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ROLE OF ABSCISIC ACID ON ANTHOCYANIN BIOSYNTHESIS UNDER DROUGHT STRESS IN *Aristotelia chilensis* (MOL.) PLANTS

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"Role of abscisic acid on anthocyanin biosynthesis under drought stress in *Aristotelia chilensis* (Mol.) plants"

Esta tesis fue realizada bajo la supervisión de la Dra. Marjorie Reyes Díaz, perteneciente al Departamento de Ciencias Químicas y Recursos Naturales, Facultad de Ingeniería y Ciencias de la Universidad de La Frontera y ha sido aprobada por los miembros de la comisión examinadora.

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quienes me han enseñado a caminar y recorrer la vida.

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Summary and thesis outline

Drought is the most important stress factor for plants, where abscisic acid (ABA) plays a crucial role to cope with the stress. It is well reported that drought stress increases *9-cis-epoxycarotenoid dioxygenase* (*NCED*) gene expression, which it is an important ABA biosynthetic pathway gene, triggering higher ABA levels in plant subjected to drought stress. Thus, it has been suggested that ABA might be involved on anthocyanin biosynthesis under drought stress. Anthocyanins are plant secondary metabolites, which may help to plants to counteract oxidative damage generated by drought stress as antioxidant; however, there is no evidence to sustain such hypothesis.

Aristotelia chilensis (Mol.), also known as Maqui, is an endemic berry in Chile belonging to the Elaeocarpaceae family. *A. chilensis* is considered as a pioneer species, colonizing and growing on stressed and disturbed environments, thus being an interesting model for studying abiotic stress resistance mechanism.

Therefore, the following hypothesis was proposed: "Higher ABA levels produced by induction of *nine-cis-epoxycarotenoid dioxygenase* (*NCED*) gene expression triggers anthocyanin biosynthesis due to the induction of *UDP-glucose: flavonoid 3-O-glucosyltransferase* (*UFGT*) gene expression in *Aristotelia chilensis* (Mol.) plants under drought stress".

The aim of this work was to study the role of abscisic acid on the regulation of anthocyanin biosynthesis in *Aristotelia chilensis* (Mol.) under drought stress.

First, we described the importance of ABA and anthocyanin biosynthesis in plants subjected to drought stress, and their relationship throughout the interaction of ABA and a microRNA (microRNA156) for anthocyanin biosynthesis. Here, we proposed a molecular model where ABA triggers anthocyanin biosynthesis in drought stressed plants.

A drought stress experiment allows us to determine that *A. chilensis* plants were subjected to severe stress at day 20 after water restriction. At the same time with this severe drought stress coincided with the highest ABA and anthocyanin levels in fully-expanded leaves.

Thus, to determine the role of ABA on anthocyanin biosynthesis in drought stressed *Aristotelia chilensis* plants, we applied fluridone (ABA inhibitor biosynthesis), and subsequent ABA at day 20 of water restriction (when plants were subjected to severe drought stress). In this experiment, we found that ABA regulates anthocyanin biosynthesis through the *AcUFGT* expression in drought stressed plants.

In the last chapter, general discussion has been developed, where the main conclusions were that 1) a negative effects of drought stress on plant growth were ameliorated by ABA and anthocyanin biosynthesis that importantly contributed to drought stress tolerance.

2) That fluridone was an effective ABA inhibitor in *A. chilensis* stressed plant, and also that ABA application was able to recover both endogenous ABA concentrations in fluridone treated plants as well as increase total anthocyanin and also inducing a different anthocyanin profile.

Finally, this thesis leads to the first step in the induction mechanism of anthocyanin biosynthesis under drought stress. However, it will be necessary in future

studies to further explore the molecular mechanisms for ABA downstream processes. These processes will allow us a target task for breeders to manage and modify anthocyanin concentrations in plant organs and consequently increase the plant tolerance to drought stress.

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CHAPTER 1

General Introduction

1.1 Introduction

Currently, about 40% of Earth's surface corresponds to land under drought stress (United Nations, 2014). The lack of water availability (drought stress) is considered the most important stress factor for plants, due to the fact that it is involved in important physiological processes (Tadeo and Gómez-Cadenas, 2008). Drought stress can limit photosynthesis and plant growth. Plants have developed complex mechanisms for preventing water loss and counteracting oxidative stress due to drought stress. Abscisic acid (ABA) synthesis, non-enzymatic compounds, and stomatal closure are some responses to drought stress in plants (Moreno, 2009; Zhang et al. 2001). It has been reported that drought stress can modify anthocyanin concentration as well as the anthocyanin profile, promoting the synthesis of tri-hydroxylated anthocyanins (Ojeda et al. 2002; Castellarin et al. 2007; Bucchetti et al. 2011). Thus, Castellarin et al. (2007) reported that tri-hydroxylated anthocyanins such as delphinidin and malvidin are better compared to di-hydroxylated anthocyanin; mitigating oxidative stress due to antioxidant power, which depends on the numbers of hydroxyl groups in anthocyanin chemical structure. Therefore, these tri-hydroxylated anthocyanins increase drought stress tolerance. It is well know that drought stress induces anthocyanin level accumulation due to up-regulation of anthocyanin pathway key genes such as *dihydroflavonol 4-reductase (DFR)*, *UDP-glucose:flavonoid 3-O-glucosyl transferase* (*UFGT*) and transcription factors such as Myeloblastosis A1 (MybA1) and Myeloblastosis 5A (Myb5A) (André et al. 2009; Borsani et al. 2010; Castellarin et al. 2007; Santesteban et al. 2011). However, the induction mechanism of this higher anthocyanin concentration is still unclear (Ferrandino and Lovisolo, 2013: Petrussa et al. 2013; Murcia et al. 2017). On the other hand, drought stress increases *9-cis-epoxycarotenoid dioxygenase* (*NCED*) gene expression, which encodes a key enzyme in ABA biosynthesis pathway (Tuteja et

al. 2007; Trivedi et al. 2016). Thus, higher *NCED* expression increases ABA concentration in the xylem sap and plant organs such as fruits and leaves of different species (Luchi et al. 2001; Zhang et al. 2009). According to Peuke (2016), ABA concentration in leaves is more variable than in other plant organs. Even more, there is evidence that in young leaves, ABA had higher levels than in fully-expanded leaves of *Coleus blumei* and *Xanthium strumarium* (Raschke and Zeevaart, 1976; LaMotte et al. 2002); on the contrary, in *Pisum sativum*, *Triticum aestivum* and *Arabidopsis thaliana*, fully-expanded leaves showed higher ABA levels compared with young leaves under drought stress (Zdunek and Lips, 2001; Zhang et al. 2012; Chen et al. 2013). Therefore, plant organs accumulate endogenous ABA in different ways in response to drought stress. Some authors have suggested that higher anthocyanin concentration under drought stress could be due to ABA concentration increase (Jiang and Joyce; 2003; Deluc et al. 2009; Bucchetti et al. 2011). For example, Nagira et al. (2006) showed that osmotic stress in *Torenia fournieri* plants elevated endogenous ABA levels before anthocyanin biosynthesis induction. Therefore, they suggested that changes in the endogenous ABA concentration might play an important role in the anthocyanin biosynthesis induction. Thus, González-Villagra et al. (2017) have proposed a model, where they explain how ABA could be involved in anthocyanin biosynthesis through the regulation of a microRNA (156), which increases the expression of anthocyanin biosynthesis genes. However, other authors have suggested that different factors might have a higher influence on anthocyanin concentrations than endogenous ABA (Gagné et al. 2011; Kondo et al. 2014). Antolín et al. (2006) reported that ABA and anthocyanin concentration (based on fresh weight) increased in *Vitis vinifera* cv. Tempranillo fruits under drought stress. However, there was no difference in anthocyanin content on a berry basis, between drought stress and well watered treatments. Therefore, whether

ABA is responsible for increasing anthocyanin concentration under drought stress is still controversial. Besides, there are few reports regarding the changes on endogenous ABA levels that link with the anthocyanin biosynthesis induction. Understanding the inductor mechanism responsible of higher anthocyanin concentration under drought stress might represent a powerful tool to manage and modify anthocyanin concentration in plant organs. Therefore, it is greatly important to know whether ABA is responsible for the increase of anthocyanin biosynthesis under drought stress.

Maqui (*Aristotelia chilensis* Mol.) is an endemic berry in Chile belonging to Elaeocarpaceae family. It is an evergreen tree, distributed from Illapel (Coquimbo Region) to Chiloé (Los Lagos Region) (Hoffman et al., 2005). The *A. chilensis* is a pioneer species, colonizing and growing on stressed and disturbed environments, being an interesting model for studying its abiotic stress resistance mechanism (Fredes et al. 2014). On the other hand, this endemic species has been of a great interest for farmers and consumers for its antioxidant action due to high anthocyanin concentration. Currently, commercial crops are being established, forcing the development of morpho-phenological, physiological, and genetic diversity studies to establish agronomic parameters, and to develop selection and breeding strategies (Fredes et al. 2014; Vogel et al. 2014). Therefore, *A. chilensis* is an adequate model to study ABA and anthocyanin accumulation.

The aim of this work was to study the role of abscisic acid on anthocyanin biosynthesis in drought stressed *Aristotelia chilensis* plants, evaluating the induction of genes related to their biosynthesis.

1.2 Hypotheses

Currently, it is known that drought stress increases anthocyanin concentration due to anthocyanin pathway key gene up-regulation. However, the inducing mechanism of this higher anthocyanin concentration is unknown. On the other hand, it has been suggested that abscisic acid could be responsible for anthocyanin biosynthesis genes regulation under drought stress. However, whether abscisic acid is responsible for increasing anthocyanin concentration under drought stress is still controversial.

Therefore, the following hypothesis is proposed:

Higher ABA levels produced by induction of *nine-cis-epoxycarotenoid dioxygenase* (*NCED*) gene triggers anthocyanin biosynthesis due to the induction of *UDP-glucose: flavonoid 3-O-glucosyltransferase* (*UFGT*) gene in *Aristotelia chilensis* (Mol.) plants under drought stress.

1.3 General objective:

To study the role of abscisic acid on the regulation of anthocyanin biosynthesis in *Aristotelia chilensis* (Mol.) under drought stress.

1.4 Specific objectives:

1. To evaluate the effect of drought stress on endogenous abscisic acid, total and profile of anthocyanin in *Aristotelia chilensis* (Mol.) plants.

2. To evaluate expression changes of *nine-cis-epoxycarotenoid dioxygenase* (*NCED*) and *UDP-glucose: flavonoid 3-O-glucosyltransferase* (*UFGT*) genes in *Aristotelia chilensis* (Mol.) plants under drought stress.

3. To compare the effect of an endogenous abscisic acid inhibitor and subsequent exogenous abscisic acid applications on total anthocyanin in *Aristotelia chilensis* (Mol.) plants under drought stress.

CHAPTER 2

Evaluating the involvement and interaction of abscisic acid and miRNA156 in the induction of anthocyanin biosynthesis in drought-stressed plants

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Evaluating the involvement and interaction of abscisic acid and miRNA 156 in the induction of anthocyanin biosynthesis in drought-stressed plants

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Abstract

Drought stress is the main cause of agricultural crop loss in the world. However, plants have developed mechanisms that allow them to tolerate drought stress. At cellular level, drought stress induces changes in metabolite accumulation, including increases in anthocyanin levels due to upregulation of the anthocyanin biosynthetic pathway. Recent studies suggest that the higher anthocyanin content observed under drought stress could be a consequence of a raise in the abscisic acid (ABA) concentration. This plant hormone crosses the plasma membrane by specific transporters, and it is recognized at the cytosolic level by receptors known as pyrabactin resistance (PYR)/regulatory component of ABA receptors (PYR/RCARs) that regulate downstream components. In this review we discuss the hypothesis regarding the involvement of ABA in the regulation of microRNA 156 (miRNA156), which is upregulated as part of dehydration stress responsiveness in different species. The miRNA156 upregulation produces a greater level of anthocyanin gene expression, forming the multienzyme complex that will synthesize an increased level of anthocyanins at the cytosolic face of the rough endoplasmic reticulum (RER). After synthesis, anthocyanins are transported from the RER to the vacuole by two possible models of transport: 1) Membrane Vesicle-mediate Transport (MVT), or 2) Membrane Transporter-mediated Transport (MTT). Thus, the aim was to analyze the recent findings on synthesis, transport and the possible mechanism by which ABA could increase anthocyanin synthesis under drought stress potentially throughout microRNA 156 (miRNA156).

Keywords: anthocyanin transporter · phytohormone · microRNA156 · pre-vacuolar compartments

2.1 Introduction

Water is the most important factor for plant growth, since it is a major component of the plant body and it is involved in fundamental physiological processes. Thus, a limitation in water availability (drought) is a major stress factor for plant growth and development, and therefore reproductive yield (Levitt 1980; Tadeo and Gómez-Cadenas 2008; Moreno 2009). According to a recent United Nations' World Water Development Report (2014), a third of the world's population lives in countries or regions with significant drought stress, and it is predicted that by 2025 this will increase by up to two thirds. It is estimated that drought stress is the main cause of agricultural crop loss in the world as drought can reduce the average expected crop yields by more than 50% (Boyer 1982; Pessarakli 2010). Plants have developed physiological and molecular mechanisms that allow them to tolerate drought stress or slow the rate of its impact on plant physiology. The most important physiological mechanism is the regulation of stomatal closure. Stomatal closure in response to drought stress can limit its severity by preventing water loss through these specialized structures (Zhang et al. 2001). At the cellular and molecular levels, drought stress generates an increase in the expression of genes that encode enzymes for the production of secondary metabolites such as osmolytes, proteins with protective functions, and enzymatic and non-enzymatic antioxidants, and thus accumulation of these gene products at the cytoplasmic level (Taiz et al. 2016).

