **Ab** | 12.4±0.1 **Aa** | 119±9 **Ab** | 45.1±6.2 **Bb** | 124±6 **Bd** | 71±6 **Ab** | 9.3±0.1 **Aa** | 2.3±0.5 **Aa** |
| 9 | | 17.3±0.3 **Aa** | 12.6±0.4 **Aa** | 110±10 **Ab** | 49.5±3.3 **Bb** | 120±10 **Bd** | 94±11 **Ab** | 9.6±1.3 **Aa** | 2.6±0.1 **Aa** |
| 18 | | 17.2±1.3 **Aa** | 11.9±0.7 **Aa** | 119±1 **Ab** | 48.2±2.5 **Ab** | 166±11 **Bc** | 75±1 **Bb** | 7.3±0.1 **Bb** | 2.8±0.3 **Aa** |
| 28 | | 18.4±0.5 **Aa** | 12.4±0.2 **Aa** | 114±1 **Ab** | 50.8±1.7 **Ab** | 192±11 **Bb** | 123±7 **Ba** | 9.8±0.2 **Aa** | 2.7±0.1 **Aa** |
| 38 | | 17.9±0.4 **Aa** | 13.4±0.3 **Aa** | 130±2 **Aa** | 60.5±0.9 **Aa** | 241±15 **Aa** | 82±5 **Bb** | 9.8±0.2 **Aa** | 2.9±0.1 **Aa** |

The values represent the average of three replicates ± SE.

Different lowercase letters indicate statistically significant differences among N treatments for the same cultivar.

Different uppercase letters indicate statistically significant differences between cultivars for the same N treatment ($p \leq 0.05$).

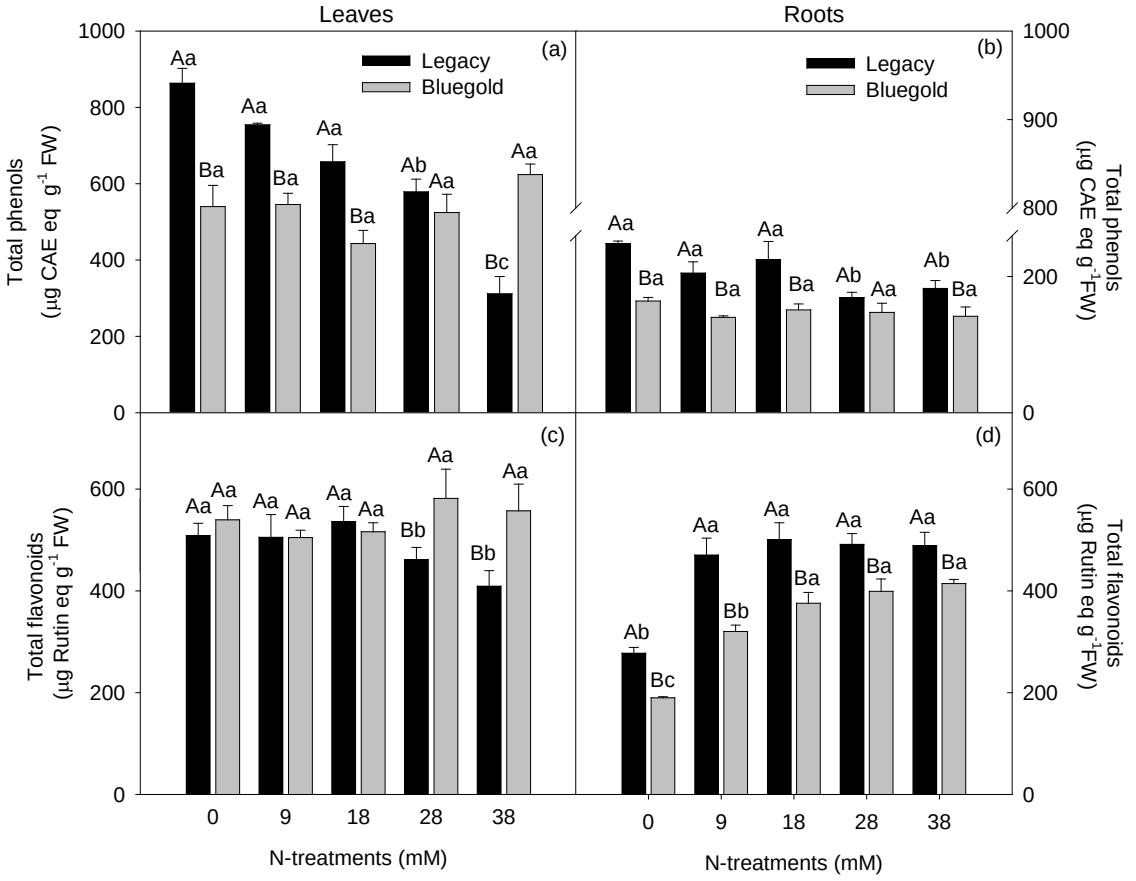


**Figure 3.2.** Total phenols and flavonoids in leaves and roots of highbush blueberry cultivars grown under different N treatments at the short-term. The values represent the average of three replicates ± SE. Different lowercase letters indicate statistically significant differences among N treatments for the same cultivar. Different uppercase letters indicate statistically significant differences between cultivars for the same N treatment ($p \leq 0.05$).

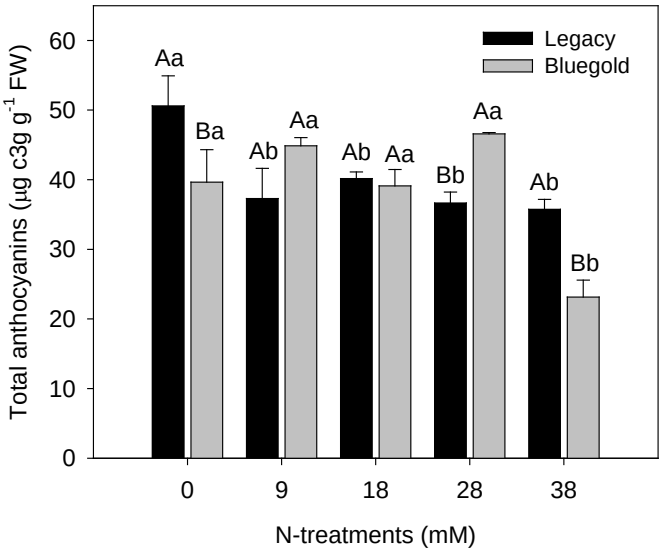


**Figure 3.3.** Anthocyanins in leaves of highbush blueberry cultivars grown under different N treatments at the short-term. The values represent the average of three replicates ± SE. Different lowercase letters indicate statistically significant differences among N treatments for the same cultivar. Different uppercase letters indicate statistically significant differences between cultivars for the same N treatment ($p \leq 0.05$).

Even though leaf flavonoids in Bluegold were not affected significantly by the N supply, they were lowered by almost 20% in Legacy at the highest N application dose (Figure 3.2c). By contrast, in roots of both cultivars, total flavonoids increased due to N supply. In fact, N-treated plants exhibited 2-fold higher flavonoid concentrations than N-starved plants (Figure 3.2d). Under N addition, Legacy roots had 18% greater flavonoid concentration than Bluegold roots at all N treatments (Figure 3.2c). Furthermore, anthocyanins concentration in Legacy leaves was highest at 0 N and decreased by 29% under N supply. In Bluegold leaves, anthocyanin accumulation was not affected up to 28 mM N, but was reduced by about 42% at the highest N dose (Figure 3.3).

## 3.4 Discussion

Nitrogen (N) is an essential nutrient for photosynthesis, plant growth and development. It is also involved in the regulation of secondary metabolites synthesis mainly phenolic compounds (Ruan *et al.,* 2010; Åkerström *et al.,* 2009). In this study the effects of different N supply on chemical, physiological and biochemical features of highbush blueberry cultivars at the short-term were evaluated. It is widely reported that leaves and fruits of blueberry exhibit a particularly high content of phenolic compounds (Ribera *et al.,* 2010; Ehlenfeldt and Prior, 2001, respectively), which could be affected by N supply as previously informed for tomato, Arabidopsis and chamomilla at short-term (Kováčik *et al.,* 2007; Olsen *et al.,* 2008; Løvdal *et al.,* 2010).

Our results demonstrated that N treatments slightly influenced N concentration in leaves of both cultivars (Table 3.1). Even though Bluegold showed usually higher leaf N concentration than Legacy; all N contents reported here are within the normal range mentioned by Bañados *et al.* (2012) for blueberry.

On the other hand, our findings indicated that photosynthesis was reduced in Legacy under N starvation without any increase of lipid peroxidation (Figure 3.1, Table 3.1). Possible explanations for a decrease of $CO_2$ assimilation at 0 N treatment in leaves could be: i) a reduced stomata aperture, lowering carbon fixation in the mesophyll tissue (Wong, 1979), ii) a decline of the stomatal conductance (Radin and Ackerson, 1981) or iii) a decrease in proteins as RUBISCO (Lawlor, 1993). Moreover, studies have demonstrated that N deficiency can decrease the content of photosynthetic pigments, thereby reducing the photosynthetic performance of plants (Huang *et al.* 2004).

Conversely, photosynthesis was significantly reduced in Bluegold at the highest N level (Figure 3.1), which was accompanied by an increase of oxidative damage (Table 3.1). Nitrogen excess is frequently associated to ammonium ($NH_4^+$) toxicity in vascular plants (Hachiya *et al.,* 2012). The negative impacts of high $NH_4^+$ accumulation in plant tissues might be a consequence of $H^+$ consumption by the conversion of ammonia ($NH_3$) to $NH_4^+$ causing uncoupling of the $H^+$ gradient into biological membranes (Bloom *et al*., 1992), which could drive the photosynthesis impairment. In addition, Pompelli *et al.* (2010) indicated that high N addition levels could increase

ROS production in cell membranes due to failure in the photosynthesis. Otherwise, it is well recognized that N stress (excess or deficiency) could trigger ROS production (Asada and Takahashi, 1987; Ramalho *et al.,* 1998) affecting cell membranes integrity of chloroplasts and then the photosynthetic machinery (Buchanan *et al.,* 2000).

Non-enzymatic and enzymatic antioxidant defenses could help to protect the photosynthetic apparatus against N-induced oxidative stress. It is noteworthy that the results regarding the effect of N nutrition on phenolic content are controversial. Thus, some reports have indicated that N supply did not influence the phenolics accumulation (Mogren *et al.,* 2006; Løvdal *et al.,* 2010), whereas Logan *et al.* (1999) detected low content of phenolics in plants grown under N deprivation. Contrarily, high N levels in the growth media have been associated with a reduction of both, phenolic compounds content and antioxidant capacity in vascular plants (Liu *et al.,* 2010; Royer *et al.,* 2013). In our study, leaves of Legacy showed a decrease in phenols (Figure 3.2a), including flavonoids (Figure 3.2c) and anthocyanins (Figure 3.3) as a consequence of increasing N additions. In fact, there was a significant inverse correlation between total phenols and N concentration in Legacy leaves ($r = -0.609$, $p \leq 0.05$). These findings agree with the hypothesis of Bryant *et al.* (1983) sustaining that plants growing under high N levels allocate N resources to the formation of new tissues and not for phenolics synthesis. In addition, we found that root flavonoids content increased in response to N supply (Figure 3.2d), mainly in Legacy, and we observed that leaf antioxidant activity was maintained at all N treatments in both cultivars (Table 3.1). Likewise, SOD activity was increased under N deprivation and at high N doses in Legacy leaves, counteracting oxidative stress at the greater N addition levels (Table 3.1). On the other hand, the enzyme activity was steadily raised in Bluegold leaves at increasing N supply. In this cultivar, such activation appears to be responsible of the maintenance of lipid peroxidation at the basal levels (up to 28 mM N), but not under the highest N treatment (Table 3.1). This behavior denotes the greater sensitivity of Bluegold to N excess compared to Legacy.

Thus, although both cultivars exhibited similar patterns for N accumulation at the short-term, we observed dissimilar plant performance between them at N starvation or excess. Based on our results about photosynthesis and oxidative stress, further studies

are required to confirm the sensitivity to either N starvation in Legacy or N excess in Bluegold at the long-term.

We conclude that blueberry cultivars differ in its sensitivity to N starvation or excess at the short-term as demonstrated by dissimilar effects of variable N supply on photosynthesis and antioxidant performance. In this regard, SOD activity seems to be more implicated than non-enzymatic antioxidant compounds in the protection against oxidative stress induced by N excess. It is remarkable that N excess can trigger a considerable decrease of leaf phenolic compounds (including anthocyanins) depending on the blueberry cultivar, which can reduce not only plant productivity, but also its nutraceutical value, and then the crop profitability.

### 3.5. Acknowledgments

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# Chapter 4

## Leaf nitrogen thresholds ensuring high antioxidant features of *Vaccinium corymbosum* cultivars

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# Leaf nitrogen thresholds ensuring high antioxidant features of *Vaccinium corymbosum* cultivars

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## Abstract

Deficiency or excess of nitrogen (N) supply can promote formation of reactive oxygen species in plants, inducing oxidative stress. Otherwise, plants may enhance phenolics biosynthesis and antioxidant capacity under N deficiency, but this effect is plant species-dependent. There is no information about influence on phenolics and antioxidant activity in highbush blueberry (*Vaccinium corymbosum* L.), in which quality and commercial importance depend on high phenolics concentration. We studied the effect of variable N supply (0 to 38 mM) on N uptake and antioxidant responses in two highbush blueberry cultivars (Legacy and Bluegold) hydroponically grown at the long-term. Nitrogen leaves concentration was enhanced for both cultivars at increasing N supply. Bluegold decreased $CO_2$ assimilation at 0 N treatment, possibly due to both, insufficient N concentration and a decline in superoxide dismutase (SOD) activity. In contrast, SOD was activated in Legacy at 0 N, and interestingly only this cultivar maintained $CO_2$ assimilation rates across all N treatments. Both cultivars showed higher phenolics and antioxidant activity levels at 9 mM. Despite the differential responses among the cultivars, we propose a threshold of 15 g N $kg^{-1}$ DW to ensure high antioxidant activity and quality in blueberry leaves.

**Keywords:** Antioxidant responses; phenolic compounds; photosynthesis; superoxide dismutase; *Vaccinium corymbosum*

## 4.1. Introduction

Nitrogen (N) is an essential macronutrient for plant growth and development (Marschner, 1986). It is a constituent of the photosynthetic apparatus, enzymes, proteins and pigments. Nitrogen deficiency or excess can negatively influences the plant metabolism. Nitrogen deficient growth media results in decreased contents of photosynthetic pigments (chlorophyll and carotenoids), thereby reducing the photosynthetic performance (particularly $CO_2$ assimilation) (Huang *et al.*, 2004). A strategy in some plant species growing under N deprivation is to recycle N from amino acids and proteins for new tissue growth (Kováčik *et al.*, 2007). By contrast, N toxicity is commonly associated with plant tissue ammonium ($NH_4^+$) accumulation. Depletion of C compounds that serve as a substrate for amino acid production has been reported in *Arabidopsis* plants grown under excessive N supply (Hachiya *et al.*, 2012).