Drought stress also induces changes in the accumulation of another group of secondary metabolites, anthocyanins, which are responsible for the red, purple, and blue colors of plant tissues (Taiz and Zeiger 2002; Schwinn et al. 2016), mostly fruits and leaves (Roby et al. 2004; Bucchetti et al. 2011; Zhang et al. 2017). Anthocyanins also accumulate in response to biotic and other abiotic stresses, and therefore are thought to

play a key role in the survival of stressed plants (Steyn et al. 2002). Under drought stress, anthocyanins have a role in osmotic regulation, contributing to the maintenance of cell turgor pressure and thus tolerance to a water deficit (Chalker-Scott 1999). However, anthocyanins may also have other regulatory roles in the event of a drought stress (Hughes et al. 2013). Anthocyanins are synthesized in cytoplasm by a multienzyme complex, associated with the cytoplasmic face of the rough endoplasmic reticulum (RER), via the phenylpropanoid pathway, and stored in the vacuole, but their cellular transport is not well known (Winkel-Shirley 1999; Winkel-Shirley 2004; Sun et al. 2012ab). Under drought stress, the accumulation of anthocyanins appears to be under complex regulatory control at both spatial and temporal levels and thus the inductive mechanisms of anthocyanin synthesis remains unresolved (Castellarin et al. 2007a; Ollé et al. 2011). Abscisic acid (ABA) is a plant hormone that regulates plant growth, development such as seed dormancy, floral induction, and is involved in abiotic stress responses such as drought stress, salinity and cold (Finkelstein 2013; Li et al. 2017a). Under these abiotic stresses, ABA regulates the activation of antioxidant enzymes and also reduces stomatal aperture (Choudhary et al. 2011; Guajardo et al. 2016). The aim of this review was to analyze, summarize and evolve the recent findings on synthesis, transport and the possible mechanism by which ABA interacts, directly or indirectly, with anthocyanin biosynthesis and, potentially, microRNA 156 (miRNA156) under drought stress.

2.2 Overview of biosynthesis and transport of anthocyanins

Anthocyanins belong to a large family of secondary metabolites known as flavonoids. This family consists of compounds such as flavones, flavonols and isoflavones. The basic anthocyanin structure consists of two aromatic rings bound by a

three-carbon bridge, and attached groups, such as hydroxyl and methoxy groups, as well as adducts, which generate the various kinds of anthocyanins (Winkel-Shirley 2006; Boudet 2007). Anthocyanins are synthesized via the phenylpropanoid pathway (Winkel-Shirley 1999) and stored in the vacuole (Sun et al. 2012a; Li et al. 2017b), but their cellular transport is not well known. The phenylpropanoid pathway of anthocyanin synthesis has been well characterized (Fig. 1), and there are several reviews that describe it in detail (Jaakola et al. 2002; Winkel-Shirley 2006; Vogt 2010; Teixeira et al. 2013). Anthocyanin synthesis occurs mainly in epidermal cells of different organs such as stem, leaves, flowers, and fruits (Jackson et al. 1992; Huits et al. 1994; Bae and Kim 2006; Ahmed et al. 2009; Gould et al. 2009). Although some authors (Pelletier and Shirley 1996; Buer and Muday 2004; Buer et al 2007) have indicated that roots and tissues grown in the dark are largely incapable of synthesizing significant levels of anthocyanins because the biosynthetic enzymes are all light-dependent, other authors (Buer et al. 2007; Neufeld et al. 2011) have demonstrated that in *Galax urceolata* and *Ipomoea batatas* root tissues anthocyanin biosynthesis can occur without light. Presently, this phenomenon remains largely unexplained. At the cellular level, the cytosolic face of the RER is the primary place where synthesis of these compounds occurs via the action of a multienzyme complex (Winkel-Shirley 2004; Tian et al. 2008). However, some of the individual enzymes of the anthocyanin biosynthetic pathway have also been found to be associated with the membranes of various other organelles such as vacuoles, plastids, and also inside the cell nucleus (Winkel-Shirley 2004; Saslowsky et al. 2005; Tian et al. 2008; Toda et al. 2012).

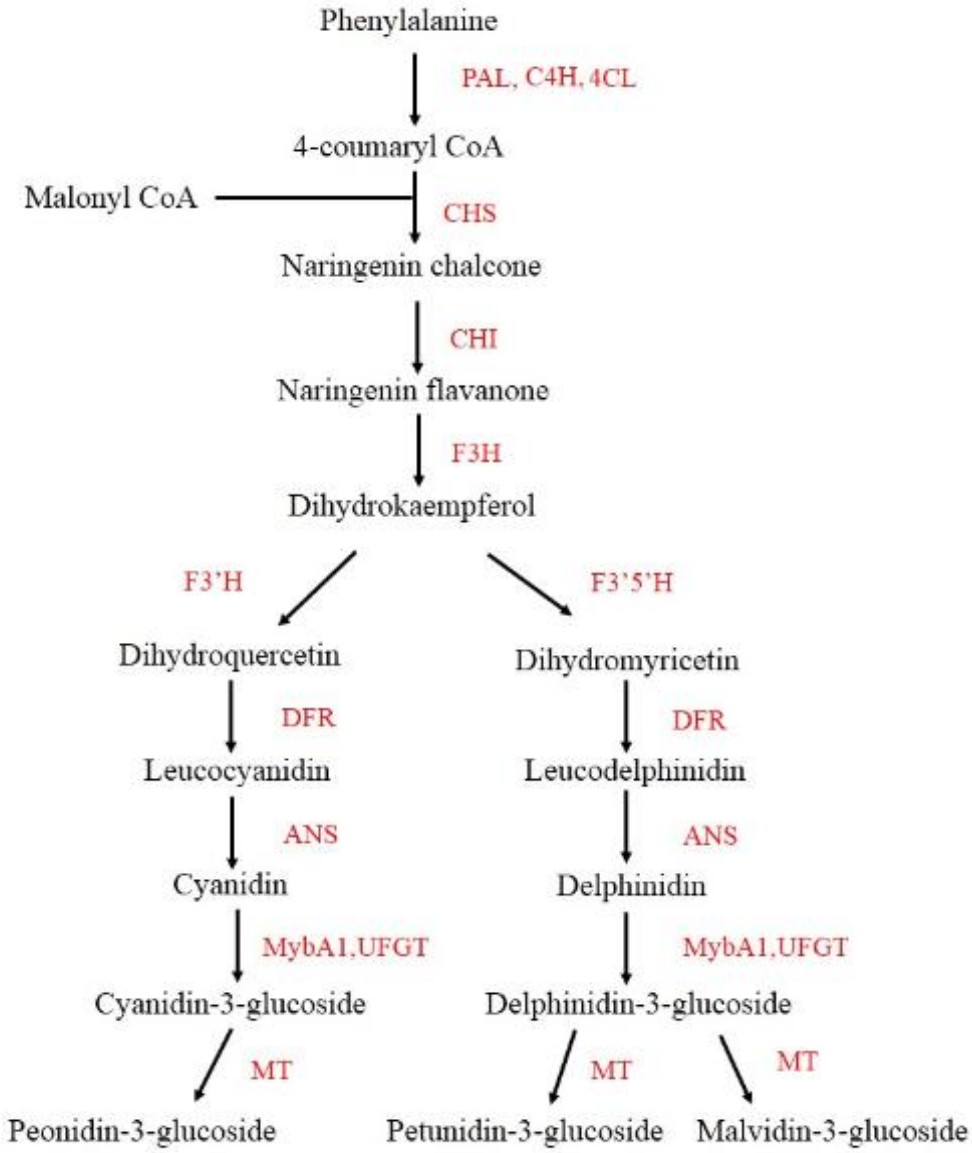


Figure 1. General phenylpropanoid pathway. PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; MybA1, myeloblastosis A1; UFGT, UDP glucose:flavonoid 3-O-glucosyltransferase; MT, methyltransferase.

As mentioned above, anthocyanins are primarily synthesized on the cytosolic face of the RER and subsequently stored in the vacuole (Sun et al. 2012a). However, it is not fully understood how anthocyanins are transported from the RER to the vacuole. Accumulation of newly biosynthesized anthocyanins in the vacuole is required to prevent their oxidation and thus maintain functional anthocyanins for a future action (Marrs et al. 1995; Verweij et al. 2008). In the vacuole, anthocyanins are stored inside bodies or structures of different sizes without defining membranes, known as anthocyanic vacuolar inclusions (AVIs) (Zhao and Dixon 2010; Zhang et al. 2006). For the anthocyanin transport from RER to vacuole, two possible models have been proposed: 1) membrane vesicle-mediated transport, and 2) membrane transporter-mediated transport (Grotewold and Davies 2008; Fig. 2).

The membrane vesicle-mediated transport (MVT) is a transport by vesicles, or structures having membranes, called pre-vacuolar compartments (PVCs), travelling from the RER to the tonoplast (Gómez et al. 2011). The transport of anthocyanins by PVCs has been described in *Vitis vinifera* (Conn et al. 2003), *Arabidopsis thaliana* (Poustka et al. 2007), and *Sorghum bicolor* (Snyder and Nicholson 1990). Anthocyanins have been shown to accumulate in the RER lumen (Poustka et al. 2007); therefore, these PVC structures could be originated within the RER lumen. PVCs can enter the vacuole by either endocytosis (Gómez et al. 2011) or directly into the vacuole by microautophagy as the vacuolar membrane engulfs anthocyanins (Chanoca et al. 2015). However, more research is needed to fully describe this input mechanism.

For the membrane transporter-mediated transport (MTT) (Fig. 2) model, two major transporter families have been suggested as being involved in this transport mechanism: the multidrug resistance-associated protein type ATP-binding cassette

(MRP-type ABC) and multidrug and toxic compound extrusion (MATE) (Zhao and Dixon 2010). The ABC transporters are proteins that can transport substrates across the membrane using energy from ATP hydrolysis (Jones and George 2002). To date only two MRP-type ABC transporters have been identified in anthocyanin transport, *ZmMrp3* in *Z. mays* and *VvMrp1* in *V. vinifera* (Goodman et al. 2004; Francisco et al. 2013). The second major anthocyanin transporter family, MATE (Yazaki 2005), is the family of multidrug efflux transporters involved in the detoxification of xenobiotics, organic acids, and secondary metabolites. Activity of these transporters depends on a H^+ gradient across the tonoplast generated by V-ATPase and H^+-pyrophosphatase (Klein et al. 1996). Gómez et al. (2009) and Zhao et al. (2011) identified genes encoding MATE transporters located in the tonoplast: *anthoMATE1* and *anthoMATE3* genes in *V. vinifera* and *MtMATE2* in *M. truncalata.* Through observations obtained with confocal microscopy, Gómez et al. (2009) and Zhao et al. (2011) suggested that small vesicles carrying anthocyanins were associated with MATE transporters.

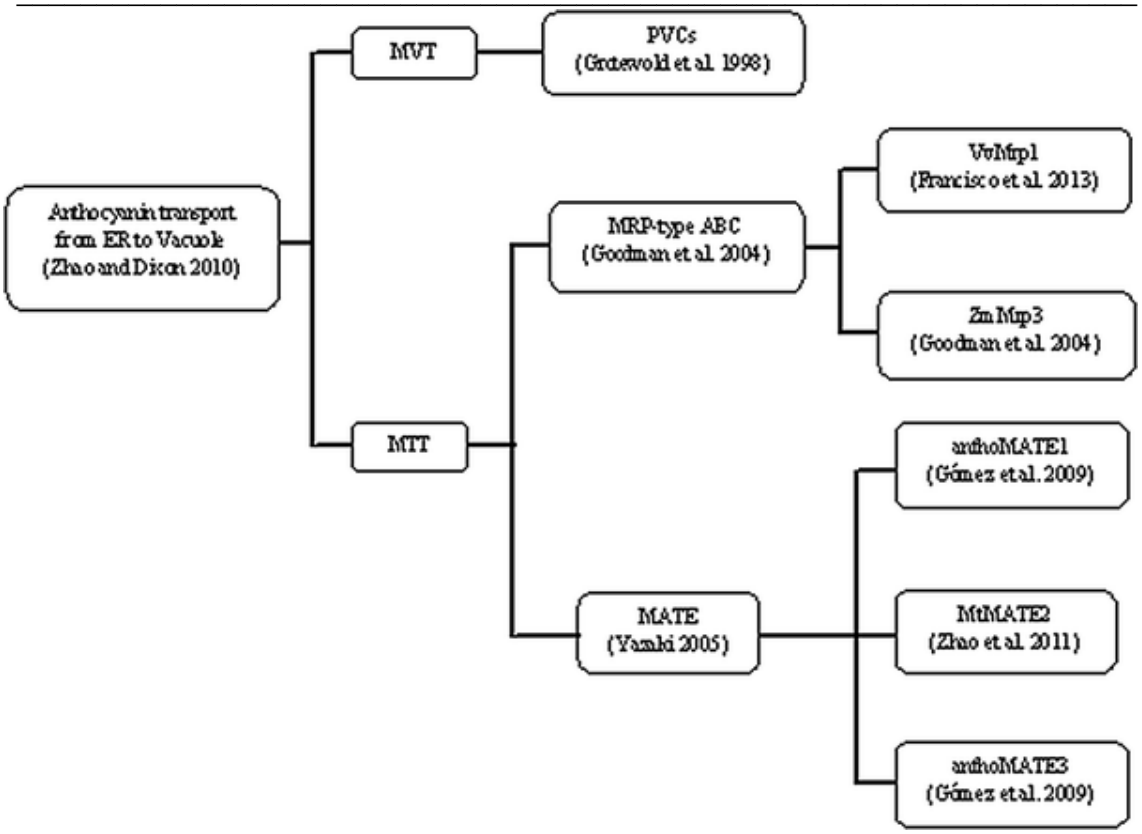


Figure 2. Anthocyanin transport from RER to Vacuole. There are two possible models of anthocyanin transport: membrane vesicle-mediate transport (MVT) and membrane transporter-mediated transport (MTT). The MVT refers to transport by vesicles or structures filled with anthocyanins inside that have a membrane, these structures are commonly named pre-vacuolar compartments (PVCs). The MTT refers to transport by transporters located at the tonoplast. Two major transporter families have been proposed as being involved in this transport mechanism: the multidrug resistance-associated protein type ATP-binding cassette (MRP-type ABC), and the multidrug and toxic compound extrusion (MATE) protein family.