Nitrogen deficiency or excess increase the production of reactive oxygen species (ROS) in plants, which results in lipid peroxidation of cell membranes (Asada *et al.*, 1987). In order to alleviate the oxidative damage induced by N-stress, enzymatic antioxidant systems such as superoxide dismutase (SOD) could be activated in plant tissues (Asada *et al.*, 1987). Nevertheless, according to Huang *et al.* (2004), N deficiency may also reduce SOD activity, exacerbating lipid peroxidation in rice (*Oryza sativa*). Otherwise, non-enzymatic antioxidant compounds (e.g. phenolics) have been induced in N-starved plants, leading an enhancement of the antioxidant activity in mustard (*Brassica juncea*) (Li *et al.*, 2008).

It is noteworthy that N supply may also impact the biosynthesis of some aromatic amino acids (Fritz *et al.*, 2006) such as phenylalanine, tyrosine, and tryptophan, which are substrates for the synthesis of phenolic compounds, which can involved in antioxidant responses. Hence, plants growing at a high N supply generally have decreased levels of phenolic compounds (Nybakken *et al.*, 2013), and the N excess might be diverted into amino acid and protein synthesis. Conversely, N-deprived plants showed an increased C resources allocation towards phenolic compounds production (Bryant *et al.*, 1983). Nevertheless,

enhanced phenolics (e.g. anthocyanin) concentration in response to N supply has also been reported (Okamoto *et al.,* 2003).

Therefore, to the date, the available literature is contradictory regarding N effects on phenolics accumulation and antioxidant capacity (Okamoto *et al.,* 2003; Mogren *et al.,* 2006). Even though most related reports have showed an inverse relationship between N availability and phenolics concentration in plant tissues, a decrease in N supply for improving antioxidant capacity could be used as a strategic tool to enhance both the quality and the profitability of some crops, minimizing environmental impact of N (Larbat *et al.,* 2012).

Highbush blueberry (*Vaccinium corymbosum* L.) is an important crop in Southern Chile that contains high concentration of phenolic acids, flavonols and anthocyanins (Ribera *et al.,* 2010; Ehlenfeldt *et al.,* 2001), which exhibit noticeable antioxidant capacity.
Currently, N fertilization commonly applied as ammonium to blueberry orchard varies between 20 and 140 kg $ha^{-1}$ (Hanson, 2006). It has been reported that blueberry plants are sensitive to N excess and common values for leaf N concentration ranged from 15 to 21 g $kg^{-1}$ DW (Bañados *et al.,* 2012).

Despite that in previous results we observed a differential response on physiological and antioxidant parameters to N fertilization during 4 days (Yañez-Mansilla *et al.,* 2014), there are not studies about N concentration in leaves that maintain a high antioxidant performance in blueberries at the long-term. We hypothesized that there is N concentration threshold that ensure a high phenolic concentration and antioxidant capacity without detrimental effects on plant performance. It is also important to highlight that behind the beneficial effects of these compounds, their increase in vegetative tissues of the plant (eg. leaves) subsequently can determine the levels of its accumulation in fruits. The aim of this work was to evaluate N uptake and antioxidant responses in two highbush blueberry cultivars hydroponically grown under variable N levels at the long-term.

## 4.2 Materials and Methods

### *4.2.1. Experimental conditions*

A nutrient solution assay was carried out during 4 weeks using two highbush blueberry (*V. corymbosum* L.) cultivars (Legacy and Bluegold) with contrasting tolerance to abiotic stresses including aluminum (Al) and manganese (Mn) toxicities and high UV-B radiation (Reyes-Díaz *et al.*, 2010; Rojas-Lillo *et al.*, 2013). The choice of harvest time was made to evaluate long-term N effects in order to determine whether the responses obtained on the short-term (Yañez-Mansilla *et al.*, 2014) are time-dependent. Two-year-old plants were provided by the commercial farm "Berries San Luis" located in Lautaro, Araucanía Region, Chile. During the time course of the experiment, greenhouse environmental conditions were: temperature 25/20°C (day/night), photoperiod 16/8 h (light/dark) and 70% relative humidity. Before beginning the assay, plants were conditioned during 72 hours in plastic boxes filled with 10 L of modified Hoagland solution (Hoagland and Arnon, 1950). After conditioning, plants were transferred to boxes filled with Hoagland solution without N for 72 hours.

Later on, plants were grown under the following N treatments: 0, 9, 18, 28 or 38 mM N. The N treatments were applied based on differential $NH_4NO_3$ levels in the culture media, and they were chosen in order to exceed the N sufficiency level associated with adequate photosynthetic performance and plant growth at the short-term (Yañez-Mansilla *et al.*, 2014). Nutrient solutions were replaced every 7 days and aerated during the course of experiment with an aquarium pump. The solution pH was adjusted to 4.8 daily using 0.1 M HCl.

At the end of the experiment, *in vivo* carbon dioxide ($CO_2$) assimilation was measured as described below. In addition, at the end of the experiment, fully expanded leaves from the second node as well as roots were collected, snap-frozen in liquid $N_2$ and then stored at -20ºC for lipid peroxidation or at -80ºC for other biochemical analyses. Additional samples were dried for determining total tissue N concentration.

### *4.2.2. Tissue N concentration*

The N concentration in leaves and roots was analyzed by the Kjeldahl method as described by Sadzawka *et al.* (2004).

### *4.2.3. $CO_2$ assimilation measurements*

Carbon dioxide ($CO_2$) assimilation was measured (between 09:00 and 10:00 AM) in intact leaves attached to the second node using a portable photosynthesis system (LI-6400, LI-COR Bioscience, Inc., Lincoln, Nebraska, US & Canada) provided by a leaf chamber with a controlled light source (300 μmol $m^{-2}$ $s^{-1}$) as described by (Reyes-Díaz *et al.,* 2011). In the leaf chamber the reference $CO_2$ concentration was 360 ppm, with a flow rate of 200 mL $min^{-1}$, 80% relative humidity and 20 ± 2 °C temperature.

### *4.2.4. Lipid peroxidation assay*

Lipid peroxidation was assessed in frozen samples by monitoring the thiobarbituric acid reacting substances (TBARS) as an index of oxidative damage in plant cells. The absorbance was measured at 532, 600 and 440 nm in order to correct the interference generated by TBARS-sugar complexes according to the modified method (Du and Bramlage, 1992).

### *4.2.5. Superoxide dismutase activity*

Frozen plant material was extracted with 50 mM potassium phosphate buffer ($K_2HPO_4$–$KH_2PO_4$), pH 7.0. Superoxide dismutase (SOD) (EC. 1.15.1.1) activity was analyzed by measuring the photochemical inhibition of nitroblue tetrazolium (NBT) at 560 nm (Giannopolis *et al.,* 1977). One SOD unit was defined as the amount of enzyme that generates a 50% inhibition of NBT reduction (Donahue *et al.,* 1997). The enzyme activity was expressed on both fresh weigh and protein basis. Protein in the crude enzyme extract was measured spectrophotometrically by the Bradford method (Bradford, 1976).

### *4.2.6. Radical scavenging activity*

The radical scavenging activity (RSA) of roots and leaves was assayed by the free radical 2.1-diphenyl-1-picrylhydrazyl (DPPH) scavenging method as described by (Chinnici *et al.,* 2004). The absorbance was measured spectrophotometrically at 515 nm using Trolox as standard.

### *4.2.7. Phenolic compound contents*

Total phenols were quantified in a spectrophotometer at 765 nm using the Folin-Ciocalteu method and chlorogenic acid as the standard (Slinkard and Singleton, 1977). Total

flavonoids were measured by the aluminum chloride colorimetric assay, using rutin as the standard (Cheng and Breen, 1991). Total anthocyanins were analyzed by the method described by (Chang *et al.*, 2002). The absorbance of anthocyanin extracts was determined in a spectrophotometer at 530 and 657 nm. Total anthocyanin content was expressed as mg of cyanidin-3-glucoside equivalent (c3g) per g FW.

#### *4.2.8. Statistical analyses*

The experiment was arranged in a completely randomized factorial design with two cultivars, five N treatments and three biological replicates. The effect of N on chemical and biochemical parameters was assessed by two-way ANOVA after the normality and homoscedasticity tests. Tukey's test was used to evaluate differences between means (at $P \leq 0.05$.) Additionally, Pearson's correlations were used to test the relationships between two response variables. Analyses were performed with Sigma Stat software v. 2.0 (SPSS, Chicago, IL).

### 4.3. Results

#### 4.*3.1 Leaf nitrogen concentration*

In general, leaf N concentration was enhanced in both cultivars with an increase in N supply (Figure 4.1A). Blueberry leaves of both cultivars exhibited the lowest N concentration (around 10 g N $kg^{-1}$ DW) under N deprivation ($P \leq 0.05$), and N doses up to 18 mM did not produce significant differences in leaf N concentration between the two cultivars. However, Bluegold had higher N concentration (21.5 g N $kg^{-1}$ DW) than Legacy (18.7 g N $kg^{-1}$ DW) at the 38 mM N treatment, and these values were about two-fold higher than those of N-starved plants.

Roots of untreated Legacy plants had 26% less N than plants grown with 9 to 38 mM N (Figure 4.1B). For Bluegold, we did not detect significant differences in root N concentration (average of 8.5 g N $kg^{-1}$ DW) at N doses up to 28 mM. Nevertheless, at the highest N supply, a significant increase in N concentration occurred in Bluegold roots. It is worth noting that Legacy roots had N concentration twice as high as the Bluegold ($P \leq 0.05$) at N doses up to 28 mM.

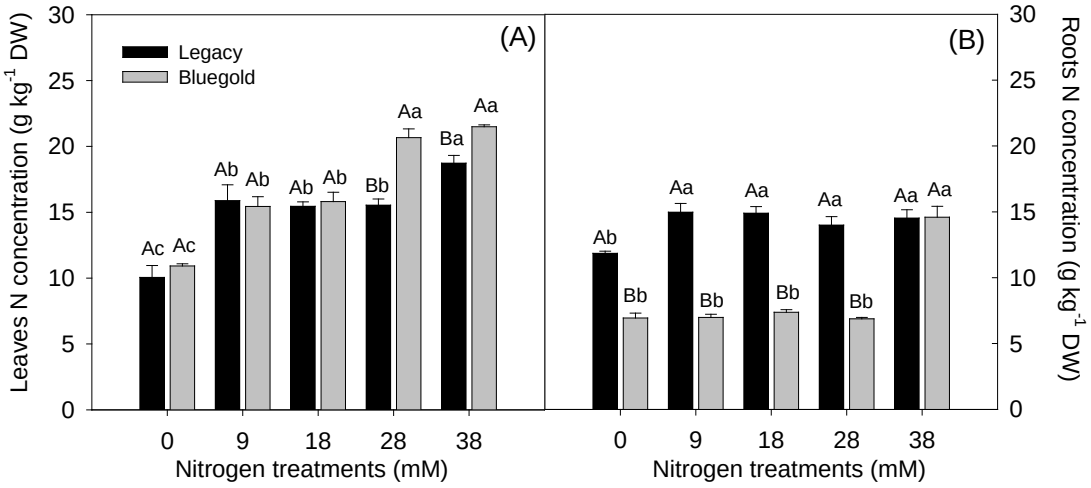


**Figure 4.1.** Nitrogen concentration in leaves (A) and roots (B) of two blueberry cultivars under different N treatments for 4 weeks. The values represent the average of three replicates + SE. Different lowercase letters indicate statistically significant differences among N treatments for the same cultivar. Different uppercase letters indicate differences between cultivars for the same N treatment.

### *4.3.2 Carbon dioxide assimilation*

No differences in $CO_2$ assimilation were found in Legacy leaves as a result of variable N supply for 4 weeks (Figure 4.2). Comparatively, when no N was applied, $CO_2$ assimilation in Bluegold was about 20% lower than that of N-treated plants ($P \leq 0.05$).

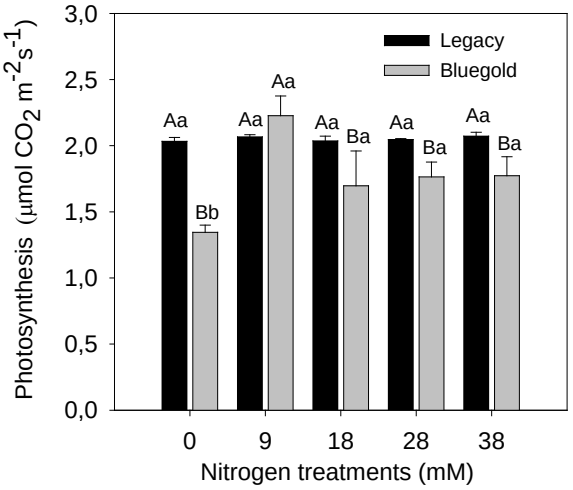


**Figure 4.2.** Nitrogen effects on $CO_2$ assimilation of two cultivars of highbush blueberries for 4 weeks. The values represent the average of three replicates + SE. Different lowercase letters indicate statistically significant differences among N treatments for the same cultivar. Different uppercase letters indicate differences between cultivars for the same N treatment.

### *4.3.3 Lipid peroxidation*

In the leaves of both cultivars, lipid peroxidation was approximately 50% lower ($P \leq 0.05$) in N-treated plants compared with those untreated (Figure 4.3A). Then, there was an inverse significant correlation between N concentration and lipid peroxidation for Legacy and Bluegold leaves ($r = -0.809$, $p \leq 0.05$; $r = -0.509$, $P \leq 0.05$, respectively). At all N treatments roots of both cultivars showed less lipid peroxidation compared to leaves (Figure 4.3B). Furthermore, 28 or 38 mM N in Legacy reduced lipid peroxidation by at least 50% in comparison with other N supplies.