2.3 Anthocyanin accumulation under drought stress

The accumulation of anthocyanin under drought stress has been studied in many plant species and different organs (Table 1). For example, Kennedy et al. (2002)

reported that the anthocyanin concentration, based on fresh weight, of drought-stressed wine grapes was significantly higher (>50%) than well-watered wine grapes (Table 1). Similar results were also reported in *V. vinifera* by Esteban et al. (2001). However, drought stress not only increases anthocyanin accumulation, but also inhibits plant growth. For example, in drought-stressed *A. thaliana* leaves, which had reduced size and biomass growth, anthocyanin concentrations were higher than in well-watered leaves (Jung 2004). Thus, is there a greater anthocyanin accumulation under drought stress because of a *de novo* anthocyanin synthesis leading to higher anthocyanin concentrations or because of the drought stress-mediated inhibition of organ growth? Castellarin et al. (2007a) and Ferrandino and Lovisolo (2013) have concluded that a higher accumulation of anthocyanins under drought stress is not due to a growth inhibition of studied organs, but rather a true upregulation of anthocyanin biosynthesis.

Table 1. Effects of drought stress on anthocyanin concentrations of different organs and in different species.

Species	Organs	Conditions	Effects	References
Vitis vinifera cv. Cabernet Franc	Fruits	Field conditions. Water was withheld (midday leaf water potential was -1.43 MPa at onset of ripening) from anthesis until the onset of ripening.	Increased concentration	Matthews and Anderson 1988.
Pisum sativum cv. *Citrina*	Leaves	Greenhouse conditions. Nutrient solution with 10% polyethylene glycol. Seedlings, 10 days old, stress applied for 7 d.	Increased concentration	Alexieva et al. 2001.
Withania somnifera	Leaves	Greenhouse conditions. Seedlings, 7 days old, were subjected to drought stress by withholding water.	Increased concentration	Sanchita et al. 2015.
Vitis vinifera cv. Cabernet Sauvignon	Fruits	Field conditions. Commercial vineyard. The irrigation was not applied until midday, leaf water potential was -1.6 MPa	Increased anthocyanin concentration	Kennedy et al. 2002.
Vitis vinifera cv. Cabernet Sauvignon	Fruits	Field conditions, the irrigation was not applied until midday, leaf water potential was -1.6 MPa	Increased concentration	Roby et al. 2004.
Cicer arietinum	Leaves	Field conditions, plants were 20 days old, drought stress was applied by withholding water.	Increased concentration	Kalefetoglu and Ekmekci

				2009.
Vitis vinifera cv. Cabernet Sauvignon	Fruits	Field conditions, water was applied when stem water potential reached -1.2 MPa, then irrigation was applied weekly for both treatments. Plants were 20 years old.	Increased concentrations	Deluc et al. 2009.
Vitis vinifera cv. Merlot	Fruits	Field conditions, treatments were applied from the onset of ripening to harvest. Water potential was kept within the interval -0.8 to -1.4 MPa for stress treatments	Increased concentration	Bucchetti et al. 2011
Vitis vinifera cv. Tempranillo	Fruits	Field conditions, treatments were applied from the onset of ripening to harvest. Water potential was kept until reaching -0.8 MPa for stress treatments	Increased concentration	Santesteban et al. 2011

However, the mechanism for induction of anthocyanin biosynthesis under drought stress remains largely unclear. Some molecular studies have facilitated the elucidation of this mechanism (Castellarin et al. 2007b; André et al. 2009). According to these studies, drought stress induces changes in the expression of several key genes involved in the anthocyanin biosynthetic pathway (Castellarin et al. 2007b; André et al. 2009; Giordano et al. 2016). In particular, drought stress induces an upregulation of expression of *CHS, Flavanone 3- hydroxylase (F3H), Flavonoid 3`,5`-hydroxylase (F3`5`H), DFR, UDP-glucose:flavonoid 3-O-glucosyl transferase (UFGT), O-methyl-transferase (OMT)*, as well as transcription factors, such as Myeloblastosis A (MYBA), Myeloblastosis 5a (MYB5a), and MYB112 (Nagabhushana and Reddy 2004; Castellarin et al. 2007a, b; André et al. 2009; Borsani et al. 2010; Martínez-Lüscher et al. 2014; Berdeja et al. 2015; Lotkowska et al. 2015). The upregulation of the expression of these structural and regulatory genes involved in the phenylpropanoid pathway results in an increased number of enzymes available for catalysis of the biosynthesis reactions and thus results in an increased number of anthocyanins at the cellular level. In fact, a correlation analysis has demonstrated a strong and positive relationship ($r^2 \geq 0.95$) between gene expression encoding biosynthetic enzymes and metabolites produced in the anthocyanin biosynthetic pathway (Castellarin et al. 2007b), demonstrating that the anthocyanin

concentration is increased due to upregulation of the phenylpropanoid pathway. Furthermore, it has been shown that drought stress not only positively influences the cumulative number of anthocyanins in plant tissues, but it also modifies the composition of anthocyanins as it specifically promotes the accumulation of tri-hydroxylated anthocyanins, due in part to a higher expression of flavonoid 3`-hydroxylase (*F3`H*) and *F3`5`H* (Deis et al. 2011; Santesteban et al. 2011). For example, Castellarin et al. (2007b) reported that in *V. vinifera* the concentrations of tri-hydroxylated anthocyanins, such as delphinidin, petunidin and malvidin, were higher under drought stress than in well-watered control treatments. However, the concentration of di-hydroxylated anthocyanins, such as cyanidin and peonidin, was similar for both control and drought stress treatments (Castellarin et al. 2007b). Therefore, there are multiple levels of regulation of anthocyanin biosynthesis under drought stress.

Previously, Chalker-Scott (1999) had suggested that anthocyanin compounds have a role in osmotic regulation by contributing to the maintenance of turgor pressure and thus tolerance to drought. However, Hughes et al. (2013) suggested that the role of anthocyanin compounds was likely not in osmotic protection because of low anthocyanin concentrations and their high metabolic cost compared to other solutes, such as proline and soluble sugars, which are typically found to be more effective in osmotic adjustment. A recent study by Sperdouli and Moustakas (2014) in *A. thaliana* suggested that anthocyanins can have an important antioxidant role under drought stress as drought-stressed leaves maintained oxidative compounds (such as malondialdehyde) within the same range as found in control leaves, thereby implying that a biochemical mechanism was in operation to cope with oxidative damage. Therefore, we can suggest, based on the above-mentioned reports, that higher expression of *F3`H*, *F3`5`H* and *UFGT* genes under drought stress will allow the accumulation of tri-hydroxylated

anthocyanin forms, giving a greater antioxidant capacity. This capacity depends in part on the numbers of hydroxyls in the anthocyanin chemical structure. Therefore, specific modification of the basic structure can be employed by the plant cell in order to help increase the defense mechanisms against reactive oxygen species (ROS).

2.4 Induction mechanism under drought stress

As discussed in the previous section, anthocyanin content is increased under drought stress due to upregulation of the expression of key genes in the phenylpropanoid pathway, although the induction mechanism is still unclear. Some authors have suggested that higher anthocyanin content under drought stress could be due to increases in the levels of ABA (Jiang and Joyce 2003; Deluc et al. 2009; Bucchetti et al. 2011). For example, McCarty et al. (1989) demonstrated that an *A. thaliana* mutant with reduced sensitivity to ABA blocks anthocyanin biosynthesis, suggesting that ABA plays an important role in the induction of anthocyanin biosynthesis. Furthermore, Fambrini et al. (1993) have demonstrated, using a *Helianthus annuns* mutant plant deficient in ABA biosynthesis, that ABA accumulation is necessary for the induction of anthocyanin biosynthesis. In another study, Nagira et al. (2006) induced osmotic stress in *Torenia* plants with sucrose (Table 2). They determined that under osmotic stress endogenous ABA levels rise significantly before the induction of anthocyanin synthesis. This led them to suggest that changes in the amount of endogenous ABA may play an important role in the induction of anthocyanin synthesis. Recently, it has been shown that treatments of *Fragaria x ananassa* fruits and *Salvia miltiorrhiza* hair roots with fluridone (an ABA biosynthetic inhibitor) resulted in a strong suppression of anthocyanin biosynthesis (Cui et al. 2012; Kadomura-Ishikawa

et al. 2015). Hence, further previous evidence supports ABA playing a direct or indirect role in the induction of anthocyanin biosynthesis under drought stress.

On the molecular level, ABA has been shown to be involved in anthocyanin biosynthesis via its ability to increase the expression levels of several key genes of the phenylpropanoid pathway. For example, Shen et al. (2014) reported that treatment of *Prunus avium* with the ABA biosynthetic inhibitor nordihydroguaiaretic acid (NDGA) downregulated the expression levels of *Myeloblastosis A* (*MYBA)*, a transcription factor that interacts and activates the promoters of the *DFR*, *ANS* and *UFGT* genes. These authors also showed that the endogenous ABA levels as well as the transcript levels of *CHS*, *chalcone isomerase* (*CHI*), *F3H*, *DFR*, *UFGT* and *MYBA* were blocked by silencing the *9-cis-epoxycarotenoid dioxygenase* (NCED) gene, which encodes a key enzyme in the ABA biosynthetic pathway. Another study by Medina-Puche et al. (2014) showed that *F. x ananassa* plants subjected to drought stress increased endogenous ABA levels as well as the expression of *MYB* and anthocyanin accumulation in fruit tissues. Finally, Li et al. (2015) showed that silencing the *8´-hydroxylase* (*CYP707A2)* gene, which encodes a key enzyme in the oxidative catabolism of ABA, further increased anthocyanin accumulation as well as endogenous ABA levels, and stimulated the expression of the transcription factor *MYBA,* all compared to the control (without silenced *CYP707A*2). Consequently, Li et al. (2015) suggested that anthocyanin synthesis is tightly regulated by endogenous ABA levels.

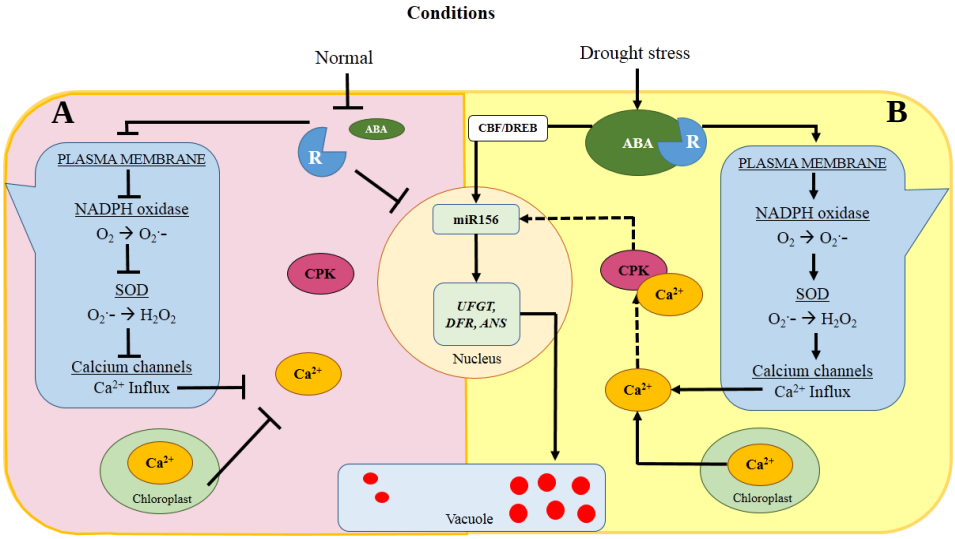
Table 2. Effect of endogenous abscisic acid on anthocyanin concentrations in plants under drought stress

Species	Evaluated organs	ABA concentration	Organ growth	Effects on anthocyanin	References
Vitis vinifera cv. Tempranillo	Fruits	Increased 2643 ng g^{-1} dw	Lower growth than well watered treatment	Increased concentration	Antolín et al. 2006
Torenia fournieri	shoots	10 ng g^{-1} fw	Not reported	Increased concentration	Nagira et al. 2006
Vitis vinifera cv. Cabernet Sauvignon	Fruits	Increased 4000 ng g^{-1} dw	Lower growth than well watered treatment	Increased concentrations	Deluc et al. 2009
Vitis vinifera cv. Cabernet Sauvignon	Fruits	Increased 500 ng g^{-1} fw	Not reported	Increased concentrations	Wheeler et al. 2009
Vitis vinifera cv. Aragonez	Fruits	Increased 1850 ng g^{-1} fw	Inhibited growth	Increased concentration	Zarrouk et al. 2012

fw= fresh weight, dw= dry weight.

Therefore, all these biochemical, hormonal and molecular studies confirm that an ABA balance is important for regulating anthocyanin biosynthesis, and thus its accumulation under drought stress. We therefore propose the following possible mechanism for the induction of anthocyanin synthesis emphasizing the participation of ABA under drought stress (Fig. 3). Under conditions without drought or other osmotic stress, ABA levels and anthocyanin concentrations in plant organs are basal (Fig. 3A). In contrast, under drought stress there is an increase in ABA biosynthesis which leads to the induction of the mechanisms discussed above for anthocyanin biosynthesis, increasing the anthocyanin concentrations above their basal levels (Fig. 3B). Drought stress augments ABA biosynthesis in roots (Davies and Zhang 1991), where it can subsequently be transported to stems and leaves by the xylem (Taiz and Zeiger 2002),

increasing ABA concentration in leaves, and/or there is an ABA biosynthesis directly in the leaf tissues. Then, the binding of newly produced and/or released ABA to its receptors must occur to trigger the downstream signaling cascade of biochemical and molecular events.



. **Fig. 3** Proposed model for ABA and miRNA156 interaction on the induction of anthocyanin biosynthesis under drought stress

Abscisic acid receptors are still the subject of critical study with currently three proposed candidates: an extracellular receptor known as G-protein coupled receptor2 (GCR2) (Pandey et al. 2009); a plastid receptor known as magnesium chelatase subunit H (CHLH) receptor (Shen et al. 2006); and a cytoplasmic receptor known as pyrabactin resistance (PYR)/regulatory component of ABA receptor (PYR/RCARs) (Park et al. 2009). However, the mechanism of GCR2 and CHLH in ABA downstream signaling is unknown; hence, their participation as ABA receptors have not yet been confirmed (Risk et al. 2009; Taiz and Zeiger 2010; Miyakawa et al. 2013). By contrast, the action of PYR/RCAR as an ABA receptor is well supported by several studies (Kharenko et al. 2013; Gonzalez-Guzman et al. 2014; Kim et al. 2014).

Furthermore, a critical review (Zhang et al. 2015) of the current status of our understanding of ABA receptors supports the idea that only the PYR/RCAR can currently be referred to as a *bona fide* ABA receptor. Finally, the recent discovery of ABA transporters in the plasma membrane (PM) supports the proposed function of PYR/RCAR receptors. These transporters belong to the ATP-binding cassette (ABC) transporter family, encoded by *AtABCG40* (Kang et al. 2010), *AtABCG25* (Kuromori et al. 2010) and *AtABCG22* genes (Kuromori et al. 2011). Therefore, when endogenous ABA levels increase in the leaves of drought-stressed plants, ABA molecules cross the plasma membrane by transporters (Boursiac et al. 2013) and bind to PYR/RCARs, triggering the downstream signaling cascade (Zhang et al. 2015). It has been clearly demonstrated that ABA binding to PYR/RCARs inhibits the type 2C protein phosphatases (PP2C) and thus results in disruption of the interaction between PP2C and sucrose non-fermenting related protein kinase 2 (SnRK2), releasing its inhibition of SnRK2. SnRK2 is activated by autophosphorylation and can then activate downstream targets such as NADPH oxidase located in PM (Sirichandra et al. 2009; Kimura et al. 2012; Boneh et al. 2012; Miyakawa et al. 2013). It has been shown that NADPH oxidase oxidates molecular oxygen (Foreman et al. 2003) and produces superoxide radical ($O_2^{\cdot}$-) (Fig. 2B). Then, with the help of superoxide dismutase (SOD), $O_2^{\cdot -}$ is rapidly converted to hydrogen peroxide (H_2O_2) in plants exposed to drought stress (Foreman et al. 2003; Hu et al. 2006; Furlan et al. 2013).

It has been widely recognized that hydrogen peroxide functions as a secondary messenger in ABA signaling (Taiz and Zeiger 2010: Taiz et al. 2016). Wang et al. (2013) showed the importance of PYR/RCARs receptor in ROS production as plants without PYR/RCARs receptors were not able to increase ROS production. Therefore, bound ABA-PYR/RCARs are essential for H_2O_2 production. The H_2O_2 induced by ABA

accumulation promotes anthocyanin biosynthesis in leaves of *O. sativa* seedlings as was shown by Hung et al. (2008). These authors reported that treatments with chemical traps for H_2O_2 effectively inhibited anthocyanin accumulation, confirming that H_2O_2 is required for anthocyanin buildup. Zhang et al. (2014) also indicated that H_2O_2 is involved in the regulation of anthocyanin synthesis, showing that inhibition of NADPH oxidase activities downregulates anthocyanin synthesis in *Malus domestica* peel. In addition, H_2O_2 can also activate calcium (Ca^{2+}) channels at the plasma membrane, promoting extracellular Ca^{2+} influx and raising the cytosolic [Ca^{2+}] (Pei et al. 2000). The production of H_2O_2 activates the NADPH oxidase releasing Ca^{2+} from calcium stores such as chloroplast, mitochondria, and rough endoplasmic reticulum (Wang et al. 2013). The importance of Ca^{2+} in the production of anthocyanin has been demonstrated by treatments with verapamil (a calcium channel blocker), which caused a reduction of anthocyanin levels in cell cultures of *Daucus carota* and *V. vinifera* (Sudha and Ravishankar 2003; Vitrac et al. 2000). More recently, analysis of the time-dependency performed by Shien et al. (2013) showed that the antagonists of Ca^{2+} strongly interfere with anthocyanin accumulation throughout downregulation of *Production of Anthocyanin Pigment 1* (*PAP1)* expression in *A. thaliana*. Therefore, both H_2O_2 and Ca^{2+} play an important role in the metabolic pathway of the proposed mechanism.

Ca^{2+} has also been recognized as an important second messenger in the signal transduction pathways of plant hormones and environmental stimuli (Zou et al. 2010: Gilroy et al. 2016). To date, two major classes of plant calcium sensors in signal transduction have been identified: calcium-binding proteins (calmodulins) and Ca^{2+}-dependent protein kinases (or CPKs) (Hong-Bo et al. 2008; Dubrovina et al. 2013). However, the participation of one of specific Ca^{2+} sensor mechanism in anthocyanin synthesis remains unknown. CPKs are one of the best characterized Ca^{2+} sensors in

plants and have been shown to be involved in the response to abiotic stresses in plants (Gao et al. 2014). CPKs are directly activated by the binding of Ca^{2+}, and their activation regulates downstream components (Harper et al. 2004). The Ca^{2+}-dependent protein kinase family consists of 34 genes in *A. thaliana* (Hrabak et al. 2003), 31 genes in *O. sativa* (Asano et al. 2005), 40 genes in *Z. mays* (Kong et al. 2013), 20 genes in *Triticum aestivum* (Li et al. 2008), and 12 genes in *Vitis amurensis* (Dubrovina et al. 2013). This calcium sensor has different locations, including the cytosol, nucleus, endoplasmic reticulum, and plasma membrane (Yoon et al. 1999; Dammann et al. 2003). In *A. thaliana*, AtCPK3, AtCPK6 (Mori et al. 2006), and AtCPK10 (Zou et al. 2010) are important in the regulation of ion channel and in ABA-regulated stomatal closure. The AtCPK11 and AtCPK32 positively regulate ABA signaling by phosphorylating stress-responsive transcription factors ABF1 and ABF4 (Choi et al. 2005; Zhu et al. 2007). The ZmCPK11 protein is involved in antioxidant enzymatic defense (Ding et al. 2013). Under drought stress, there are many CPKs whose function remains unknown. The CPKs potentially involved in anthocyanin biosynthesis are among those of unknown function, but such protein kinases could be expected to act as with better characterized CPKs and activate transcription factors, and thus upregulate anthocyanin synthesis under drought stress. Signal transduction pathways are complex, and it will require significant additional research to understand this process well. Nevertheless, it is clearly an important research target of high reward to elucidate which CPKs are associated with anthocyanin synthesis in order to have a complete understanding of the mechanism for induction.

Table 3. The microRNAs involved in responses of plants under drought.

miRNA	Expression pattern	Targets	Role	References
miRNA164	Down	NAC domain transcription factors	Lateral root development	Guo et al. 2005
miRNA398	Up	Cu/Zn superoxide dismutases	Response to oxidative stress	Trindade et al. 2010; Sunkar et al. 2006.
miRNA169	Down	CCAAT binding factor (CBF)	Nodule development	Li et al. 2008
miRNA156	Up	Squamosa Promoter Binding protein-like	Transition from juvenile to adult phase	Wang et al. 2011; Kantar et al. 2010.
miRNA171	Down	GRAS transcription factors	Floral development	Llave et al. 2002.

As mentioned above, CPKs can regulate the activity of diverse targets by phosphorylation. It has recently been reported that a protein kinase can phosphorylate human microRNAs (miRNAs), enhancing miRNA production and increasing their stability (Paroo et al. 2009; Herbert et al. 2013), thus suggesting potential for a similar mechanism in plants. Plant miRNAs are small non-coding RNAs, which consist of 20-24 nucleotides that activate or inhibit gene expression via transcriptional or post-transcriptional processes. miRNAs act by controlling expression levels of multiple genes and thus have been reported to regulate root initiation, flower development, and physiological responses to environmental stimuli (Khraiwesh et al. 2012; Eldem et al. 2013). In particular, drought stress often increases the expression of some specific miRNAs (Table 3). For example, microRNA 156 (miRNA156) was shown to be upregulated as a dehydration stress-responsive gene in *Hordeum vulgare* (Kantar et al. 2010), *Phaseolus vulgaris* (Nageshbabu et al. 2013b), *Vigna unguiculata* (Barrera-Figueroa et al. 2011), *Glycine max* (Li et al. 2011), *Panicum virgatum* (Sun et al. 2012b), *A. thaliana* (Liu et al. 2008), and *Eleusine coracona* (Nageshbabu et al. 2013a). Furthermore, Boopathi (2015) has identified that miRNA 156 is expressed in response to an increase in endogenous ABA levels. Likewise, transcription factors could be

involved in plant responses under drought stress (Agarwal et al. 2006). One group of transcription factors which includes the basic domain/Leu zipper (bZIP), MYB and MYC are activated by increased ABA biosynthesis; meanwhile, the other transcription factors may follow an ABA-independent signal transduction pathway, such as c-repeat binding factor (CBF)/drought response elements binding (DREB) proteins (Agarwal et al., 2006). However, a crosstalk between ABA-dependent and ABA-independent activation of different transcription factors have been documented in several plants (Fujita et al. 2011). Haake et al. (2002) reported that CBF/DREB can respond to an ABA-dependent signal transduction pathway. In addition, it has been reported that miRNAs associated with CBF/DREB transcription factor increase drought stress tolerance (Shi and Hussain, 2016; Candar-Cakir et al., 2016). Recent studies have also shown that CBF/DREBs can increase the expression of miRNA 156 (Hackenber et al. 2012: Artilip et al. 2016). Therefore, these findings suggest that upregulation of miRNA 156 under drought stress is driven by higher ABA levels and the downstream signaling cascade which they activate.

The functional role of miRNA156 in the adaptation of plants to drought stress has been suggested by Nageshbabu et al. (2013a) and Kantar et al. (2010). However, exactly what such a possible functional role might be under drought stress is still largely unknown. Recently, Gou et al. (2011) showed the overexpression of miRNA156 promoted anthocyanin accumulation in *A. thaliana*, whereas wild type plants accumulated significantly less anthocyanin. In addition, expression of anthocyanin synthesis and structural genes (*DFR, UFGT, ANS* and *F3`H*) was greatly increased, and their transcripts were higher by over 30-fold. Furthermore, Cui et al. (2014) confirmed the involvement of miRNA156 in drought stress tolerance through the use of target mimicry methodology where *A. thaliana* plants with blocked miRNA156 action were

extremely sensitive to drought stress and accumulated lower anthocyanins than drought stressed-plants without miRNA156 blockage. In the same experiment, Cui et al. (2014) also demonstrated that the expression levels of two genes of the phenylpropanoid pathway, *DFR* and *PAP1,* were induced in the drought-stressed plants without miRNA156 blockage, concluding that the miRNA156 pathway is contributing to drought stress tolerance via its involvement in anthocyanin biosynthesis. It has also been shown that miRNA828 affects anthocyanin accumulation during phosphate deficiency (Hsieh et al. 2009). Furthermore, it has been suggested that miRNA828 can act directly upon the transcription factors (AtMYB113, AtMYB75 and AtMYB90) that are known to be involved in anthocyanin synthesis (Hsieh et al. 2009). Finally, it was reported that miRNAs could also act directly on gene targets at the transcriptional level (Jopling et al. 2005; Orom et al. 2008). This transcriptional upregulation mechanism has been called RNA activation (RNAa) (Portnoy et al. 2011). Therefore, we further hypothesize that under drought stress higher expressions of miRNA156 may produce a greater expression of anthocyanin genes such as *DFR, UFGT, ANS or F3`H,* which form the multienzyme complex that will synthesize a higher content of anthocyanins in the cytosolic face of the RER. After synthesis on the cytosolic face of the RER, the anthocyanins would then be stored in the vacuole (Sun et al. 2012a; Li et al. 2017b).

2.5 Conclusions and future perspectives

Anthocyanins have received great attention by a number of plant and nutrition researchers. Their biosynthetic pathway, sites of synthesis, and aspects of their transport have all been well established. Our level of knowledge about the molecular response expression of key genes of phenylpropanoid pathways has increased considerably, and

this has helped to partially elucidate responses leading to accumulation of anthocyanins. The results from molecular studies and the evidence presented above suggest that under drought stress ABA interacts with anthocyanin biosynthesis and potentially throughout miRNA156 as we have proposed in the model (Fig 3). This hypothesis should hopefully guide future experimental approaches and help lead to solutions of such research challenges including a better understanding of responses under drought stress. This might improve plant defense response mechanisms against reactive oxygen species, as this represents an important goal plant tolerance to drought stress.