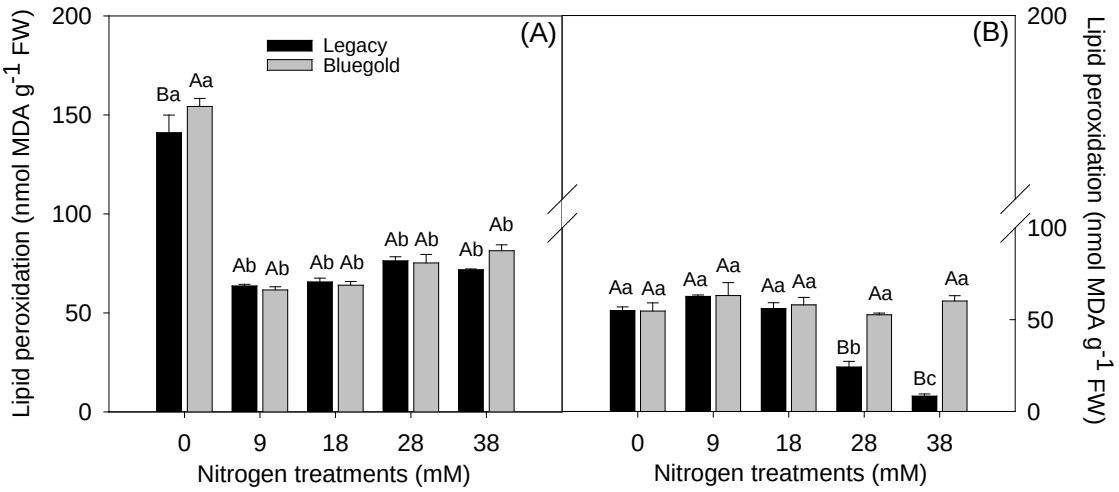


**Figure 4.3.** Lipid peroxidation in leaves (A) and roots (B) of two blueberry cultivars under different N treatments for 4 weeks. The values represent the average of three replicates + SE. Different lowercase letters indicate statistically significant differences among N treatments for the same cultivar. Different uppercase letters indicate differences between cultivars for the same N treatment.

### *4.3.4 Radical scavenging activity*

In both cultivars, no differences were found in the radical scavenging activity (RSA) of leaves at all N treatments (Figure 4.4A). However, at N supply above 18 mM, leaf RSA was higher in Bluegold than Legacy (Figure 4.4A). In roots of both cultivars, we observed a trend of decreasing RSA in treatments up to 18 mM N, followed by significantly increased RSA at 28 and 38 mM N (Figure 4.4B).

### *4.3.5 Total phenolics, flavonoids and anthocyanins concentrations*

Total phenolics concentration in Legacy leaves did not vary when plants were cultivated at N doses up to 18 mM. However, phenolics concentration decreased significantly ($P \leq 0.05$) at 28 and 38 mM N (Figure 4.5A). Thus, a 49% decrease in the concentration of total phenolics was found at 38 mM N compared to the levels found in 0 N treated plants. Likewise, a reduction of at least 25% in total phenolics concentration was observed in Bluegold leaves as a consequence of N supply above 18 mM. There was an inverse correlation between leaf concentration of N and phenolics in each cultivar (Legacy: r = -0.569, $P \leq 0.05$; Bluegold: r = -0.697, $P \leq 0.05$). In general, Legacy accumulated more phenols

than Bluegold in roots, and the lowest concentration was observed at 18 and 28 mM N in both cultivars (Figure 4.5B).

No significant effect of N on flavonoids was observed in Legacy (Figure 4.5C). In Bluegold a significant increase of these compounds was found due to N supply. In roots of both cultivars, flavonoid concentration nearly doubled at 9 mM N compared to N starved plants, staying relatively constant with a further increase in the N supply (Figure 4.5D). Total concentration of anthocyanin in leaves steadily decreased with increasing N doses in both cultivars (Figure 4.6). Therefore, an inverse correlation occurred between anthocyanin and N concentrations in leaves of Legacy and Bluegold (r =- 0.811, $P \leq 0.05$; r = -0.742, $P \leq 0.05$, respectively).

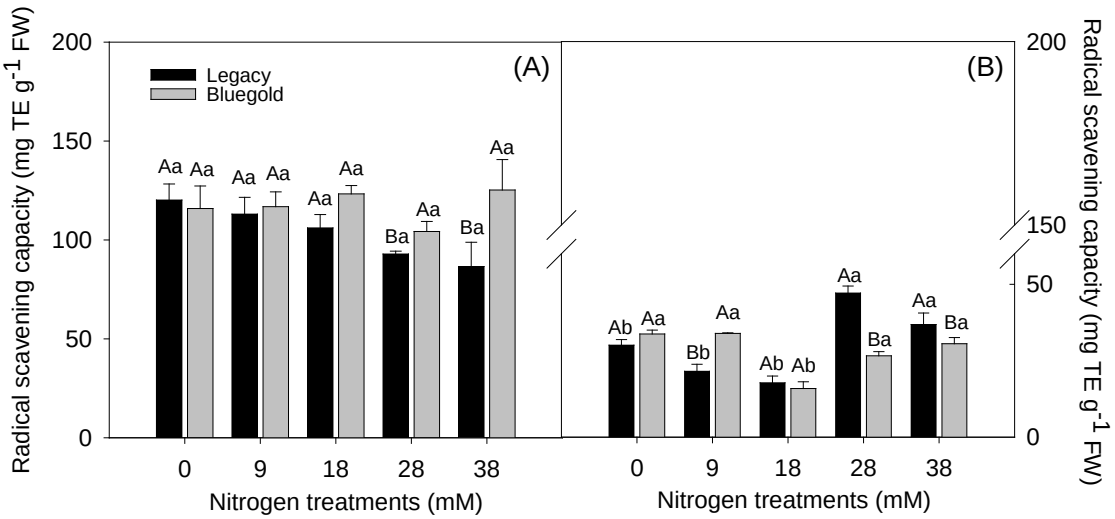


**Figure 4.4.** Antioxidant activity in leaves (A) and roots (B) of two blueberry cultivars under different N treatments for 4 weeks. The values represent the average of three replicates + SE. Different lowercase letters indicate statistically significant differences among N treatments for the same cultivar. Different uppercase letters indicate differences between cultivars for the same N treatment.

### *4.3.6 Superoxide dismutase activity*

In leaves and roots, similar trend in SOD activity was found on protein and fresh weight basis in both cultivars (Figure 4.7A-D). The highest specific SOD activity was observed at 0 and 38 mM N in leaves of Legacy and Bluegold, respectively (Figure 4.7A). The roots of both cultivars showed less SOD activity than leaves across N treatments (Figure 4.7B,D). In Bluegold roots, N application did not influence SOD activity, whereas in Legacy there was a strong decrease ($P \leq 0.05$) in the enzyme activity in treatments from 18 mM N (Figure 4.7B). In general, soluble protein concentration was not significantly altered by N treatments for both cultivars (Appendix I).

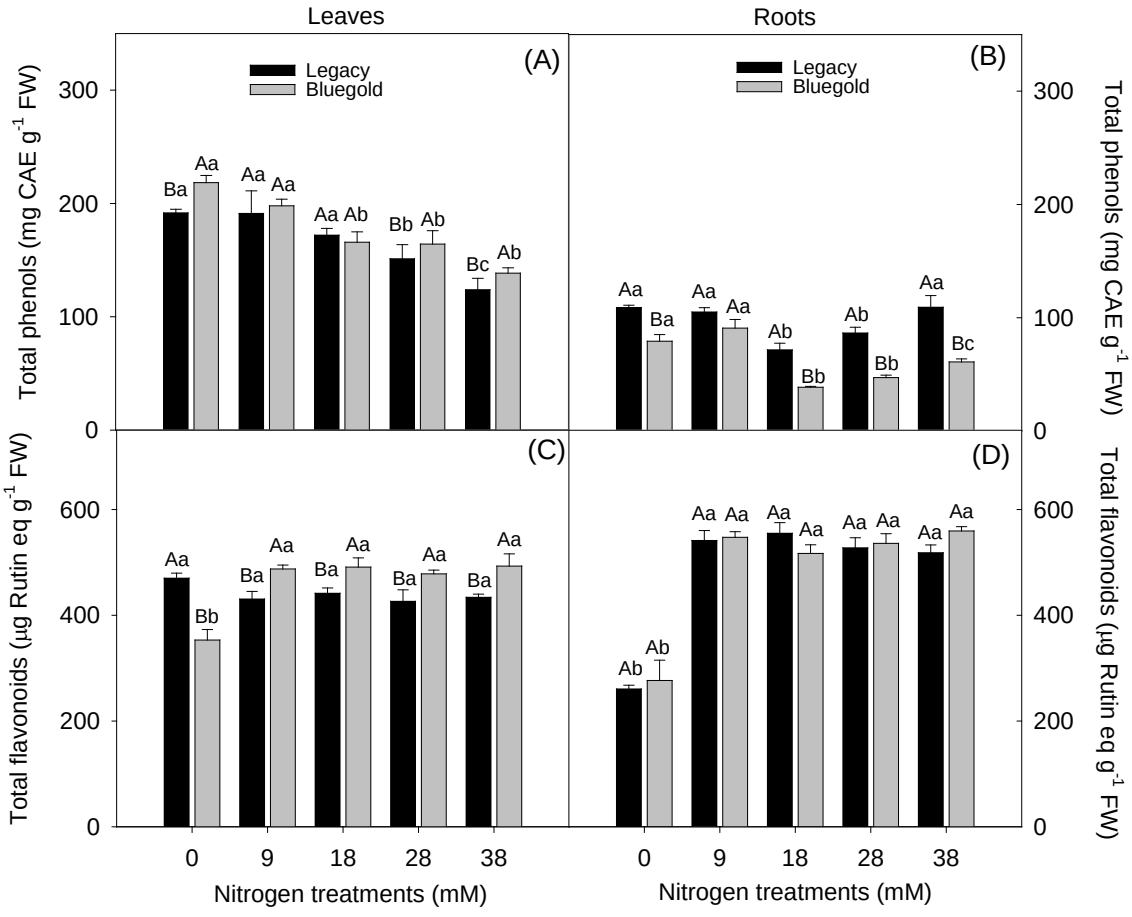


**Figure 4.5.** Total phenols (A,B) and flavonoids (C,D) of two blueberry cultivars under different N treatments for 4 weeks. The values represent the average of three replicates + SE. Different lowercase letters indicate statistically significant differences among N treatments for the same cultivar. Different uppercase letters indicate differences between cultivars for the same N treatment.

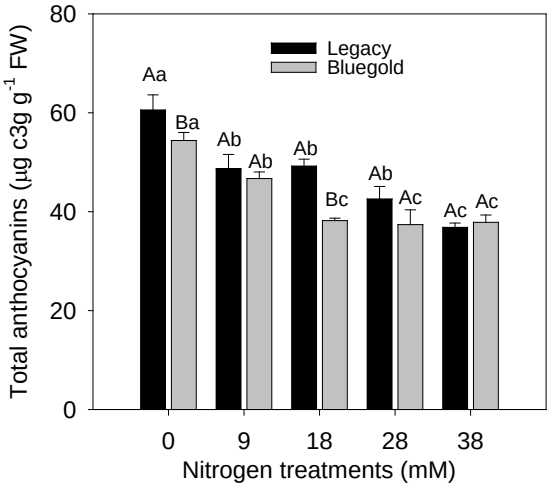


**Figure 4.6.** Total anthocyanins in leaves of two blueberry cultivars under different N treatments for 4 weeks. The values represent the average of three replicates + SE. Different lowercase letters indicate statistically significant differences among N treatments for the same cultivar. Different uppercase letters indicate differences between cultivars for the same N treatment.

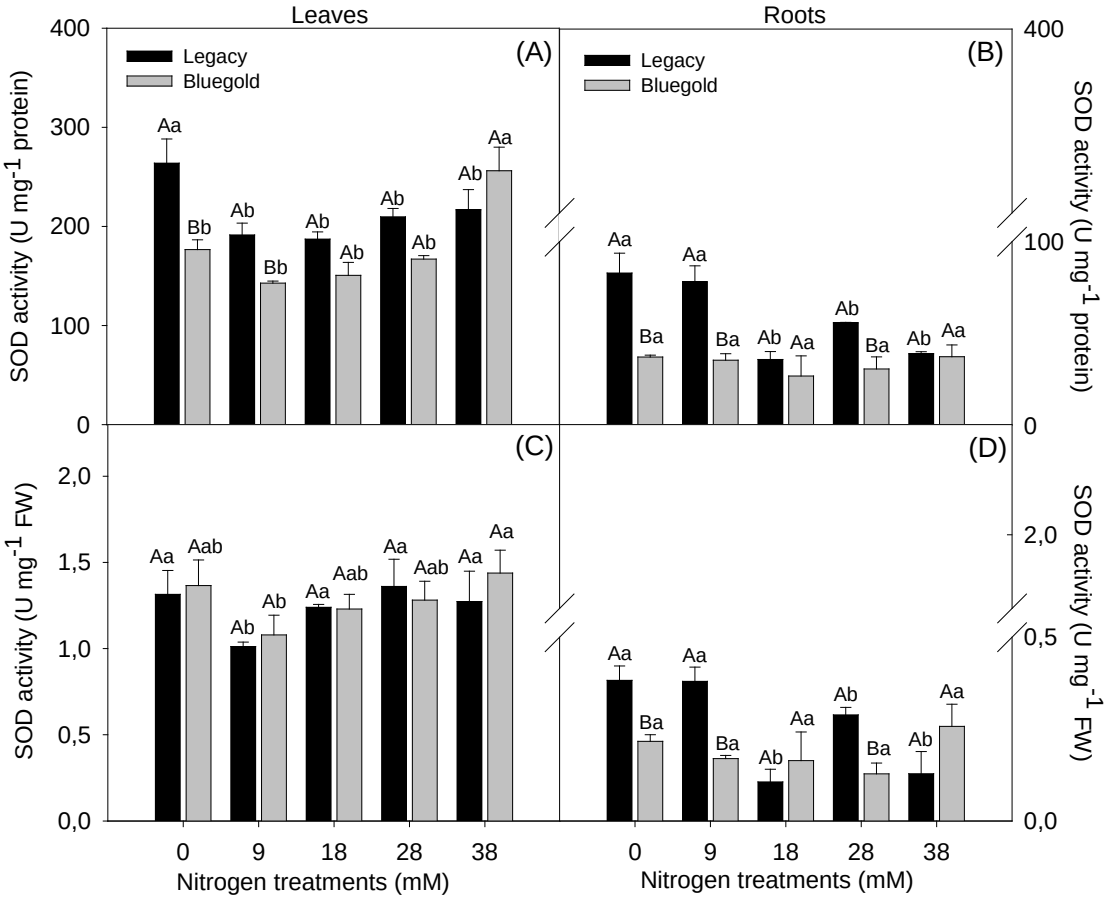


**Figure 4.7.** SOD specific activity (A,B) and fresh weight (C,D) of two blueberry cultivars under different N treatments for 4 weeks. The values represent the average of three replicates ± SE. Different lowercase letters indicate statistically significant differences among N treatments for the same cultivar. Different uppercase letters indicate differences between cultivars for the same N treatment.