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CHAPTER 3

"Age-related mechanism and its relationship with secondary metabolism and abscisic acid in *Aristotelia chilensis* (Mol.) plants subjected to drought stress"

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Age-related mechanism and its relationship with secondary metabolism and abscisic acid in *Aristotelia chilensis* plants subjected to drought stress

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Abstract

Drought is the most important stress factor for plants, being the main cause of agricultural crop loss in the world. Plants have developed complex mechanisms for preventing water loss and oxidative stress such as synthesis of abscisic acid (ABA) and non-enzymatic antioxidant compounds such as anthocyanins, which might help plants to cope with abiotic stress as antioxidants and for scavenging reactive oxygen species. *A. chilensis* (Mol.) is a pioneer species, colonizing and growing on stressed and disturbed environments. In this research, an integrated analysis of secondary metabolism in *Aristotelia chilensis* was done to relate ABA effects on anthocyanins biosynthesis, by comparing between young and fully-expanded leaves under drought stress. Plants were subjected to drought stress for 20 days, and physiological, biochemical, and molecular analyses were performed. The relative growth rate and plant water status were reduced in stressed plants, with young leaves significantly more affected than fully-expanded leaves beginning from the 5^{th} day of drought stress. *A. chilensis* plants increased their ABA and total anthocyanin content and showed upregulation of gene expression when they were subjected to severe drought (day 20), with these effects being higher in fully-expanded leaves. Multivariate analysis indicated a significant positive correlation between transcript levels for NCED (9-cis-epoxycarotenoid dioxygenase) and UFGT (UDP glucose: flavonoid-3-O-glucosyltransferase) with ABA and total anthocyanin, respectively. Thus, this research provides a more comprehensive analysis of the mechanisms that allow plants to cope with drought stress. This is highlighted by the differences between young and fully-expended leaves, showing different sensibility to stress due to their ability to synthesize anthocyanins. In addition, this ability to synthesize different and high amounts of anthocyanins could be

related to higher *NCED1* and *MYB* expression and ABA levels, enhancing drought stress tolerance.

Keywords: anthocyanins; fully-expanded leaves; maqui; phytohormone; water stress; young leaves

3.1 Introduction

Drought stress is the main cause of loss in production of agricultural crops in the world, reducing yields by more than 50% (Boyer, 1982; Pessarakli, 2010). Water stress can limit photosynthesis, plant growth, and can even cause the death of plants (Raven, 1984; Moreno, 2009). Thus, drought is considered the most important stress factor for plants. Plants have developed complex mechanisms for preventing water loss and counteracting oxidative damage, such as stomatal closure, synthesis of abscisic acid (ABA), and non-enzymatic antioxidant compounds (Zhang et al., 2001).

It has been well established that ABA plays an important role in controlling plant water balance by stomatal closure during drought stress (Finkelstein, 2013). ABA biosynthesis involves many steps, however, it has been demonstrated that drought stress increases *9-cis-epoxycarotenoid dioxygenase* (*NCED*) gene expression, which encodes an enzyme in the ABA biosynthesis pathway, considered a key regulatory step during drought stress (Tuteja et al., 2007; Maruyama et al., 2014; Trivedi et al., 2016). Higher *NCED* expression has been associated with increases in ABA concentration in plant organs such as fruits and leaves of different species (Luchi et al., 2001; Zhang et al., 2009; Finkelstein, 2013). At the cellular level, ABA binds to the ABA-receptors, increasing ROS and cytosolic calcium (Ca^{2+}) in guard cells. Both these components modulate ion channels, decreasing guard cell turgor and closing the stomata (Finkelstein, 2013; Singh et al., 2017). It has been suggested that ABA can be involved in regulation of anthocyanin synthesis; however, the molecular mechanisms for possible regulation have not yet been elucidated (Jiang and Joyce, 2003; Deluc et al., 2009; Gagné et al., 2011; Kondo et al., 2014; Murcia et al., 2017). It has been reported that drought stress induces anthocyanin accumulation due

to the up-regulation of key genes from the anthocyanin pathway such as *dihydroflavonol 4-reductase* (*DFR*), *UDP-glucose:flavonoid 3-O-glucosyl transferase* (*UFGT*) and transcription factors such as *Myeloblastosis A1* (*MybA1*) (André et al., 2009; Borsani et al., 2010; Castellarin et al., 2007a; Castellarin et al., 2007b; Santesteban et al., 2011). Anthocyanins might help plants to cope with abiotic stress as antioxidants and for scavenging reactive oxygen species (ROS), thus increasing drought stress tolerance (Agati et al., 2012; Nakabayashi et al., 2014; Sperdouli and Moustakas, 2014; Kovinich et al., 2015; Li et al., 2017). Some studies have reported that young and fully-expanded leaves of several species have differences in secondary metabolites and ABA content in response to abiotic stresses such as UV-B, low nitrogen supply, and salinity (Zdunek and Lips, 2001; Reifenrath and Müller, 2007; Ibañez et al., 2008; Chen et al., 2013). However, the effect of drought stress on the biosynthesis of secondary metabolites and ABA separately has primarily focused in fully expanded leaves (Tattini et al., 2004: Yuan et al., 2012: Ma et al., 2014; Griesser et al., 2015). Thus, information is still limited regarding an integrated analysis of secondary metabolism related to ABA focused on anthocyanins biosynthesis during leaf development that is comparing young to fully-expanded leaves under drought stress.

Aristotelia chilensis (Mol.), also known as Maqui, is an endemic berry in Chile belonging to the Elaeocarpaceae family (Hoffman et al., 2005). Maqui is an evergreen tree distributed from Illapel (Coquimbo Region) to Chiloe (Los Lagos Region) (Hoffman et al., 2005). *A. chilensis* is a pioneer species, colonizing and growing on stressed and disturbed environments, thus being an interesting model for studying abiotic stress resistance mechanisms (Hoffman et al., 2005; Fredes et al., 2012). Maqui has been of great interest for

farmers and consumers due to its antioxidant action with high anthocyanin concentrations (Fredes et al., 2014). This interest has led to the development of morpho-phenological, physiological, and genetic diversity studies to establish agronomic parameters and the development of strategies of selection and breeding (Fredes et al., 2014; Vogel et al., 2014, Bastías et al., 2016). Consequently, in this study, we investigated the effects of drought stress on anthocyanin biosynthesis and endogenous ABA levels in young and fully-expanded leaves of *A. chilensis*.

3.2 Materials and methods

3.2.1 Plant materials and experimental conditions

Plants of maqui (*Aristotelia chilensis*) obtained from *in vitro* conditions and donated by BestPlant Co. (Curico, Chile) were used in this study. One-year-old plants were transplanted to 2 L pots filled with Andisol soil and acclimated in a greenhouse (temperature: 25±3 ºC; photoperiod: light 16 h/8 h dark; humidity: 60-70%; and a mean photosynthetic active radiation (PAR) at midday of 300 µmol m^{-2} s^{-1}) for 2 weeks. Plants were then divided into two groups (20 plants for each group); daily irrigated (DI) and non-irrigated (NI). The DI plants were irrigated daily at field capacity; meanwhile, NI plants were exposed to water withholding to initiate drought stress. The experiment was carried out for 20 days. At different time points (0, 5, 10 and 20 days) of the experiment, leaf samples were collected in the morning at two different positions from the plants for physiological, biochemical and molecular analysis. The two different positions represented different leaf ages: young leaves, from middle to the top; and fully-expanded leaves, from

middle to basal leaves. Leaves were frozen in liquid nitrogen and stored at -80 ºC prior to determination of the biochemical parameters.

3.2.2 Plant growth measurement

Relative growth rate (RGR) was determined according to Hoffmann and Poorter (2002), as the mean natural logarithm-transformed dry weight (DW) at the beginning and the end of the experiment, where t1 and t2 are the times 0 and 20 days, respectively. RGR was calculated by Formula 1.

Formula 1:

$$RGR = [(\ln DW2) - (\ln DW1)/(t2 - t1)]$$

3.2.3 Plant water status

Relative water content (RWC) was determined by the method described by Rahimi et al. (2010). Two leaves were removed, weighed and immersed into double distilled water to saturate them with water for the next 24 h at 4 ºC and dark conditions. Then, leaves were oven dried to a constant weight at 60 °C. Next, the dry weights were determined. The RWC was calculated according Formula 2 (below). Leaf water potential (Ψ_{md}) was measured using a Scholander chamber Plant Moisture Stress (Model 1000, Instrument Co., Corvallis, Ore.) in the morning, following the protocol proposed by Matthews et al. (1987).

Formula 2:

$$RWC = [(\text{fresh weight} - \text{dry weight})/(\text{turgor weight} - \text{dry weight})] \times 100$$

3.2.4 Endogenous ABA determination

Endogenous ABA was quantified by the isotope dilution method, essentially as described by Liu et al. (2012) for auxin analysis, using NH_2 resin solid phase extraction (SPE) TopTip minicolumns. After methylation by diazomethane, the samples were then injected into a gas chromatograph (GC) coupled to single quadrupole mass spectrometer (MS) (GC-MS-SIM, Agilent 6890N GC System with an Agilent 7683 Automatic Liquid Sampler and an Agilent 5973 MS; column, temperatures, carrier gas and other analysis conditions were exactly as described in Liu et al. 2012) and the samples were analysed using selected ion monitoring (SIM) with Agilent Chemstation software. Deuterated-abscisic acid ([$^2H_6$]ABA) was used as internal standard (Liu et al., 2012), and it was synthesized according to Dobrev et al. (2005) yielding a product with no detectable unlabeled ABA and a major predominate ion at *m/z* 194 for the [$^2H_4$]ABA isotopomer. Endogenous ABA concentration was thus determined from the ion abundance at the base peak of each compound: the *m/z* value of 190 for plant ABA, and the *m/z* value of 194 for [$^2H_4$]ABA using the isotope dilution equation which accounts for the isotopomer distribution in the internal standard (Liu et al. 2012).

3.2.5 Lipid peroxidation

Lipid peroxidation (LP) was measured based on the formation of thiobarbituric acid-reactive substances (TBARS) according to the modified method of Du and Bramalage (1992). Absorbance was measured spectrophotometrically at 440, 532 and 600 nm (UV/VIS Unico SpectroQuest 2800) in order to correct the interference generated by

TBARS-sugars complexes. The TBARS content was expressed as nmol of malondialdehyde (MDA) per gram of dry weight (nmol MDA g^{-1} DW).

3.2.6 Antioxidant activity determination

Antioxidant activity was determined in leaves by the DPPH (2.2-diphenyl-1-picryl-hydrazyl) method described by Chinnici et al. (2004). Absorbance was measured to 515 nm (UV/VIS Unico SpectroQuest 2800). Antioxidant activity was expressed as mg of Trolox equivalent per gram of dry weight (mg TE^{-1} DW).

3.2.7 Determination of total phenols

The Folin-Ciocalteau method was used to determine total phenols (TP) (Singleton and Rossi, 1965). Absorbance was measured spectrophotometrically at 765 nm (UV/VIS Unico SpectroQuest 2800) using caffeic acid as standard.

3.2.8 Total and profile of anthocyanins

Total anthocyanins (TA) were determined as previously described by Strack and Wray (1989) by the pH differential method. Absorbance was measured at 530 and 675 nm (UV/VIS Unico SpectroQuest 2800). Total content of anthocyanins was expressed as mg of cyanidin-glucoside equivalent (C3G) per gram of dry weight. To determine the anthocyanin profile, the protocol for anthocyanidin determination was used as described by Ribera et al. (2010). Determinations were performed using a High Performance Liquid Chromatography (HPLC) system (Jasco LC-Net IIADC) equipped with a photodiode array detector (DAD) (Jasco MD 2015 Plus) and separations were done on a Kromasil Reversed-Phase (RP-18) C_{18} column (250 x 4.6 mm).

3.2.9 Total RNA isolation and cDNA synthesis

Total RNA was isolated from 200 mg of leaves by the method describe by Jaakola et al. (2001). RNA concentrations were measured spectrophotometrically using a Spectral Scanning Multimode Reader Varioskan Flash µDrop™ Plate (Thermo Scientific, Wilmington, USA). Likewise, RNA purity was determined using the A260/A280 and A260/A230 ratios. RNA quality was also evaluated visually through gel electrophoresis of the denatured RNA. First-strand cDNA was synthesized from 2 µg of total RNA from *A. chilensis* leaves, which was reverse-transcribed by M-MLV (Promega, MA, USA) following the manufacturer`s recommendations. To remove genomic DNA, the cDNA was cleaned according to Jaakola et al. (2004) using a DNA gel extraction kit (Millipore Corporation, Bedford, MA, USA).

3.2.10 Real-time quantitative PCR (qRT-PCR) analysis

Quantitative real-time (qRT-PCR) reactions were conducted in order to determine the expression patterns of *AcNCED1* and *AcUFGT* in *A. chilensis* leaves. All qRT-PCR reactions were performed using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, California) in an ABI 7300 Real-Time PCR system (Applied Biosystems, Foster City CA, USA) using the procedure described by Inostroza-Blancheteau et al. (2014). *NCED* and *Elongation Factor 1 alpha* (*EF1a)* sequences of *Vitis vininfera*, *Populus euphratica*, and *Prunus persica* were obtained from Genbank®. Sequence alignments were done using the Clustal Omega program (www.ebi.ac.uk) and primers were design using AmplifX 1.7.0. Transcripts were sequenced and confirmed in Genbank®. Finally, specific primers were design based on the sequences in AmplifX1.7.0. *Aristotelia*

chilensis UFGT primers were kindly providing by Dr. Victor Polanco from Universidad Mayor, Chile. The specific primers used in this study are shown in Table 1, which amplified 180 bp fragments. *EF1a* is a stably expressed gene that was used as the internal control. All the experiments were performed using three biological replicates. Cycling conditions were 95 ºC for 10 min, followed by 40 cycles at 94 ºC for 30 s, 60 ºC for 30 s, and 72 ºC for 30 s. Gene expression data (Ct values) were employed to quantify relative gene expression using the comparative $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001).

Table 1. Primer sequences used for quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of NCED1 and UFGT genes. The *EF1a* was used as an internal control.