### 4.4. Discussion

Blueberry leaves has been well characterized for its high antioxidant value. However, to our knowledge, there are no reports about the optimal fertilization strategies that could maximize its antioxidant richness, especially in terms of N nutrition. Controversial results regarding N fertilization effects on phenolics accumulation and antioxidant capacity in plants have been previously reported (Okamoto *et al.*, 2003; Mogren *et al.*, 2006). Nevertheless, it might be expected that a range of N concentrations in blueberry plant tissues could induce phenylpropanoid pathway and thus enhance phenolics synthesis, without deleterious effects on plant production.

Our results showed differences in physiological and biochemical features between cultivars Bluegold and Legacy as a consequence of increasing N supply at 4 weeks. Consistent with earlier studies (Bañados *et al.*, 2012), we found that leaves N concentration steadily rose at increasing N supply in both cultivars (Figure 1). It has been reported that wheat cultivars could exhibit differential N uptake (Diekmann and Fischbeck, 2005). In this context, we observed that Legacy had nearly 2-fold higher N concentration than Bluegold under N treatments, but only in roots (Figure 1B), without important variation in leaf N between the cultivars up to 18 mM N. In general, the leaf N concentrations obtained in this study (from 10 to 21 g N $kg^{-1}$ DW) were within the range of those found by Bañados *et al.* (2012).

Even though it is widely recognized that N nutrition may positively influence the photosynthetic performance of plants (Agüera *et al.*, 2010), in our study we did not detect any difference in $CO_2$ assimilation regardless of the N supply in Legacy (Figure 2). In contrast, Bluegold showed a significant decline in photosynthesis, but only when plants grown under N starvation (Figure 2), indicating that this cultivar seems to be sensitive to low leaf N concentration (10 g $kg^{-1}$ DW). Nevertheless, our previous results showed a differential responses under N starvation or excess during 4 days exposure to differential N supply (Yañez-Mansilla *et al.*, 2014), suggesting that acclimation to differential N supply can occur over time.

Our earlier studies on aluminum (Al) toxicity in cultivars of highbush blueberry showed that Legacy and Bluegold were Al-resistant and Al-sensitive, respectively (Reyes-Díaz *et al.*, 2010). These differences were explained mainly by the differential antioxidant capacity, which was higher in Legacy than Bluegold. Thus, we expected that those cultivars might also differ in their ability to tolerate N deficiency or excess. Previous studies indicated that young blueberry plants are sensitive to N excess, causing decreased growth and leaf chlorosis (Bañados *et al.*, 2012). In contrast, rice plants were sensitive to N deprivation, exhibiting a significant reduction in net photosynthesis accompanied by an increase in ROS production and lipid peroxidation (Huang *et al.*, 2004). In our experiment, symptoms of N toxicity in leaves and roots were not observed. However, N starvation led to the lowest leaf N concentration (10 g $kg^{-1}$ DW, Figure 1A), which was associated with the highest lipid peroxidation in this tissue, without difference between the cultivars (Figure 3A). Indeed, N

addition significantly reduced lipid peroxidation in leaves of both cultivars, without difference among N supply doses. This result was also detected at root level, but only in Legacy.

A decline in concentration of some antioxidant compounds occurred in spinach plants cultivated under N starvation (Logan *et al.*, 1999). Despite these findings, in our study leaf radical scavenging activity (RSA) of both cultivars did not change with variable N supply (Figure 4A). Legacy roots showed increased antioxidant capacity at the higher N addition level (Figure 4B). It may be that N concentration in roots up to 14 g $kg^{-1}$ DW could increase accumulation of the antioxidant compounds in this cultivar. In fact, this response could be partially explained by enhanced total flavonoid concentrations (Figure 5D). This increase in antioxidant capacity and flavonoids in roots was associated with decreased lipid peroxidation at the higher N doses, but only in Legacy (Figure 3B).

The N source and the dose influence the synthesis of the primary and secondary metabolites in various plant species. However, the literature is scarce in relation to the range of N concentration required to enhance the accumulation of phenolics in blueberry. In the present work, the concentration of phenolics in leaves of Legacy and Bluegold was reduced with an increase in N supplies (Figure 5A, 6). It appears that leaf N concentration of 15 g N $kg^{-1}$ could represent a critical threshold of N above which the synthesis of phenolics (mainly anthocyanins) decrease in blueberry leaves. Probably, above this critical leaf N concentration, N might be mainly shunted into amino acid and protein synthesis, and the formation of secondary compounds is decreased because the absence of N stress as supported by the lowest lipid peroxidation here observed (Figure 3A). This fact also suggest that at a N concentration below 15 g N $kg^{-1}$, both cultivars allocated C resources to the formation of secondary compounds. These findings agree with the Bryant *et al*. (1983) hypothesis of carbon/nutrient balance (CNB), claiming that plants growing under N deficiency have more secondary metabolites than those in an N-optimal environment.

Regarding the antioxidant enzyme SOD, which is widely recognized as the first line of defense against ROS (Alscher *et al.*, 2002), it was activated significantly in Legacy at 0 mM N (Figure 7A). Accordingly, the lack of N in culture media triggered the enzyme activity to combat ROS production caused by N stress (Alscher *et al.*, 2002). This may be also related to the maintenance of $CO_2$ assimilation observed there (Figure 2), which is in line with previous

results for mulberry plants (Tewari *et al.*, 2007). This enzymatic antioxidant mechanism seems to be important in Legacy for counteract the damage by N deprivation. On the contrary, in the case of Bluegold leaves, SOD activity showed the same tendency to increase, but at the highest N level (Figure 7A,C), which was associated with a substantial reduction in phenol concentration (Figure 5A). These findings might be related to compensatory antioxidant mechanisms aimed to maintain a steady ROS balance, as demonstrated by the low oxidative damage of membranes at the highest N supply (Figure 3A). Since soluble protein concentration was not affected by N treatments (Appendix I), the increase of specific SOD activity in leaves could be attributed to an activation of the enzyme under N starvation and N excess for Legacy and Bluegold, respectively.

Our study revealed differential responses to N supply between blueberry cultivars, particularly under N starvation. Despite these differences, in order to preserve elevated antioxidant capacity in leaves, a threshold of about 15 g N $kg^{-1}$ DW for both cultivars can be recommended. Thus, it is important to highlight that a high antioxidant concentration in leaves may be deliver to fruits. Further studies are required to elucidate the mechanism explaining the relationship between tissue N concentration and the regulation of the plant antioxidant system at the molecular level in blueberry plants.

## 4.5 Acknowledgments

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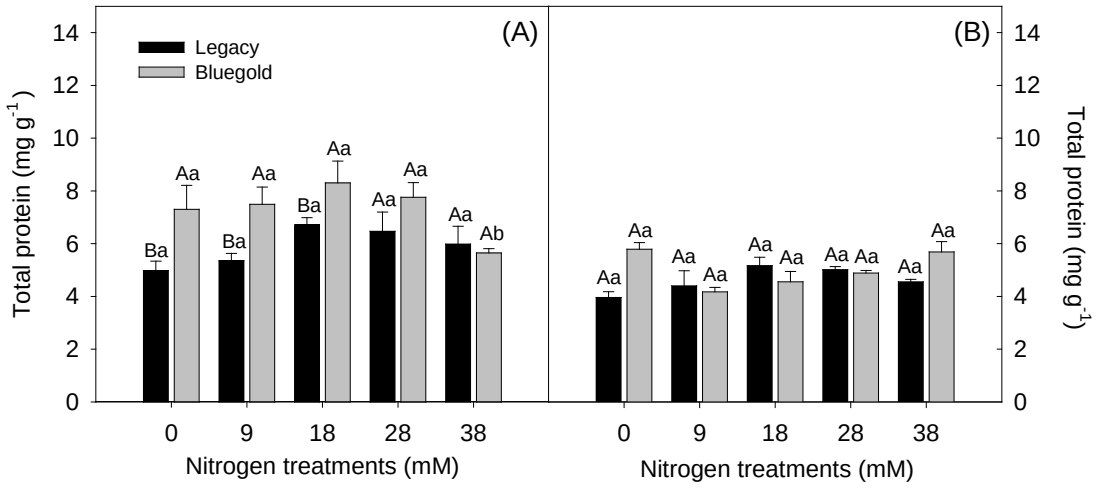


**Appendix I.** Soluble proteins in leaves (A) and roots (B) of Legacy and Bluegold cultivars under different N treatments for 4 weeks. Values represent the mean of three replicates ± SE (n=3). Different lowercase letters indicate statistically significant differences among N treatments for the same cultivar. Different uppercase letters indicate differences between cultivars for the same N treatment.

# Chapter 5

## Nitrogen fertilization effect on antioxidant capacity, phenolic composition and PAL activity of highbush blueberry (*Vaccinium corymbosum* L.)

In preparation

# Nitrogen fertilization effect on antioxidant capacity, phenolic composition and PAL activity of highbush blueberry (*Vaccinium corymbosum* L.)

## Abstract

In a soil experiment we tested N uptake and its effect on phenolic composition in blueberry leaves at increasing N treatments (0 to 80 kg N $ha^{-1}$) at long-term under controlled conditions. Later on, based on long-term results; antioxidant capacity, anthocyanidins profile and phenylalanine ammonia-lyase (PAL) activity were evaluated in terms of N concentration in a kinetic assay. In the long-term assay, the N addition increased N concentration in Legacy and Bluegold leaves. The N uptake in Legacy was higher than Bluegold under N deprived conditions. For both cultivars, lipid peroxidation was induced significantly at 40 and 80 kg N $ha^{-1}$ and this behavior was accompanied by lower photosynthetic rate at the highest N dose. Both cultivars diminished total phenols concentration when increasing N doses. Between cultivars, phenolic acids and flavonols showed opposite behavior to increasing N addition.

In the kinetic assay, without N addition we observed that Legacy accumulated more N than Bluegold across harvest days. In addition, even though $CO_2$ assimilation was not significantly altered in Legacy, Bluegold cultivated at 80 kg N $ha^{-1}$ showed a reduction in this parameter on day 28 associated with high lipid peroxidation. Legacy plants that accumulated up to 15 g N $kg^{-1}$ increased PAL activity, and this response was supported by an increase in ORAC, total phenols and anthocyanins at 28 days. Conversely, antioxidant capacity and anthocyanins steadily decreased in Bluegold that accumulated about 20 g N $kg^{-1}$ which corresponded to 80 kg N $ha^{-1}$ applied. Anthocyanidins compounds showed a differential behavior between cultivars, being higher in Legacy grown at 20 kg N $ha^{-1}$. Petunidin and malvidin were diminished in N-deprived plants of Bluegold after 7 days. Based on both experiments, the high N dose in both cultivars declined antioxidant parameters at the long-term and this behavior was supported by a decrease PAL activity in the kinetic assay. Finally, a threshold of 15 g N $kg^{-1}$ in blueberry leaves promoted antioxidant features without negative effects on physiological parameters.

**Keywords:** *Vaccinium corymbosum*, antioxidant capacity, nitrogen, PAL activity

## 5.1 Introduction

Plant nutrition is a fundamental factor that affects crop yield and quality, in particular the content and profile of compounds with antioxidant properties. Phenolic compounds are secondary metabolites which are found in leaves, flowers and fruits, which are induced and accumulated under different environmental conditions (Harbone, 1976).

Excess or deprivation of macronutrients as nitrogen (N) influences the concentration of carbon (C)-based compounds, mainly phenolics. The antioxidant activity of phenolic compounds is associated with defense and might counteract environmental stresses in higher plants (Dixon and Paiva, 1995). There are variable responses about N nutrition and its effect on phenolic compounds synthesis (Mogren *et al,* 2006; Bongue-Bartelsman and Phillips, 1995). Although it is known that high N doses decline phenolic compounds levels and N deficiency leads to high antioxidant capacity, there are few studies that indicate the N concentration that ensure a high phenolic compounds concentration (Yañez-Mansilla *et al.,* 2015) and the related enzymes of phenylpropanoid pathtway in plant tissues. Indeed, phenylalanine ammonia-lyase (PAL) is a key enzyme of phenylpropanoid pathway and it is influenced by N fertilization (Kováčik *et al.,* 2007). An increase of PAL activity has been reported in N deprived plants accompanied by high phenolics levels. Thereby, L-phenylalanine amino acid is deaminated by PAL to give cinnamic acid and ammonia (Razal *et al.,* 1996; Jones and Hartley, 1999). As consequence, cinnamic acid skeleton acts as precursor of the phenolic compound families (Singh *et al.,* 1997).

Highbush blueberry (*Vaccinium corymbosum* L.) is an ericaceous plant and has been turned into an important fruit crop activity in Chile (Espinoza *et al.*, 2009; ODEPA, 2011). In terms of N fertilization, it has been widely reported that high N application doses reduces blueberry growth and yield (Bañados *et al.,* 2012; Strik *et al.,* 2014) and low N application increase fruit production (Vargas and Bryla, 2015). Phenolic acids, flavonols and anthocyanins are the main source of antioxidants in fruits and leaves of blueberry (Prior *et al.,* 1998). Indeed, the phenolics profile in blueberry tissues are mainly by chlorogenic acid, rutin, quercetin, delphinidin and cyanidin which have a high oxygen radical antioxidant capacity (ORAC) value (Ehlenfeldt and Prior, 2001; Ribera *et al.,* 2010; Zheng and Wang, 2003). Some phenolics have been evaluated in *Vaccinium* plants subjected to N additions. Witzell and Shevtsova (2004) found that N application declined the concentrations of

chlorogenic and *p*-coumaric acid as well as arbutin. In addition, quercetin-3-glucoside and catechin were not significantly affected by N in leaves. On the other hand, anthocyanidins profile was not altered by N additions in fruits of *Vaccinium myrtillus* (Åkerström *et al.*, 2009). However, in previous results, we reported that N excess can trigger a significant decrease of leaf phenolic compounds at hydroponic conditions. Besides, we observed that 15 g N $kg^{-1}$ in blueberry leaves maintain a great antioxidant capacity without negative effects on physiological parameters (Yañez-Mansilla *et al.*, 2015).

Although N fertilization could influence the synthesis and accumulation of phenolic compounds as well as antioxidant features in blueberry plants (Yañez-Mansilla *et al.*, 2014), it is necessary to evaluate the effect of N concentration in leaves on phenolics profile and its relationship with PAL enzyme activity. The aim of this study was to investigate the effect of increasing N doses on N uptake and phenolics profile in blueberry cultivars grown on an Andisol under greenhouse conditions at the long-term. In addition, according to the long-term results we selected N doses for assessing the anthocyanins profile, PAL activity and antioxidant features in leaves of blueberry cultivars in a soil kinetic assay.

## 5.2. Materials and Methods

### *5.2.1 Long-term assay: N effect on the concentration and profile of phenolic compounds*

In a soil pot experiment two years-old blueberry plants (cv. Legacy and Bluegold) were used for evaluating N uptake and its effect on phenolic compounds concentration and composition during five weeks. Plants were cultivated on an acid Andisol, which was characterized by the methodology described by Sadzawka *et al.* (2004) (Appendix II). Before starting the assay, plants were conditioned in soil without fertilizer addition for two weeks. Greenhouse environmental conditions were: temperature 25/20°C (day/night), photoperiod of 16/8 h (light/dark) and 70% relative humidity. Nitrogen fertilization doses (as urea) were 0, 20, 40 or 80 kg N $ha^{1}$. Prior to harvest, *in vivo* $CO_2$ assimilation measurements were recorded and relative plant growth was determined. Fresh samples corresponding to completely expanded leaves were stored at -20 or -80ºC for biochemical (lipid peroxidation and total phenols) and HPLC (phenolic acids and flavonoids) analyses; subsamples were dried for N concentration determination in leaves.

### *5.2.2 Kinetic study: N concentration in leaves and its influence on antioxidant capacity, phenolic compounds and PAL activity*

According to long-term results, three N doses were selected to conduct a kinetic soil pot experiment. During growth period, environmental conditions, plant conditioning and N source were similar to those described for the long-tem assay (see above). For each cultivar (Legacy and Bluegold), 27 plants were divided into 3 groups, each of them corresponding to 0, 20 and 80 kg N $ha^{-1}$ treatments. Samples of the second youngest completely expanded leaves were harvested on 7, 14 and 28 days for analyses. For each time of harvest, *in vivo* $CO_2$ assimilation measurements were recorded and relative plant growth was determined. Besides, N concentration, lipid peroxidation, ORAC, total phenols and anthocyanins as well as anthocyanidins profile by HPLC and PAL activity were analyzed.

### *5.2.3 Nitrogen concentration in leaves*

Nitrogen concentration was determined by the Kjeldahl method (Sadzawka *et al.*, 2004).

### *5.2.4 Measurements of $CO_2$ assimilation*

Assimilation of $CO_2$ was measured using a portable photosynthesis system (LI-6400, LI-COR Bioscience, Inc., Lincoln, Nebraska, US & Canada) as described by Reyes-Díaz *et al* (2011).

### *5.2.5 Grown parameters*

Growth was analyzed by determining the change in fresh weight of 3 plants from the beginning (W1) to the end of the treatment (W2) for each harvest time. Growth was expressed as the mean relative growth rate (MRGR) from the mean natural logarithm-transformed weight plants: MRGR= (lnW2)–(lnW1)/(t2– t1) (Hoffmann and Poorter, 2002).

### *5.2.6 Oxidative damage*

To determine the influence of different N doses on the lipid peroxidation, the thiobarbituric acid reacting substances (TBARS) assay was performed according to Du and Bramlage (1992). Thus, the absorbance was spectrophotometrically measured at 532, 600 and 440 nm in order to correct the interference caused by TBARS–sugar complexes.

### *5.2.7 Oxigen radical antioxidant capacity (ORAC)*

The ORAC assay was carried out in leaves extracts previously described by Cao *et al* (1997) and values were expressed as micromol trolox equivalents (TE) per g FW.

### *5.2.8 Quantification of phenolic compounds*

Total phenols were determined at 765 nm by the Folin-Ciocalteu method (Slinkard and Singleton, 1977). The results were expressed as milligrams of chlorogenic acid (CAE) equivalent per g FW. Total anthocyanins were analyzed by the method described by Chang *et al* (2002). The absorbance of anthocyanin extracts was measured in a spectrophotometer at 530 and 657 nm. Total anthocyanin content was expressed as milligrams of cyanidin-3-glucoside (c3g) equivalent per g FW.

### *5.2.9 Phenolic acids, flavonols and anthocyanidins concentration by HPLC-DAD*

Quantitative analyses of blueberry phenolic compounds in leaves were conducted in a high performance liquid chromatography (HPLC) system Jasco (LC-Net II/ADC) using a Kromasil reversed-phase (RP)-18 column (250 x 4.6 mm i.d.) equipped with a photodiode array detector (DAD) (Jasco MD 2015 Plus). Long-term HPLC-DAD analyses of phenolic acids and flavonols were performed as described previously by Ruhland and Day (2000) with minor modifications. Phenolic acids such as chlorogenic, caffeic, ferulic and *p*-coumaric and the flavonols quercetin, myricetin, and rutin were used as standards. In the kinetic assay, anthocyanidins profile was performed as described previously by Nyman and Kumpulainen (2001). Delphinidin, cyanidin, peonidin, petunidin, y malvidin were used as standards.

### *5.2.10 PAL activity*

Phenylalanine ammonia-lyase (PAL) activity was measured spectrometrically at 290 nm by the production of cinnamic acid (CA) for 120 min at 38 ºC. The specific enzyme activity was expressed as nmol of cinnamic acid produced per hour and per milligram of protein (Zucker, 1965). In addition, soluble protein content in the enzyme extract was determined at 595 nm by Bradford (1976) method.

### *5.2.11 Experimental design and statistical analysis*

Both experiments were arranged as a completely randomized design and all determinations were based on three replicates each. Pearson correlation was used to assess the relationships between two response variables. Analyses were performed using Sigma Stat software v. 2.0 (SPSS, Chicago, IL, USA). Long-term assay was designed with two cultivars and four N treatments. Chemical and biochemical data were analyzed by two-way ANOVA after the normality and homoscedasticity tests. Tukey's test was used to evaluate differences between means ($P \leq 0.05$). Two cultivars, three N treatments and three harvest time were used for the kinetic assay. Data were subjected to a three-way analysis of variance ANOVA. Tukey's test was used to evaluate differences between means ($P \leq 0.05$).

## 5.3 Results

### *5.3.1 Long term-assay: N effects on chemical, physiological and antioxidant features of blueberry cultivars*

Nitrogen concentration was raised between 20 and 30% in leaves at the highest N treatment for both Legacy and Bluegold respectively, compared with 0 kg N $ha^{-1}$ (Table 5.1; $P \leq 0.05$). Legacy showed significantly higher N concentration than Bluegold at 0 N treatment. Otherwise, no differences were observed for Legacy in $CO_2$ assimilation rate due to N supply up to 40 kg $ha^{-1}$. However, a significant decline was found in this parameter in plants that accumulated 20 g N $ha^{-1}$ DW ($P \leq 0.05$), that corresponded to the highest N treatment. Besides, Bluegold showed a significant decay in its $CO_2$ assimilation in non-fertilized plants and in those at high N addition level. Not significant differences were obtained for relative growth in both cultivars under N supply levels tested in this assay ($P > 0.05$; Appendix III). Lipid peroxidation was elevated by at least 2-fold at leaves N concentration above 18 g $kg^{-1}$, that corresponded to 40 and 80 kg $ha^{-1}$ N applied to both cultivars. Under N deprived conditions Bluegold exhibited approximately 2-fold MDA levels higher than Legacy.

Total phenols in Bluegold diminished at all N doses, resulting in 2.4-fold difference between 0 and 80 kg N $ha^{-1}$ treatments. In addition, phenols levels were more affected in

Legacy by N treatments being 4.2-fold lower when compared 0 and 80 kg N $ha^{-1}$ (Table 5.1).

**Table 5.1.** Chemical and biochemical features in Legacy and Bluegold leaves at increasing N doses. Values represent the mean of three replicates ± SE (n=3). Different upper case letters indicate statistically significant differences between cultivars for the same N treatment ($P \leq 0.05$). Different lower case letters indicate statistically significant differences between N treatments for the same cultivar ($P \leq 0.05$).

| **N treatments (kg $ha^{-1}$)** | **N concentration (g $kg^{-1}$ DW)** | | **$CO_2$ assimilation (µmol $CO_2$ $m^{-2}$ $s^{-1}$)** | | **Lipid peroxidation (nmol MDA $g^{-1}$ FW)** | | **Total Phenols (mg CAE $g^{-1}$ FW)** | |
|---|---|---|---|---|---|---|---|---|
| | *Legacy* | *Bluegold* | *Legacy* | *Bluegold* | *Legacy* | *Bluegold* | *Legacy* | *Bluegold* |
| 0 | 16.2±0.9 **Ab** | 13.8±0.8 **Bc** | 1.81±0.1 **Ba** | 2.45±0.2 **Ab** | 45±1 **Bb** | 86.9±5 **Ab** | 308±18 **Ba** | 233±25 **Aa** |
| 20 | 17.7±0.1 **Bb** | 15.1±0.5 **Bb** | 1.86±0.1 **Ba** | 2.83±0.1 **Aa** | 29±6 **Bc** | 52.5±4 **Ac** | 200±21 **Ab** | 180±5 **Ab** |
| 40 | 18.5±0.5 **Aa** | 18±1.7 **Aa** | 1.76±0.1 **Ba** | 2.92±0.1 **Aa** | 124±11 **Aa** | 100±7 **Ba** | 169±22 **Bb** | 106±12 **Ac** |
| 80 | 20.2±0.7 **Aa** | 20.2±0.3 **Aa** | 1.31±0.1 **Bb** | 2.13±0.1 **Ac** | 140±13 **Aa** | 106±7 **Ba** | 73±10 **Ac** | 96±14 **Ac** |

### *5.3.2 Profile of phenolic compounds in leaves of blueberry cultivars subjected to N fertilization at the long-term*

In terms of phenolic acids and flavonols evaluated by HPLC-DAD a varied response due to N treatments in leaves of both cultivars was observed (Table 5.2). Despite that feluric acid was not affected at all tested N doses, chlorogenic, caffeic and cumaric acids decreased their concentration by increasing N treatments in Legacy. In addition, quercetin concentration was not altered by N treatments, but rutin and mirecetin showed approximately 50 and 40% higher levels in plants without N fertilization than in those fertilized.

On the other hand, chlorogenic acid, cumaric acid and rutin improved their concentration when N was applied in Bluegold plants. The concentration of these compounds was significantly greater in Bluegold compared with Legacy.

**Table 5.2.** Phenolic compounds profile (µg $g^{-1}$ FW) by HPLC analyses in Legacy and Bluegold leaves at different N treatments (kg $ha^{-1}$). Values represent the mean of three replicates ± SE (n=3). Different upper case letters indicate statistically significant differences between cultivars for the same N treatment ($P \leq 0.05$). Different lower case letters indicate statistically significant differences between N treatments for the same cultivar ($P \leq 0.05$).

| N treatments | | Phenolic acids (µg $g^{-1}$ FW) | | | | Flavonols (µg $g^{-1}$ FW) | | |
|---|---|---|---|---|---|---|---|---|
| (kg $ha^{-1}$) | Cultivars | Chlorogenic | Caffeic | Cumaric | Feluric | Rutin | Mirecetin | Quercetin |
| 0 | *Legacy* | 9041±569 **Aa** | 1364±201 **a** | 230±19 **Aa** | 129±22 **Aa** | 2848±214 **Ba** | 297±23 **Aa** | 34,5±3 **a** |
| 20 | | 3574±207 **Bb** | 399±19 **b** | 143±27 **Bb** | 122±11 **Aa** | 1492±69 **Bb** | 168±12 **Ab** | 37±4 **a** |
| 40 | | 6942±522 **Bb** | 736±57 **b** | 142±22 **Bb** | 128±6 **a** | 1439±95 **Bb** | 177±4 **Ab** | 37±5 **a** |
| 80 | | 7352±792 **Bb** | 605±30 **b** | 141±22 **Bb** | 130±6 **a** | 1446±83 **Bb** | 176±50 **Ab** | 41±4 **a** |
| 0 | *Bluegold* | 8576±226 **Ab** | n.d | 264±29 **Ac** | 114±7 **Aa** | 9177±638 **Ab** | 98±7 **Bb** | n.d |
| 20 | | 10264±711 **Aa** | n.d | 365±49 **Ab** | 130±18 **Aa** | 8259±760 **Ab** | 129±6 **Ba** | n.d |
| 40 | | 11641±1200 **Aa** | n.d | 321±45 **Ab** | n.d | 7640±797 **Ab** | 100±2 **Bb** | n.d |
| 80 | | 9870±863 **Aa** | n.d | 420±17 **Aa** | n.d | 12215±476 **Aa** | 105±**Bb** | n.d |

n.d: not detected

### *5.3.3 Kinetic assay:* Chemical and physiological parameters under N treatments

In Legacy and Bluegold cultivars, three N treatments (0, 20, 80 kg N $ha^{-1}$) were tested during three times of N exposure. Nitrogen concentration in Legacy leaves increased up to 20 g $kg^{-1}$ DW in plants growing under 20 and 80 kg N $ha^{-1}$ on day 28 (Figure 5.1a). Conversely, Legacy N-starved plants accumulated in average 15 g N $kg^{-1}$ during the experiment. In Bluegold, 0 and 20 kg N $ha^{-1}$ did not raise leaves N concentration, except on day 14. Nevertheless, a significant increase of N concentration in leaves was found at each harvest time in plants supplied with 80 kg N $ha^{-1}$ (Figure 5.1b). In addition, a statistically significant difference in leaves N concentration was found between cultivars at 0 kg N $ha^{-1}$ during the time course of the assay ($P \leq 0.05$). Even though the $CO_2$ assimilation rate Legacy was not affected by N treatments, a reduction in this parameter was observed in Bluegold plants growing at 0 and 80 kg N $ha^{-1}$, but only on days 14 and 28 (Figure 5.2a,b).

Non significant differences were obtained for relative growth in both cultivars under the N supply levels tested in this assay ($P > 0.05$; Appendix III).

The addition of 80 kg N $ha^{-1}$ increased MDA levels in both cultivars compared with 0 and 20 kg N $ha^{-1}$. Comparatively, lipid peroxidation was 60% more pronounced in Bluegold than in Legacy plants supplied with 80 kg N $ha^{-1}$ on day 28 (Figure 5.2c,d; $P \leq 0.05$).