Gene	Forward primer (5` to 3`)	Reverse primer (5` to 3`)
NCED1	AAA GAC CCG GTT CGC GTA CT	TCT GAA TTG GGG TCT CTG GGA A
UFGT	TTC CAG GAA TGT CTC AAG TA	CAA AGG AGT TTA TGA AGA CT
EF1a	CTC CTG GGC ATC GTG ACT TT	CCA AGG GTG AAA GCA AGC AA

3.2.11 Experimental design and data analysis

A complete randomized design was used with five replicates for each treatment and time. The results are expressed as mean and standard error of the mean (±SE) for each treatment. All data passed the normality and equal variance Kolmogorov-Smirnov tests. Means were analyzed using a three-way ANOVA, where the factors were time, leaf age and treatment. The Tukey multiple comparison test at $p \leq 0.05$ was used. Sigma Stat 3.5 (SYSTAT

Software Inc.) was used to performed the statistical analysis. Relationships among variables were examined using Pearson correlation analysis at a significance level of $P < 0.05$. The resulting p-values were corrected using one R script displayed by the Rbio software (www.biometria.ufv.br). A Principal Component Analysis was performed to reduce the dimensionality of the data set and identify the variables that explained a higher proportion of the total variance (Minitab® 17 statistics program, Minitab Inc., Philadelphia).

3.3 Results

3.3.1 Growth and plant water status during drought stress

After 20 days under water limiting conditions it was observed that the RGR was strongly affected by drought stress, where stressed (NI) plants displayed a reduction of 71% in the growth rate compared to well-watered (DI) plants at the end of the experiment (Fig. 1). A 42% RGR reduction was observed after 10 days of drought treatment, reaching its lowest growth on the 20th day. When plants were subjected to severe drought stress, leaf water potential (Ψ_w) decreased significantly through the experiment, where young leaves were significantly more affected than fully-expanded leaves from 5th day of drought stress (Fig. 2A). In this parameter, young leaves of stressed plants decreased their Ψ_w around 6-fold with respect to control plants at the end of the experiment; meanwhile, fully-expanded leaves of NI plants reduced their Ψ_w around 5-fold regarding to fully-expanded leaves of DI plants at the same time (Fig. 2A). Concerning RWC, DI and NI plants maintained their RWC values during the first days of the experiment, decreasing significantly from 10th day.

Young and fully-expanded leaves of NI plants showed a decrease of about 40-45% in their RWC compared to their control plants at the end of the experiment (Fig. 2B).

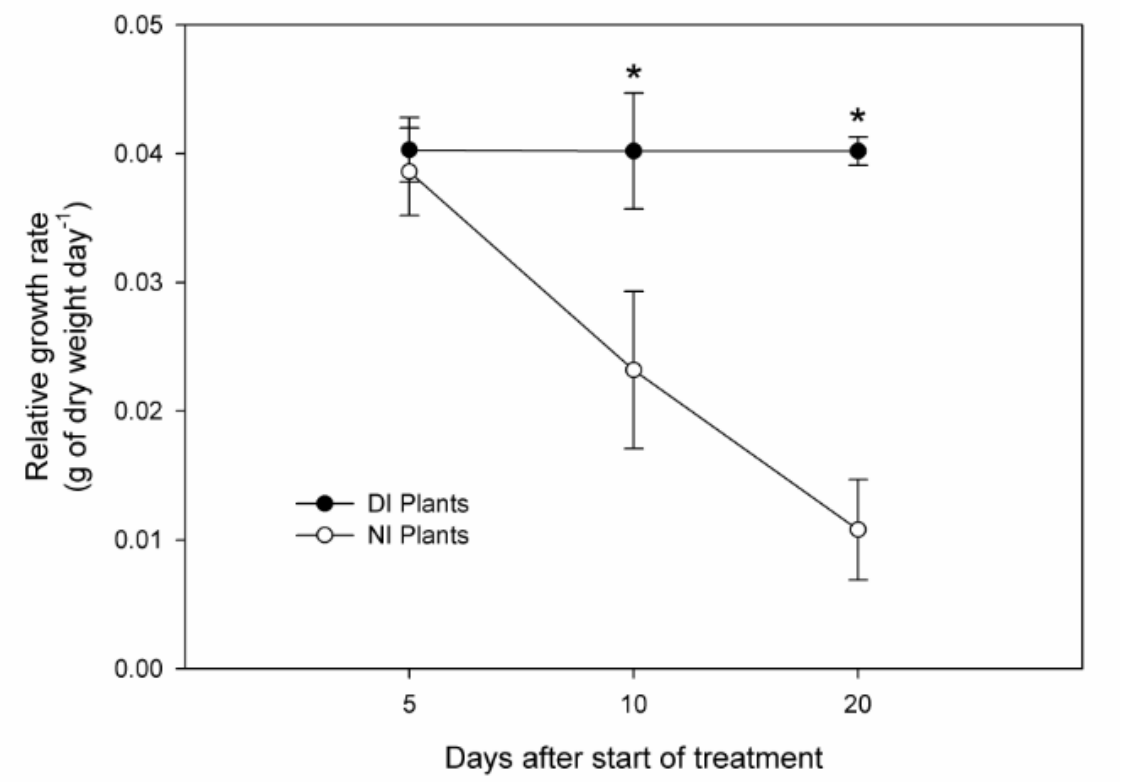

Figure 1. Relative growth rates of *Aristotelia chilensis* plants grown under two water treatments; Daily-irrigated (DI) and Non-irrigated (NI). All values represent averages of three biological replicates ±SE. *Asterisks* indicate significant differences between treatments for the same day ($P \leq 0.05$).

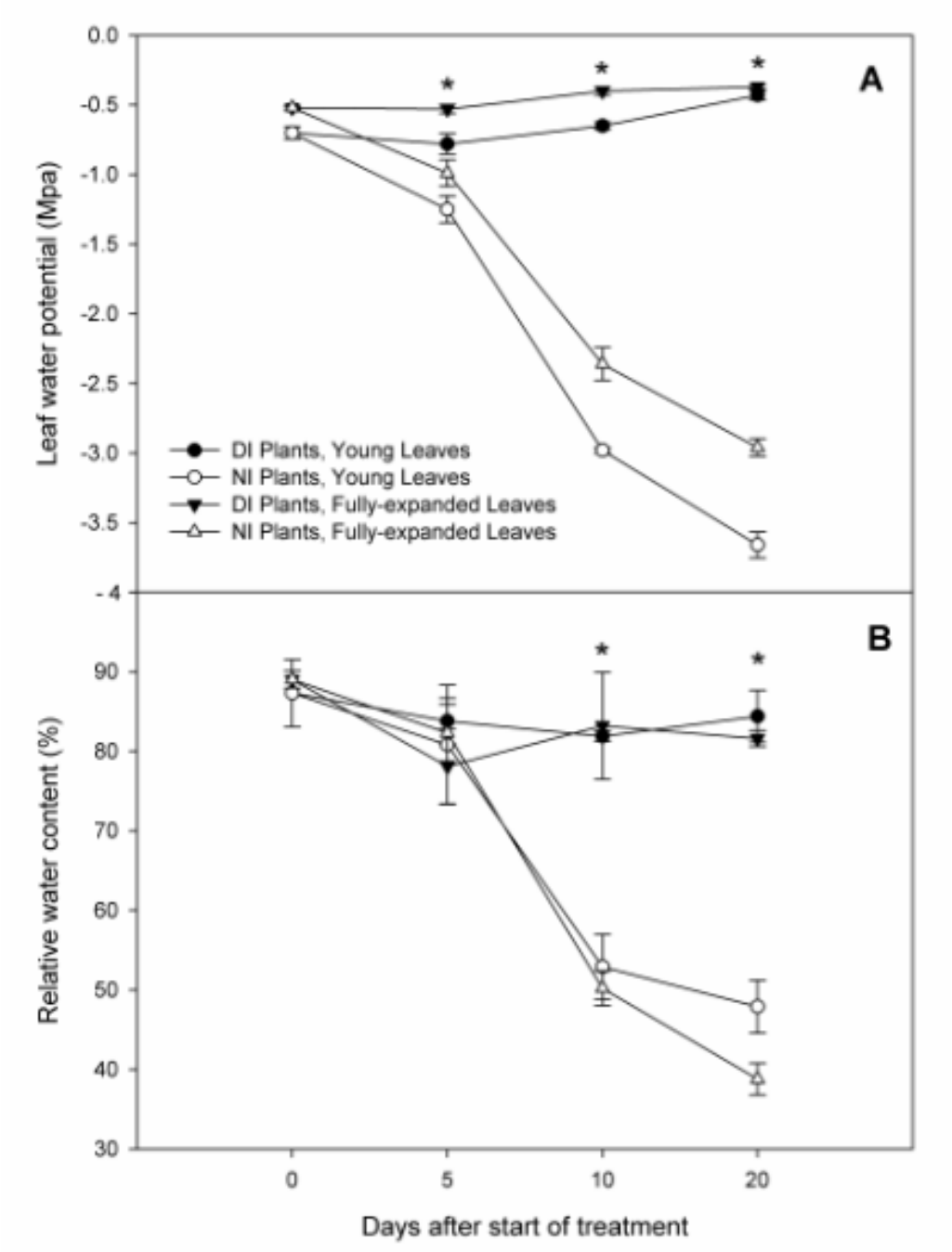

Figure 2. Plant water status; (A) Leaf water potential and (B) Relative water content in young and fully-expanded leaves of *Aristotelia chilensis* plants grown under two water treatments; Daily-irrigated (DI) and Non-irrigated (NI). All values represent averages of

three biological replicates ±SE. *Asterisks* indicate significant differences between treatments for the same day and leaf age ($P \leq 0.05$).

3.3.2 ABA levels under drought stress

Throughout the experiment period, significant differences ($p \leq 0.05$) were observed in the endogenous ABA levels in leaves between NI and DI plants from 5th day, after withholding (Fig. 3). When plants were subjected to severe drought stress (day 20), ABA levels of young and fully-expanded leaves increased significantly to reach around 6-fold with respect to control plants. Meanwhile, young and fully-expanded leaves of well-watered plants maintained their endogenous ABA levels relatively constant around 2.3 μg g^{-1} DW. When we compared young and fully-expanded leaves of NI plants, we observed significant differences between their ABA levels, being higher in fully-expanded leaves (about 20%) than in young leaves (Fig. 3).

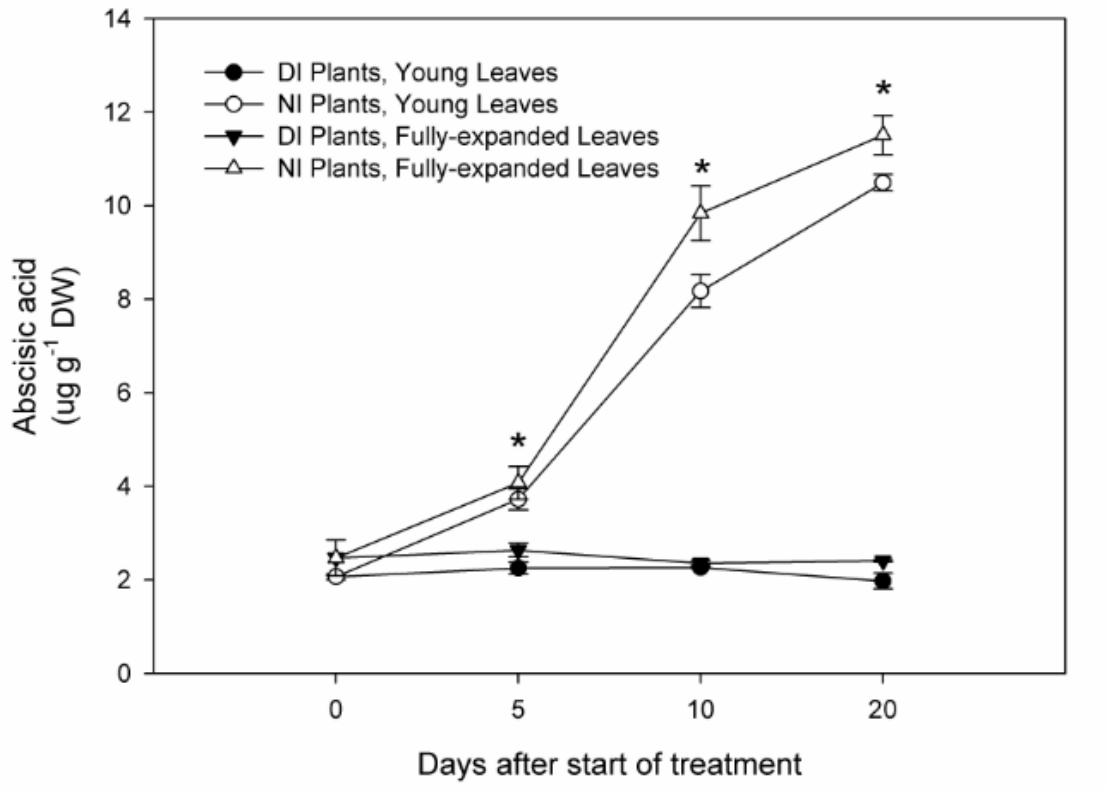

Figure 3. Endogenous abscisic acid in young and fully-expanded leaves of *Aristotelia chilensis* plants grown under two water treatments; Daily-irrigated (DI) and Non-irrigated (NI). All values represent averages of three biological replicates ±SE. *Asterisks* indicate significant differences between treatments for the same day and leaf age ($P \leq 0.05$).

3.3.3 Lipid peroxidation

Lipid peroxidation (LP) in leaves of stressed plants, including young and fully-expanded leaves, showed a significant increase throughout the experiment (Fig. 4). Young and fully-expanded leaves increased their LP levels about 50% on the 20th day of drought stress, with young leaves affected earlier than fully-expanded leaves, showing an increase in their LP levels from the 5th day (Fig. 4).