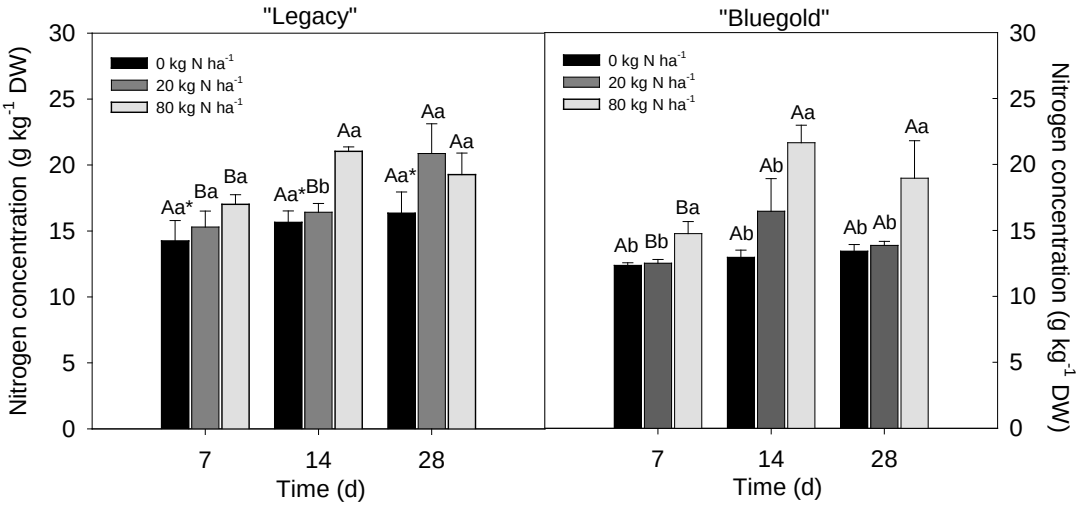


**Figure 5.1.** Nitrogen concentration in blueberry leaves at increasing N doses and different times (days). Values represent the mean of three replicates ± SE (n=3). Different upper case letters indicate statistically significant differences between exposure times for the same cultivar and same the N treatment ($P \leq 0.05$). Different lower case letters indicate statistically significant differences between N treatments for the same cultivar and exposure time ($P \leq 0.05$). Asterisks (*) showed differences among cultivars for the same N treatments and time.

### 5.3.3.1 The effect of N treatments on antioxidant features and PAL activity

Figures 5.3, 5.4 and 5.5 present the influence of N treatments on antioxidant performance, anthocyanidins profile and PAL activity in leaves of Legacy and Bluegold. An enhanced antioxidant capacity on day 7 resulted in non-fertilized plants of Legacy that accumulated up to 15 g $kg^{-1}$ on average compared with N-treated plants. Indeed, this effect

was 27% higher on day 28 (Figure 5.3a). For Bluegold, ORAC value was higher after day 7 in plants cultivated without N and 20 kg N $ha^{-1}$ compared with those plants grown at 80 kg N $ha^{-1}$ (Figure 5.3b).

A significant decline of total phenols was found on day 28 in Legacy plants growing at the highest N addition compared to those non N fertilized (Figure 5.3c; $P \leq 0.05$). An enhancement in phenols concentration was showed at the end of assay in Bluegold plants that accumulated about 15 g N $kg^{-1}$ (0 or 20 kg N $ha^{-1}$ added) compared with those that accumulate up to 20 g N $kg^{-1}$ (80 kg N $ha^{-1}$ added) (Figure 5.3d). In addition, at 0 kg N $ha^{-1}$, we found a significant difference in phenols concentration between cultivars and time ($P \leq 0.05$).

Although during the experiment total anthocyanins did not present variation under the different N treatments, an increase was found in Legacy on day 28 in N starved plants (Figure 5.3e; $P \leq 0.05$). On the other hand, a diminished anthocyanins concentration after 14 days with 80 kg N $ha^{-1}$ was found in Bluegold (Figure 5.3f). In general, we did not observe significant variation in the anthocyanins profile through time course for Legacy. Nevertheless, we found that delphinidin, cyanidin, peonidin and malvidin concentration were significantly higher (at least 35%) in plants supplied with 20 kg N $ha^{-1}$ than with those plants treated with 0 or 80 kg N $ha^{-1}$ for each time (Figure 5.4a,c,e,i).

On the other hand, the addition of the highest N treatment for Bluegold raised cyanidin and delphinidin concentrations, being respectively 20% and 30% greater than plants without N supply for each time (Figure 5.4b,d). In addition, after day 7 petunidin and malvidin declined their concentration in N deprived plants (Figure 5.4h,j).

Figure 5.5 shows phenylalanine ammonia (PAL) activity on protein and fresh weight basis for blueberry leaves at increasing N treatments and different harvest times. In general, a reduction in PAL activity based on protein was observed in Legacy across the assay at N addition compared with non-fertilized plants (Figure 5.5a).

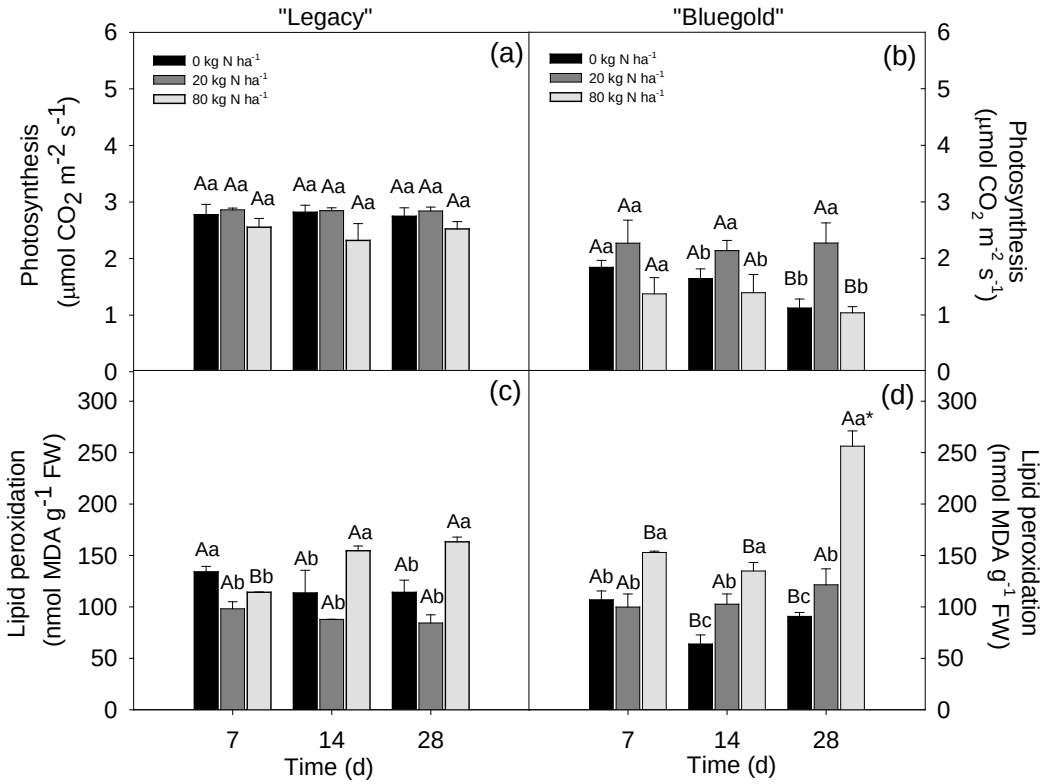


**Figure 5.2.** Nitrogen effects on $CO_2$ assimilation (a,b) and lipid peroxidation (c,d) of Legacy and Bluegold leaves at different times (days). Values represent the mean of three replicates ± SE (n=3). Different upper case letters indicate statistically significant differences between exposure times for the same cultivar and the same N treatment ($P \leq 0.05$). Different lower case letters indicate statistically significant differences between N treatments for the same cultivar and exposure time ($P \leq 0.05$). Asterisks (*) show differences among cultivars for the same N treatments and time.

Bluegold exhibited an enzyme activity about 35% higher on day 28 than on day 7 in N deprived conditions. Comparatively, at 20 kg N $ha^{-1}$, PAL activity tended to increase in Legacy across the time, but in Bluegold a reduction was found at the end of the experiment. PAL activity on basis of fresh weight was 40% higher in Legacy N starved plants on the day 28 compared with fertilized plants (Figure 5.5c). Meanwhile, we found a reduction of PAL in Bluegold subjected to 20 kg N $ha^{-1}$ during the assay (Figure 5.5d). Additionally, N fertilization (20 or 80 kg N $ha^{-1}$) for both Legacy and Bluegold leaves increased significantly soluble protein concentration during the experiment compared with N deprived plants (Figure 5.5e,f).

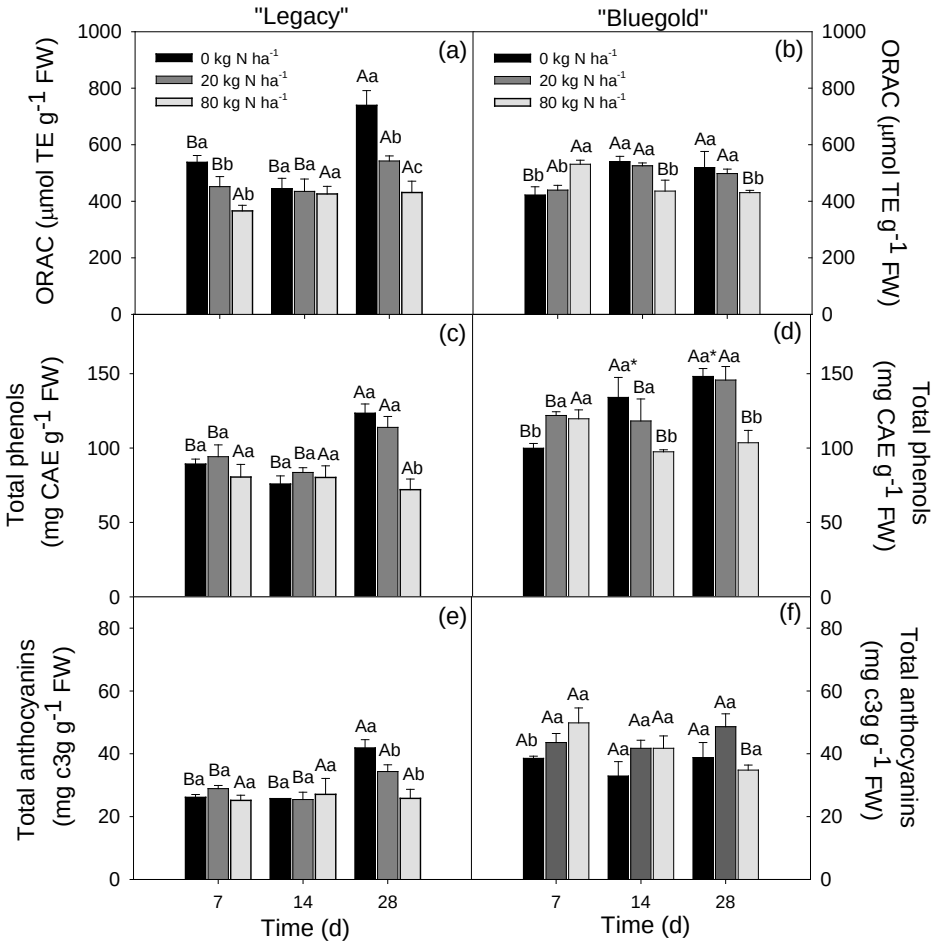


**Figure 5.3.** Antioxidant capacity (ORAC), total phenols and anthocyanins in Legacy and Bluegold leaves at increasing N treatments and different times. Values represent the mean of three replicates ± SE (n=3). Different upper case letters indicate statistically significant differences between exposure times for the same cultivar and the same N treatment ($P \leq 0.05$). Different lower case letters indicate statistically significant differences between N treatments for the same cultivar and exposure time ($P \leq 0.05$). Asterisks (*) show differences among cultivars for the same N treatments and time.

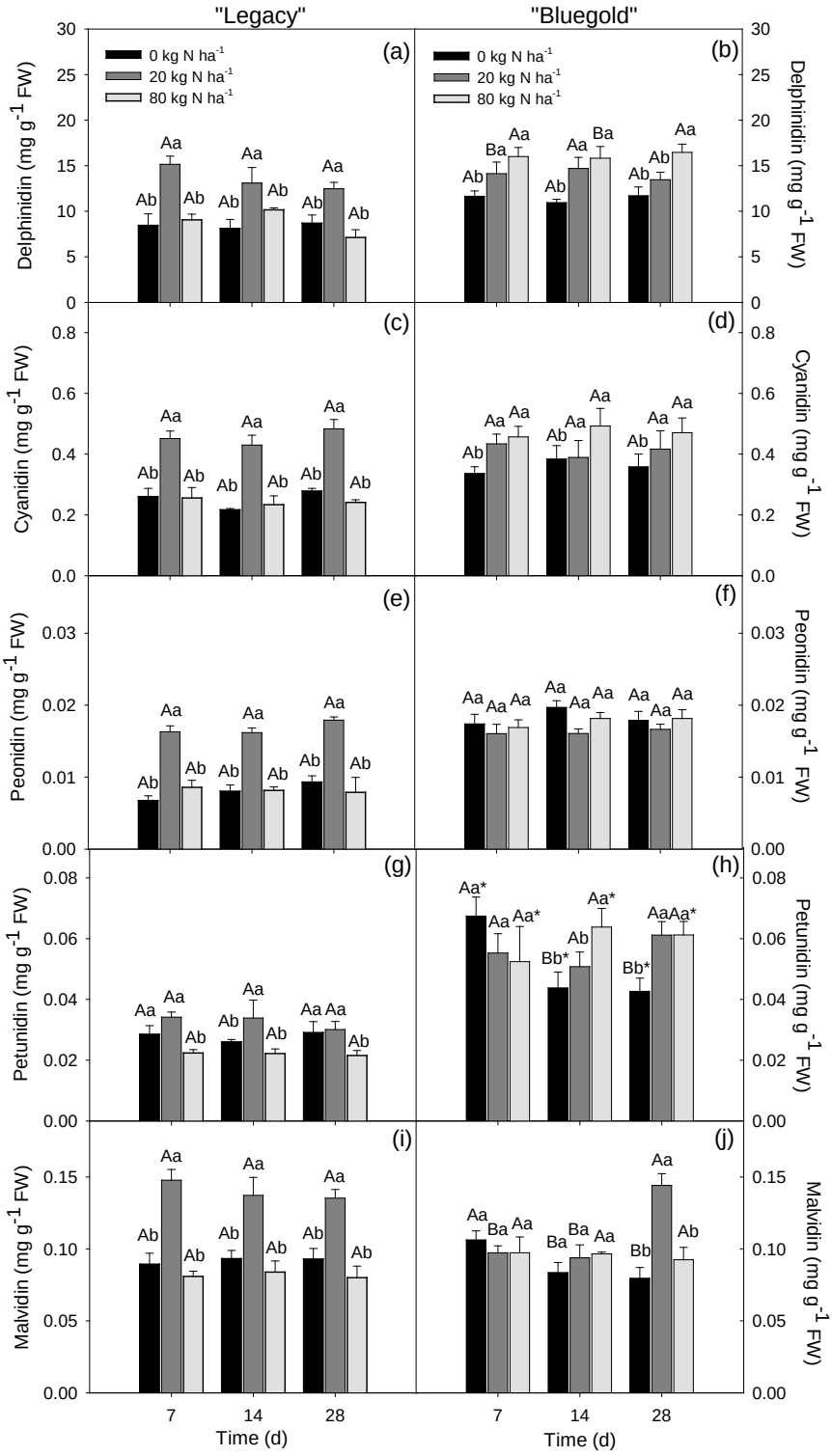


**Figure 5.4.** Anthocyanidins concentration by HPLC-DAD analyses in blueberry leaves at different N treatments and days harvest. Values represent the mean of three replicates ± SE (n=3). Different upper case letters indicate statistically significant

differences between exposure times for the same cultivar and the same N treatment ($P \leq 0.05$). Different lower case letters indicate statistically significant differences between N treatments for the same cultivar and exposure time ($P \leq 0.05$). Asterisks (*) show differences among cultivars for the same N treatments and time.

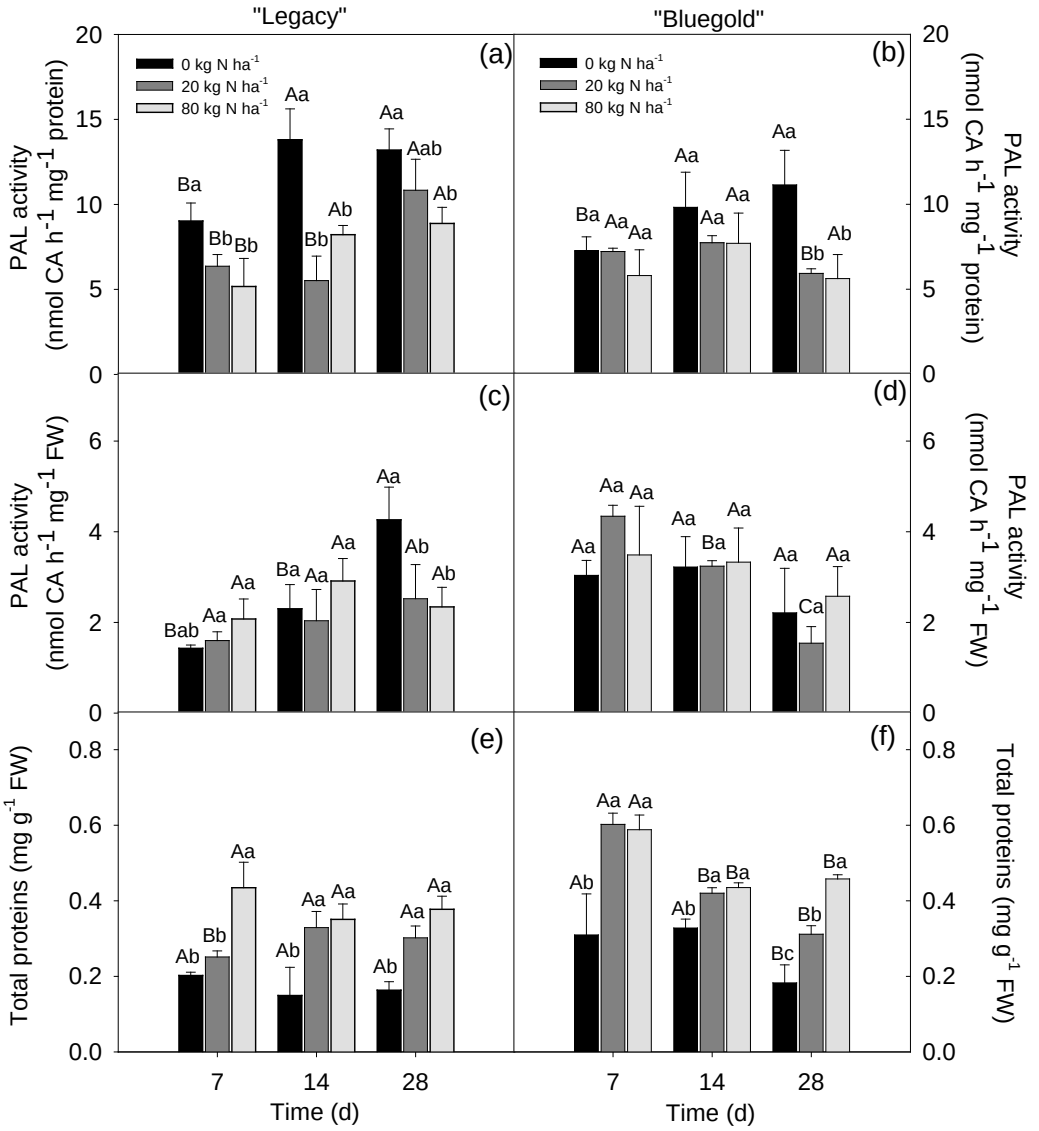


**Figure 5.5.** PAL activity based on protein (a,b), fresh weight (c,d) and soluble proteins (e,f) in Legacy and Bluegold cultivars at increasing N treatments and different times. Values represent the mean of three replicates ± SE (n=3). Different upper case letters indicate statistically significant differences between exposure times for the same cultivar and same N treatment ($P \leq 0.05$). Different lower case letters indicate statistically

significant differences between N treatments for the same cultivar and exposure time ($P \leq 0.05$).

## 5.4 Discussion

Blueberry leaves and fruits are a great source of phenolic compounds (Riihinen *et al.*, 2008). Nitrogen fertilization for blueberry has only been evaluated in relation to fruit production and yield (Bañados *et al.*, 2012. However, it has not been assessed in terms of phenolic compounds composition and related enzymes of phenylpropanoid pathway. In this study, we evaluated the N uptake effects on phenolics profile in two blueberry cultivars at long-term. Later, from the results of the long-term assay based on beneficial and harmful N effects, we studied anthocyanidins profile, PAL activity and antioxidant capacity in leaves of blueberry cultivars in a kinetic assay.

In the long-term study, N concentration about 20 g N $kg^{-1}$ in leaves of both cultivars showed negative impact on $CO_2$ assimilation which was followed by a significant increase in MDA levels indicating that N (80 kg N $ha^{-1}$) induced oxidative damage (Table 5.1). Differential accumulation of phenolics between Legacy and Bluegold cultivars in response to N addition at the long term was observed. In this sense, N addition diminished total phenols (Table 5.1) as well as phenolic acids and flavonols (Table 5.2) in Legacy. These observations agree with Witzell and Shevtsova (2004) study where some cinnamic acids (e.g. chlorogenic acid) diminished their concentration under N additions in *Vaccinium myrtillus* leaves. On the other hand, the high phenolics levels (mainly chlorogenic acid as well as rutin in our study) at long-term with 20 g N $kg^{-1}$ DW in Bluegold leaves was observed. According with Kováčik and Klejdus (2014), chlorogenic acid could be a stress protective compound in those N stress plants and the physiological damage is considerer like in our results (Table 5.1).

On the other hand, under N deprived conditions, Legacy leaves accumulated about 16 g N $kg^{-1}$ which triggered high total phenols (Table 5.1) and this response was according with elevated phenolic acids and flavonols levels (Table 5.2). The great phenolics

concentration observed at this N concentration might be reducing oxidative damage and thus maintaining $CO_2$ assimilation in Legacy leaves.

In the kinetic assay, the N uptake was greater in Legacy than in Bluegold reaching 15 and 13 g N $kg^{-1}$ under N deprived conditions respectively, which is considered an N deficient condition according to Hart *et al* (2006) classification. Whereas N deficiency had no negative impact on blueberry yield (Strik *et al.,* 2014), in our kinetic assay we found that N concentration of about 15 g N $kg^{-1}$ in leaves does not reduce $CO_2$ assimilation and did not altered MDA levels. Otherwise, due to N excess, reduction of growth in blueberry plants at vegetative stage was observed by Bañados *et al* (2012). Even though we did not find negative effects on relative growth at high N addition for both cultivars (IV), our results show that Bluegold leaves exhibited a high lipid peroxidation level at elevated N supply, being significantly more pronounced than in Legacy on day 28. Furthermore, N stress (deficiency or toxicity) for Bluegold might be inducing the degradation of photosynthetic enzymes, thus reducing $CO_2$ assimilation (Huang *et al.,* 2004; DaMatta *et al.,* 2002). This response was previously observed under hydroponic conditions (Yañez-Mansilla *et al.,* 2014), and we confirm the sensitivity of Bluegold to N excess here.

Kinetic assay also showed that N concentration of 15 g N $kg^{-1}$ DW maintained high ORAC, phenols and anthocyanins levels, but these parameters decreased at higher N concentrations in leaves mainly at 28 days (Figure 5.3a,c,e). In addition, Legacy leaves showed a negative inverse correlation between N concentration and ORAC values were observed on day 28 ($r = -0.669$). Anthocyanidins have great antioxidant properties and are synthesized differentially in plants when they are subjected to abiotic stress (Ali *et al.,* 2012; Kovinich *et al.,* 2014). To date the role of N on anthocyanins biosynthesis and its accumulation in plant tissues remains unclear, and in this respect Åkerström *et al.* (2009) did not find effects of N fertilization over anthocyanins concentration in *Vaccinium* fruits. In the kinetic study, we observed differential responses in anthocyanidins profile between cultivars to the different N treatments (Figure 5.4). Thus, delphinidin, cyanidin, peonidin and malvidin concentrations were greater in Legacy leaves at 20 kg N $ha^{-1}$ compared with 0 or 80 kg N $ha^{-1}$. This result was in accordance to those reported by Okamoto *et al.* (2003) in

grape skins, where moderate N additions induced a high anthocyanin compounds concentration compared with either unfertilized or heavily fertilized plants. Furthermore, the concentration of delphinidin and cyanidin in Bluegold were higher at 80 kg N $ha^{-1}$, particularly at the end of the assay. Among the principal anthocyanins aglycones, delphinidin has a great ORAC activity followed by cyanidin (Wang *et al.,* 1997). In our study, these aglycones (delphinidin and cyanidin) could have increased their concentration as a response to N excess and also be playing a role in protecting against ROS overproduction. However, their contribution for decline lipid peroxidation is limited in leaves (Figure 5.2d).

An increase in phenols concentration has been associated with high PAL activity in N starved plants (Cheng and Breen, 1991; Chen *et al.,* 2006), but there are no reports indicating a relation between N concentration and PAL activity in blueberry leaves. On fresh weigh and protein basis, PAL activity was raised in Legacy plants that accumulated about 15 g N $kg^{-1}$ in their leaves on day 28 (Figure 5.5a). Despite that at this N concentration in leaves soluble proteins concentration remained at low levels in those plants without N fertilization (Figure 5.5e), the greatest cinnamic acid production were observed (Figure 5.5a,c). As consequence, higher levels of cinnamic acid would be derivated into accumulation of phenolic acids and flavonols at the long-term or total anthocyanins in kinetic assay (Table 5.2; Figure 5.3). Comparable responses have been observed in strawberry and vineyards, which showed an enhanced PAL activity supported by high levels of phenols compounds (Cheng and Breen, 1991; Hilbert *et al.,* 2003). On the other hand, PAL activity decreased in N fertilized plants of Legacy, and this behavior was supported by low total phenols and anthocyanins at the end of assay (Figure 5.3).

In Bluegold, PAL activity on protein basis showed lower levels at N additions on day 28 (Figure 5.5b). These results are consistent with those observed in tomato (Bongue-Bartelsman and Phillips, 1995) and grapevine (Soubeyrand *et al.,* 2014) where PAL enzyme subjected to N additions declined its activity and also phenolic compounds. Moreover, Bluegold leaves showed higher PAL activity on protein basis and similar PAL activity on fresh weight in N-fertilized plants at the end of assay (Figure 5.5d). Considering

the decline of soluble proteins level at N deprived conditions (Figure 5.5f), the high PAL activity observed at 28 days protein basis suggests that this enzyme was activated in N deprived conditions. This fact could explain an increase of total phenols concentration in the kinetic assay (Figure 5.3b) or great values of individual phenolic acids and flavonols at the long-term (Table 5.2).

Finally, high N fertilization induced detrimental effects on physiological parameters in blueberry cultivars, being Bluegold more sensitive than Legacy. Although high N fertilization is related to yield and production of crops, we found that leaves that accumulated 15 g N $kg^{-1}$ ensure a suitable antioxidant capacity, phenols concentration and PAL activity. High N application doses on crops with high antioxidant capacity in leaves and fruits like blueberry should be evaluated in futures studies and thereby maintains their nutraceutical properties.

## 5.5 Acknowledgments

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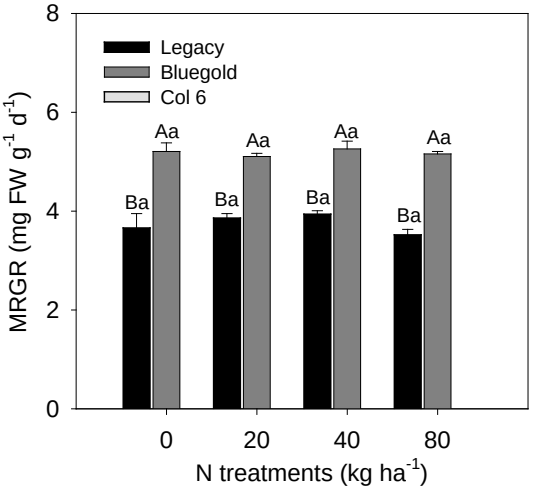
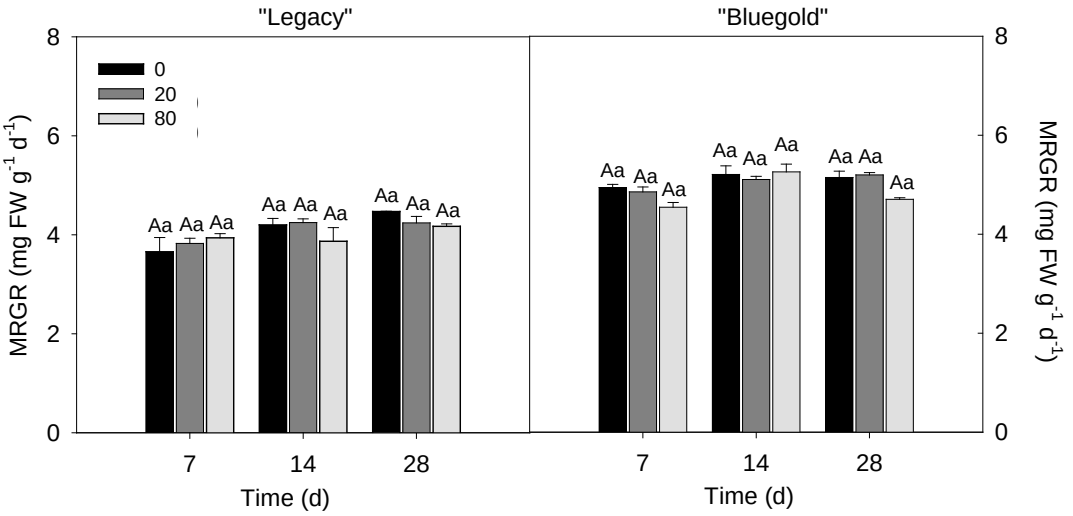
**Appendix II.** Soil chemical properties (Metrenco Series) used in both assays (long-term and kinetic) before N treatments applied.

| Soil analysis | |
|---|---|
| N (mg/kg) | |
| P (mg/kg) | 8 |
| K (mg/kg) | 207 |
| pH (water) | 5.41 |
| Organic matter (%) | 11 |
| K (cmol+/kg) | 0.53 |
| Na (cmol+/kg) | 0.04 |
| Ca (cmol+/kg) | 6.35 |
| Mg (cmol+/kg) | 0.68 |
| Al (cmol+/kg) | 0.03 |
| Saturación de Al (%) | 0.39 |
| CICE (cmol+/kg) | 7.63 |
| S. Bases (cmol+/kg) | 7.6 |




**Appendix III.** Mean relative growth rate (MRGR) of blueberry at increasing N treatments at the long-term. Values represent the mean of three replicates ± SE (n=3). Different upper case letters indicate statistically significant differences between cultivars for the same N treatment ($P \leq 0.05$). Different lower case letters indicate statistically significant differences between N treatments for the same cultivar ($P \leq 0.05$).