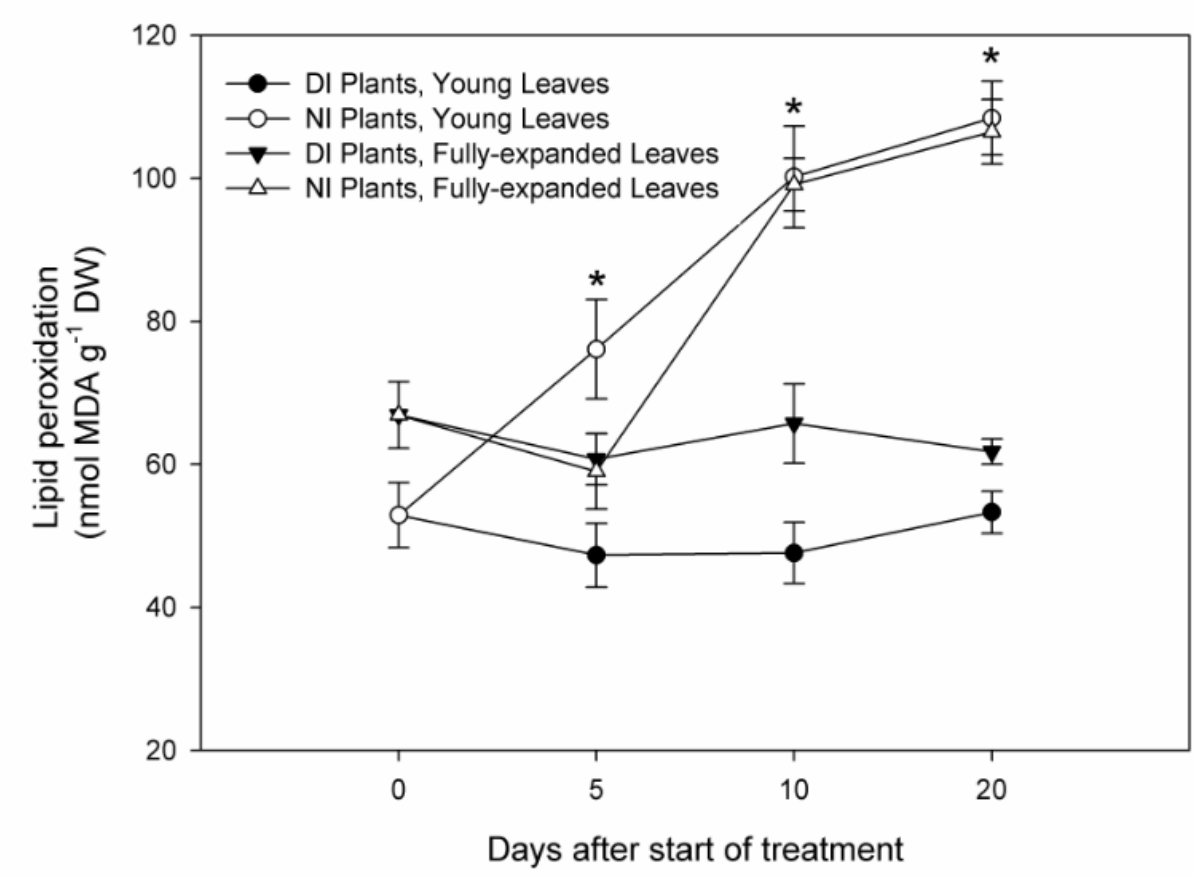

Figure 4. Lipid peroxidation in young and fully-expanded leaves of *Aristotelia chilensis* plants grown under two water treatments; Daily-irrigated (DI) and Non-irrigated (NI). All values represent averages of three biological replicates ±SE. *Asterisks* indicate significant differences between treatments for the same day and leaf age ($P \leq 0.05$).

3.3.4 Antioxidant activity and total phenols

The antioxidant activity **(**AA) only showed statistically significant differences in young leaves from the 10th day of drought stress ($p \leq 0.05$), where it increased about 35% with respect to control leaves of the same leaf age (Fig. 5A). In contrast, fully-expanded leaves of stressed plants only showed an increase at the end of the experiment. An increase of

about 22% was found in total phenols of young and fully-expanded leaves from the 10th day of drought stress (Fig. 5B).

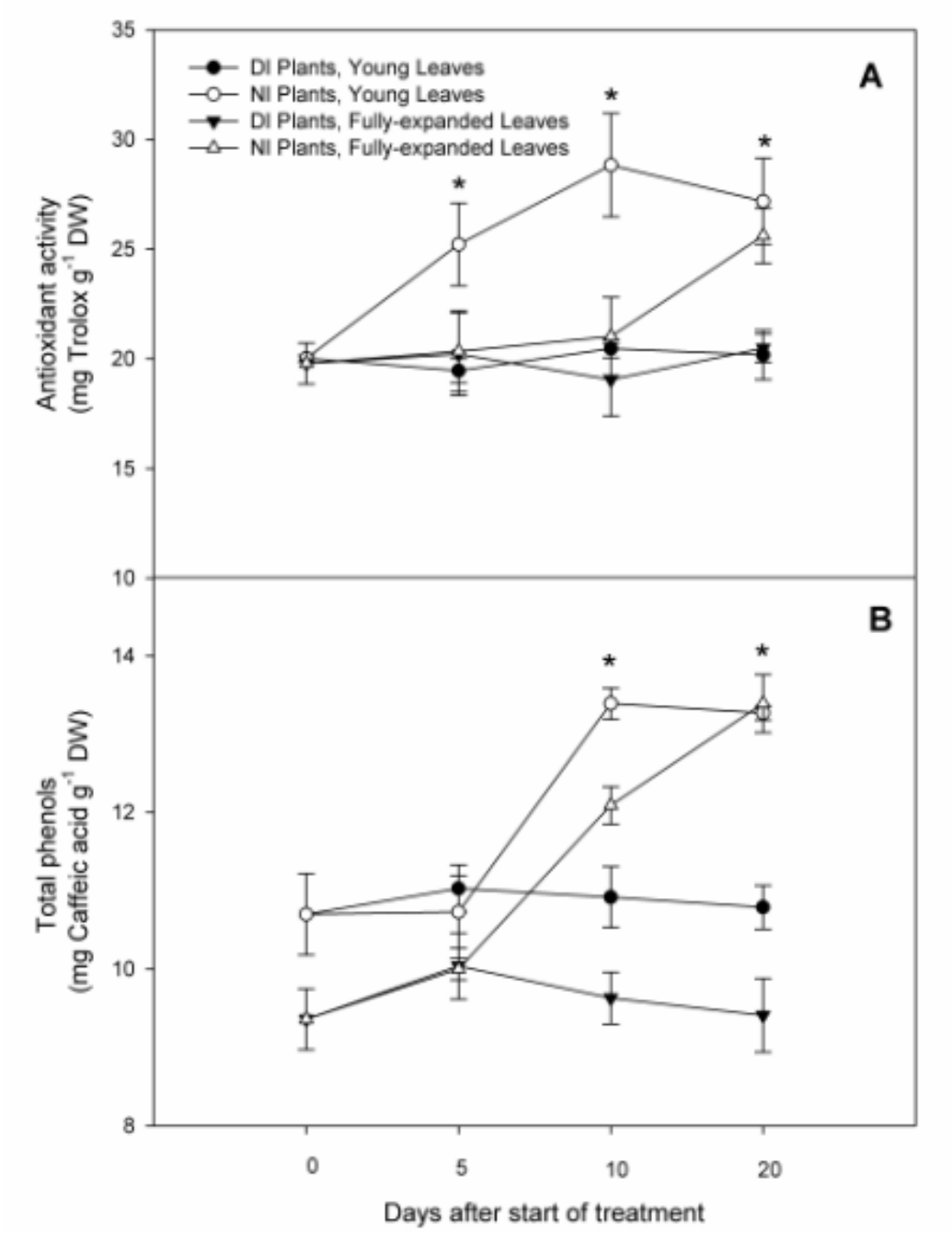

Figure 5. Antioxidant activity (A) and total phenols (B) in young and fully-expanded leaves of *Aristotelia chilensis* plants grown under two water treatments; Daily-irrigated

(DI) and Non-irrigated (NI). All values represent averages of three biological replicates ±SE. *Asterisks* indicate significant differences between treatments for the same day and leaf age ($P \leq 0.05$).

3.3.5 Total anthocyanin content and profile

The total anthocyanin (TA) content increased in young and fully-expanded leaves of stressed plants from the 10th day of the experiment with respect to their well-watered plant leaves. Fully-expanded leaves of NI plants had a significantly ($p \leq 0.05$) higher TA content compared to young leaves of the same plants (Fig. 6). The TA content was higher in fully-expanded leaves at day 20 of drought stress, where it had increased 7-fold compared to control plant leaves of the same leaf age. Meanwhile, young leaves of NI plants increased their TA content 2-fold compared to DI plants. Moreover, the anthocyanidin profile was different throughout the experiment. HPLC analysis showed that cyanidin was present in both leaf types in control and stressed plants during the experiment. However, cyanidin was increasing from the 10th day in young leaves and from the 5th day in fully-expanded leaves of stressed plants, remaining constant in control plants throughout the experiment (Fig. 7b). The highest cyanidin increment was found at the 20th day of the experiment, where cyanidin increased 17-fold in young leaves of NI plants compared to their DI plants. On the other hand, we detected malvidin only in fully-expanded leaves from the 5th day of the experiment. However, there was a significant increase (30-fold) in fully-expanded leaves compared to well-watered plants at the end of the experiment (Day 20). Interestingly, delphinidin was only detected in fully-expanded leaves of stressed plants at the 20th day of the experiment, when plants were subjected to severe drought stress (Fig. 7a)

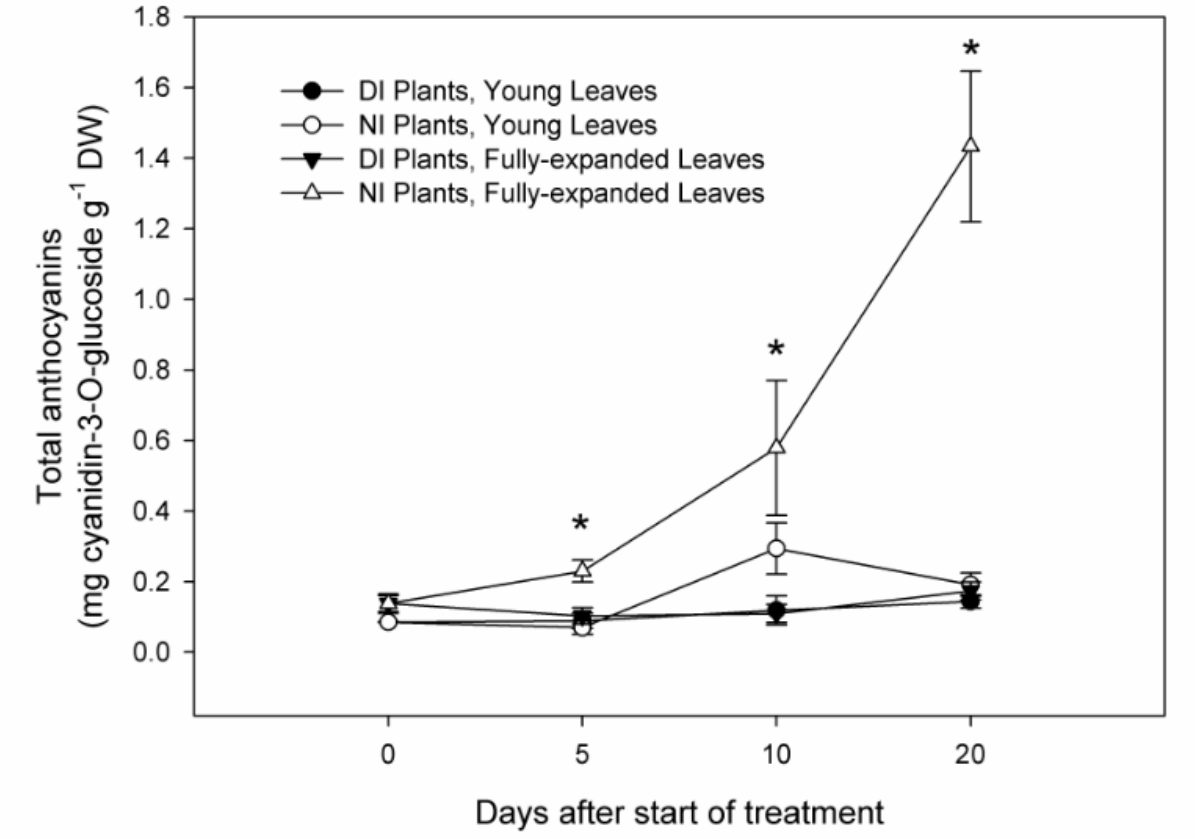

Figure 6. Total anthocyanins in young and fully-expanded leaves of *Aristotelia chilensis* plants grown under two water treatments; Daily-irrigated (DI) and Non-irrigated (NI). All values represent averages of three biological replicates ±SE. *Asterisks* indicate significant differences between treatments for the same day and leaf age ($P \leq 0.05$).