**Appendix IV.** Mean relative growth rate (MRGR) of blueberry at increasing N treatments and time (kinetic assay). Values represent the mean of three replicates ± SE (n=3). Different upper case letters indicate statistically significant differences between exposure times for the same cultivar and same N treatment ($P \leq 0.05$). Different lower case letters indicate statistically significant differences between N treatments for the same cultivar and exposure time ($P \leq 0.05$).

# Chapter 6

## General discussion, concluding remarks and future directions

## 6.1 General discussion

It is well known that the addition of mineral nutrients is fundamental to growth and ensure yield of plants. However, the large N application doses on crops specifically to those with high antioxidant capacity in leaves or fruits would affect negatively plant growth (Hanson and Retamales, 1992), secondary metabolites as well as quality (Ali *et al.,* 2012; Ehret *et al.,* 2014). Plant species of great agronomic interest as highbush blueberry have not been studied yet in relation to N fertilization and phenolic composition accumulation. In the last decades, highbush blueberry has become an economically important fruit crop in Southern Chile, because it is a rich source of antioxidant compounds such as phenols, flavonols and anthocyanins with antioxidant properties for human health. (Prior *et al.*, 1998; Ribera *et al.,* 2010).

In the present study we evaluated the effect of N concentration on the phenolic composition, antioxidant capacity and phenylalanine ammonia-lyase (PAL) activity of highbush blueberry (*Vaccinium corymbosum* L.). We divided the study on two main experiments (hydroponic and soil experiments, respectively). First, we studied the effect of increasing N supply on the photosynthetic and antioxidant performance of highbush blueberry cultivars grown at the short-term (Chapter 3). Then, on basis of the short-term results, we performed a second assay for assessing the impact of plant N concentration on the antioxidant responses in blueberry at the long-term (Chapter 4). Later on, using an Andisol we tested the N uptake and its effect on phenolic concentration in blueberries leaves at increasing N treatments during five weeks. Finally, antioxidant capacity, anthocyanidins profile and phenylalanine ammonia PAL activity were evaluated in terms of N concentration in leaves in a kinetic assay at selected N treatments (Chapter 5). Based on results of this thesis we established a N threshold concentration in leaves that ensure high nutritional quality of blueberry plants.

We evaluated antioxidant capacity and concentration of phenolic compounds of blueberry cultivars (Legacy and Bluegold) cultured hydroponically under different N doses. The results indicated that increasing N supply decline antioxidant capacity, phenolic compounds concentration as well as physiological traits for Legacy and Bluegold at short and the long-term. Overall, N concentration increased up to 20 g N $kg^{-1}$ DW in leaves at increasing N applications in both cultivars. In addition, we observed an inverse relation between non-enzymatic antioxidant defenses and N accumulation in the experiments. For instance, Legacy leaves declined their levels of total phenols, flavonoids and anthocyanins at the highest N treatment compared to N starved plants. Our findings agreed with previous works (Liu *et al.,* 2010; Royer *et al.,* 2013, among others) where high N levels in the growth media have been associated with a reduction of both the phenolic compounds content and the antioxidant capacity. In this sense, our results are in agreement with Carbon-Nutrient Balance (CNB) theory where under N excess condition C-based skeleton are mainly destined for amino acids or proteins production and in a lesser extent phenolic compounds (Bryant *et al.,* 1983).

On the other hand, we found in both cultivars an increase of lipid peroxidation probably due to ROS overproduction triggered by either N deficiency or excess at the short or long-term. Photosynthesis was reduced under N starvation or high N addition level (38 mM) for Legacy and Bluegold, respectively, and this behavior was accompanied by high lipid peroxidation at the short-term. Furthermore, Bluegold showed sensitivity to N starvation and recovery to N excess at the long-term assay. In this sense, SOD might be activated in response to N excess and then maintain oxidative damage at basal levels but its activation occurs at the long-term. Thus, SOD activation would be a compensatory antioxidant response (Ramalho *et al.,* 1998; Alscher., 2002; Cai *et al.,* 2008) in those plants where phenolic compounds are diminished to N excess.

Nitrogen concentration in the plant tissues is an indicator of N nutritional status for crops (Errecart *et al.,* 2012; Lemaire and Gastal, 2009). Taken together, our results show that 15 g N $kg^{-1}$ DW in both Legacy and Bluegold leaves maintain high antioxidantcapacity and phenolics levels. In addition, below this N concentration there is not deleterious effects on physiological and biochemical features for blueberry plants. However, above 15 g N $kg^{-}$

[1] DW, N might be mostly derived into amino acids, proteins and biomass production, thus reducing the production of phenolic compounds as described above. Additionally, over 20 g N $kg^{-1}$ DW in leaves, blueberry plants are susceptible to N toxicity, as demonstrated by the oxidative damage observed here.

Thus, hydroponic assays support the hypothesis presented in this Thesis where an N concentration of 15 g N $kg^{-1}$ DW promotes high antioxidant capacity and phenolics concentration in blueberry leaves. However, there are no studies in the soil-plant system intended to evaluate the impact of N uptake on antioxidant and photosynthetic attributes of blueberry plants. Based on hydroponic results, we expect confirm an N concentration threshold that mantain high phenolic concentration and PAL activity in leaves of blueberry plants cultivated on an Andisol.

Soil assays reaffirm the sensitivity of blueberry to N excess. In fact, MDA levels were significantly elevated in leaves that contained until 20 g N $kg^{-1}$ DW. This N concentration in leaves would be affecting negatively $CO_2$ assimilation observed here. These findings are according with Bañados *et al.* (2012) where high N fertilization doses (100 kg N $ha^{-1}$) applied to blueberry at vegetative stage generate chlorosis symptoms due to N stress. We emphasized the sensitivity of Bluegold plants to high N concentration at hydroponic or soil conditions, which was firstly evidenced by low $CO_2$ assimilation.

On the other hand, phenolics profiles responded differentially to N depending of the blueberry cultivars. Thus, Legacy plants that accumulated over 15 g N $kg^{-1}$ DW diminished their phenolic acids and flavonols concentrations as well as total phenols. Conversely, an increase of chlorogenic acid and rutin was presented for Bluegold; however, this improvement was not reflected in total phenols that were significantly lowered at high N concentration in plant tissues. Our long-term soil assay support that low N addition maintains high levels of phenolic concentration in blueberry leaves, but a decline in antioxidant capacity occurred in those plants cultivated at high N fertilization.

In a soil kinetic assay, we also found that N concentration of about 15 g N $kg^{-1}$ DW in Legacy leaves does not reduce $CO_2$ assimilation and did not alter MDA levels. Otherwise, Bluegold leaves exhibited a high MDA content at the highest N treatment, being significantly more pronounced than in Legacy. The great levels of MDA could be induced

by an overproduction of ROS due to N excess in the tissues. It has been also reported that N excess (mainly ammonium) in leaves diminish the photosynthetic rate in blueberry (Claussen and Lens, 1999). We hypothesized that an N concentration of about 20 g N $kg^{-1}$ DW in Bluegold leaves may cause uncoupling of the electron transport on the photosystems and, in turn, low NADPH and ATP production as well as high ROS accumulation. Thus, these facts would be inducing a failure on photosystems, which could trigger a reduced $CO_2$ assimilation in Bluegold. This response was observed at hydroponic and soil conditions (see above).

In this assay, we also confirmed the efficiency of Legacy which at 15 g N $kg^{-1}$ DW maintains high antioxidants features. Conversely, antioxidant parameters such as ORAC, total phenols and anthocyanins were negatively affected at higher N concentration in leaves. In fact, Legacy leaves showed a negative inverse correlation between N concentration and ORAC ($r = -0.669$).

In addition, anthocyanidins profile exhibited differential responses between cultivars to the different N treatments. Anthocyanidins are main phenolic compounds in blueberry leaves and fruits (Lätti *et al.,* 2008; Norberto *et al.,* 2013; Veberic *et al.,* 2015), and the role of anthocyanins against N stress in plants remains unclear. The main anthocyanidins (delphinidin, cyanidin, peonidin and malvidin) were greater in Legacy leaves that accumulated 15 g N $kg^{-1}$ DW. Furthermore, at 20 g N $kg^{-1}$ DW delphinidin and cyanidin concentrations increased in Bluegold, possibly for declining ROS due to N excess. However, their contribution for decline an elevated lipid peroxidation was limited in Bluegold leaves.

In general, enzymes of phenylpropanoid pathway exhibit a high activity under environmental stress in plant tissues (Kovacik *et al.,* 2007). An increase in PAL activity has been associated with high phenols concentration in N starved plants (Cheng and Breen, 1991; Sanchez *et al.,* 2000; Chen *et al.,* 2006), but there are no reports indicating a relation among N concentration, PAL activity and phenols accumulation in blueberry leaves. PAL activity was high in Legacy leaves that accumulated about 15 g N $kg^{-1}$ DW. Consequently, higher levels of cinnamic acid could derivate into accumulation of flavonols at the long-term or total anthocyanins in kinetic assay. PAL activity in Bluegold leaves was also activated at this N concentration, and this fact was accompanied by an increase of total

phenols content at the long-term assay. Comparable responses have been observed in strawberry and vineyards, which showed an enhanced PAL activity accompanied by high levels of phenols compounds (Hilbert *et al.,* 2003). Conversely, PAL activity decreased in N fertilized plants of both cultivars, and this behavior was supported by a reduction of total phenols and anthocyanins

We have emphasized that our findings are according to CNB theory. As expected, we observed both the significant increase of proteins concentration (see chapter 5) when N was acquired excessively (20 g N $kg^{-1}$ DW) and the limited carbon assimilation into phenolic compounds at this N uptake level. In contrast, higher amounts phenolic compounds were accumulated by plants that accumulated about 15 g N $kg^{-1}$ DW in leaves, supporting the hypothesis of this study. Thus, we found an N threshold of about 15 g N $kg^{-1}$ DW in blueberry leaves that increase the content of antioxidant phenolic compounds. Whereas that the role of N and its influence on individual anthocyanidins content is unclear (Hilbert *et al.,* 2003) our findings demonstrate that anthocyanidins reached high levels at this N concentration, mainly in Legacy.

In summary, our research show differential behavior between cultivars being Bluegold sensitive to N deficiency or excess exhibiting low photosynthetic rate likely influenced by high lipid peroxidation compared with Legacy. However, the most important finding was related to an N concentration of about 15 g N $kg^{-1}$ DW, which trigger high antioxidant capacity, phenolic compounds and elevated PAL activity (Figure 6.1). Conversely, a higher N concentration (e.g. 20 g N $kg^{-1}$ DW) has a negative effect on antioxidant capacity, phenolic compounds and PAL activity for both cultivars.

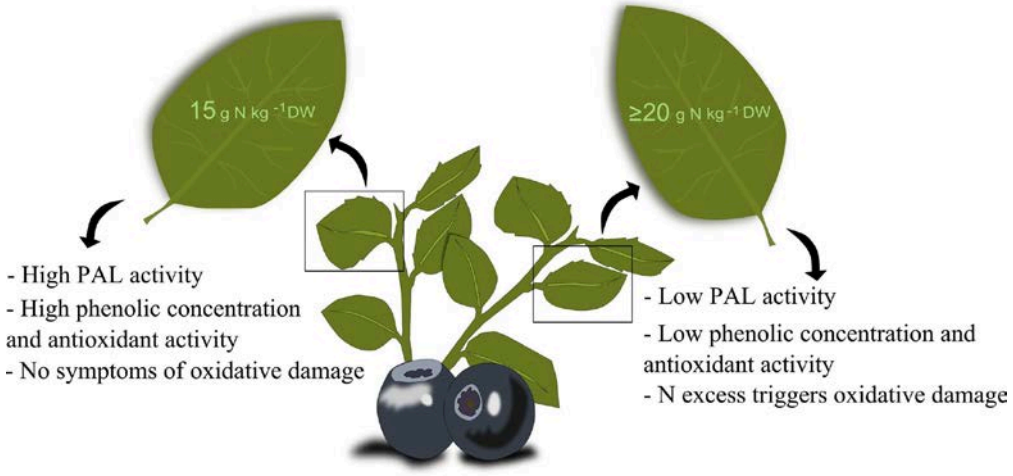



Figure 6.1. Nitrogen concentration threshold as related to antioxidant phenolic compounds and PAL activity in blueberry leaves.

## 6.2 Concluding remarks

- Taking to account the results obtained here, variable N doses at hydroponic and soil conditions showed differential responses between blueberry cultivars related to antioxidant capacity, profile and concentration of phenolic compounds.

- A threshold of N concentration in leaves of 15 g N $kg^{-1}$ DW would be recommended as adequate N nutrition status in blueberry cultivars. It enhances PAL activity and maintains elevated phenolic compounds concentration as well as great antioxidant performance, without detrimental effect on photosynthesis.

## 6.3 Future directions

- It is remarkable that N excess trigger a considerable decrease of phenolic compounds in blueberry leaves, which reduce the antioxidant quality at both the short- and the long-term, and then the crop profitability. Therefore, the effect of N nutrition on highbush blueberry fruits should be evaluated over multiple seasons in order to enhance the accumulation of phenolic compounds under field conditions.

- Further studies are also necessary to better understand the impact of N nutrition on phenolic compounds families (e.g flavonols and anthocyanins) and the enzymes that regulate their synthesis and accumulation.

- The reduction of N fertilization doses is highly desirable at the soil-plant system due to low N inputs decrease the N losses to the environment and, based on our findings, the antioxidant performance could be maximized in blueberry leaves.

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