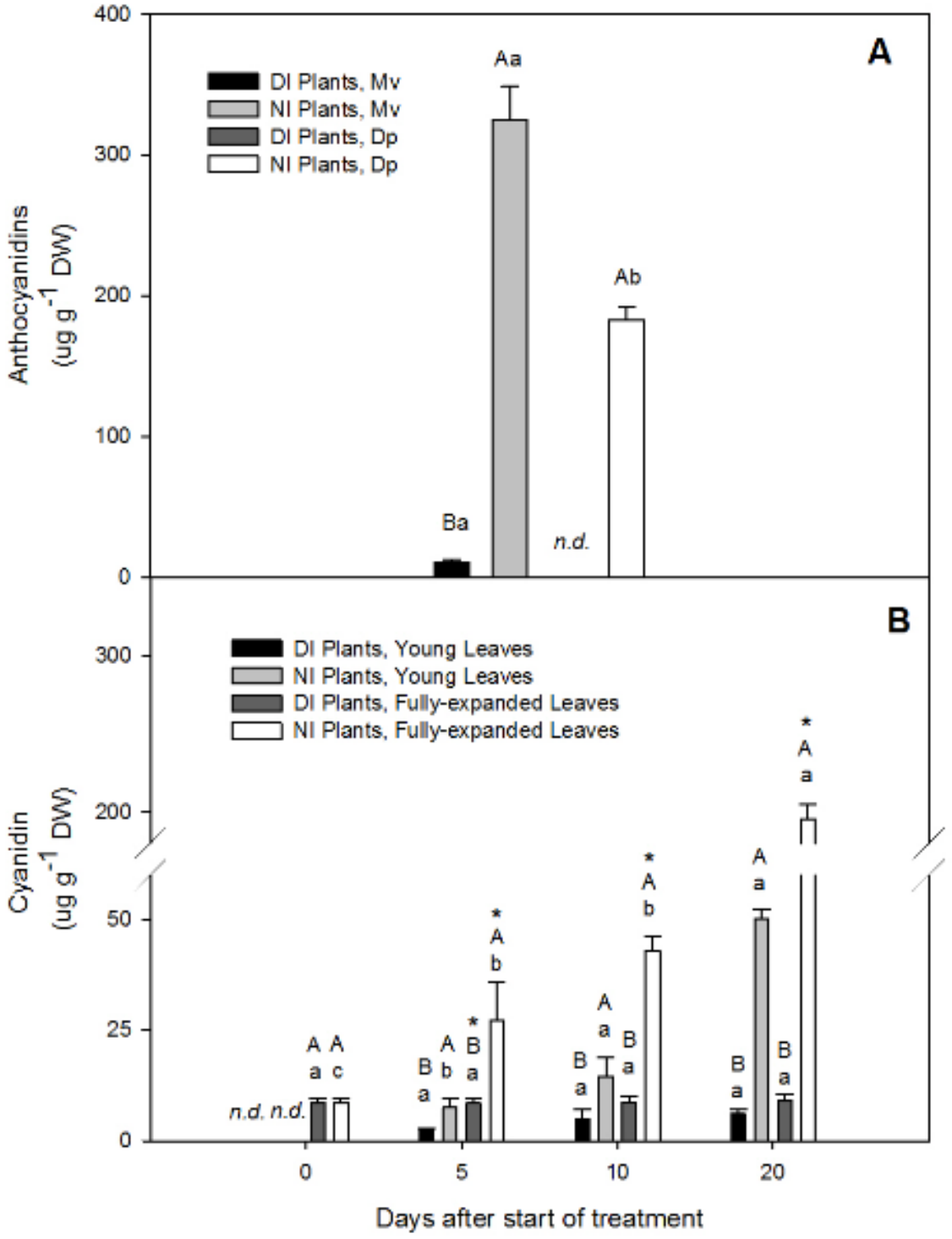

Figure 7. Anthocyanidins (A) of fully-expanded leaves, and cyanidin (B) obtained by HPLC in young and fully-expanded leaves of *Aristotelia chilensis* plants grown under two

water treatments; Daily-irrigated (DI) and Non-irrigated (NI). Mv=Malvidin. Dp=Delphinidin. n.d.=non-detected. All values represent averages of three biological replicates ±SE. Different upper case letters indicate significant differences between treatments for the same day and leaf age, different lower case letters among days for the same treatment and leaf age, and asterisks between leaf age for the same treatment and day ($P \leq 0.05$).

3.3.6 Gene expression analysis under drought stress

The expression *NCED1* and *UFGT*, involved in ABA and anthocyanin biosynthesis pathways, respectively, was investigated in *A. chilensis* in response to drought stress by qRT-PCR. The *NCED1* gene showed a basal expression during the first days of the experiment (until the 5th day) in young and fully-expanded leaves of stressed plants (Fig. 8A). After that, *NCED1* expression was enhanced 8-fold at the 10th day of the experiment likely as a consequence of intensified drought stress severity. *NCED1* reached the highest expression at the 20th day of the experiment, when drought stress was more severe. Meanwhile, control plants remained constant in their NCED1 expression level throughout the experiment. We observed that fully-expanded leaves of stressed plants had higher *NCED1* expression during the experiment as compared to young leaves. On the other hand, *UFGT* expression increased significantly (2-fold) in fully-expanded leaves of stressed plants from the 10th day of drought stress with respect to the control (Fig. 8B). Fully expanded leaves of stressed plants showed the highest *UFGT* expression (5-fold) at the 20th day of drought stress with respect to control leaves. Meanwhile, *UFGT* expression in young

leaves of stressed plants did not change significantly during the experiment, with exception being at the 20th day where a slight increase was observed (Fig. 8B).

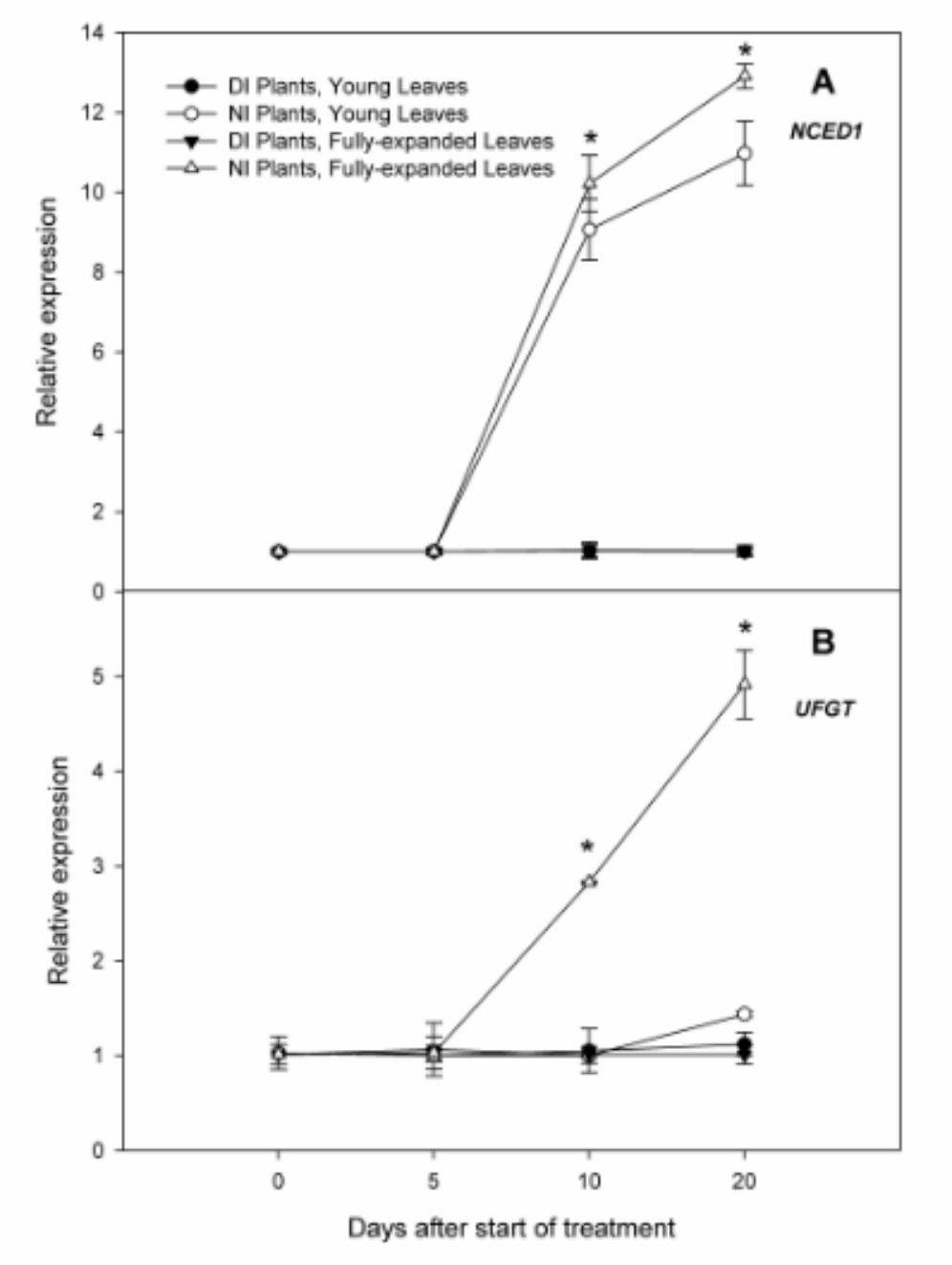

Figure 8. qRT-PCR analysis of *NCED1* (A) and *UFGT* (B) mRNA levels in young and fully-expanded leaves of *Aristotelia chilensis* plants grown under two water treatments;

Daily-irrigated (DI) and Non-irrigated (NI). Three independent biological replicates ±SE were used for this study. All data were normalized to geometric mean value from *AcEF1a* internal control. *Asterisks* indicate significant differences between treatments for the same day and leaf age ($P \leq 0.05$).

3.3.7 Correlation analysis of all traits measured

Pearson correlation analysis was performed to determine the level of association between traits measured in *A. chilensis*. First, by using data only from control treatments in the correlation analysis, we did not find any significant correlation between the variable measured (data not shown). Nevertheless, when Pearson correlation analysis was performed with all traits measured from drought stressed plants 14 significant correlations ($P < 0.05$) were observed. The correlation coefficients are presented in a correlation matrix (Fig. 9). Among the significant correlations, we found seven strong positive correlations between transcript levels of NCED and UFGT and ABA levels ($r = 0.98$) and total anthocyanins ($r = 0.97$), respectively. By contrast, we found that plant water status variables (Ψ_w and RWC) were negatively correlated with most of the metabolite and transcript data sets (Fig. 9).

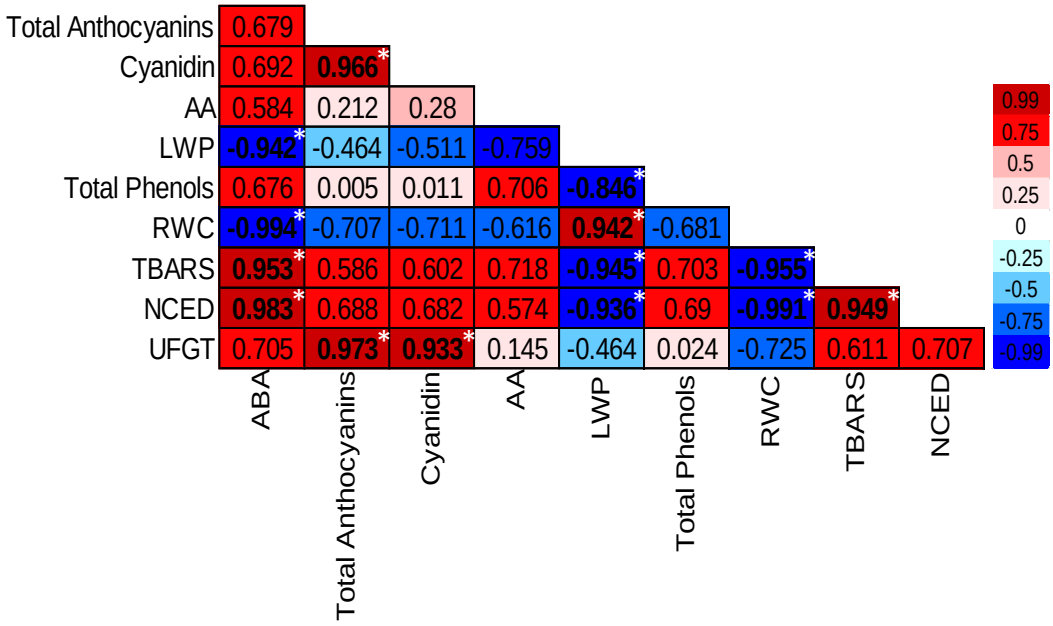

Figure 9. Correlation matrix based on Pearson coefficients derived from physiological, metabolic and transcript data from *Aristotelia chilensis* in young and fully-expanded leaves grown under Non-irrigated (NI) treatment for 20 days. Correlation coefficients are presented in colors, and the significant ones are indicated in bold (P). In addition, the asterisk represents significances based on p-value corrected by FDR correction (Bonferroni-Hochberg). Abbreviations: Abscisic acid (ABA), total anthocyanins (TA), cyanidin (Cy), antioxidant activity (AA), leaf water potential (LWP), total phenols (TP), relative water content (RWC), Lipid peroxidation (TBARS), 9-cis-epoxycarotenoid dioxygenase (NCED), UDP-glucose:flavonoid 3-O-glucosyl transferase (UFGT).

3.3.8 Principal component analysis

All measured variables were used to perform the analysis of the principal components. Through the PCA results, it was possible to observe a clear influence of the drought stress

modulating some parameters of the stressed plants. Interestingly, all the control treatments remained in the same group. The first component (PC1) explained 75.8% of the variation and the second component (PC2) only 17.2%, which shows that PC2 did not distinguish the plants under control and drought stress. This result turned our attention to the separation explained by PC1 (Fig. 10A). We observed that the analysis of principal components separated three groups, whose were also confirmed by Euclidean distance. Group I, includes all control plants and plants subjected to drought stress for 5 days; group II, includes the plants (young and fully-expanded leaves) with 10 days under drought stress and the plants that went through 20 days of stress; and group III composed only by fully-expanded leaves of drought stressed plants at 20^{th} day. When we analyzed the variables that contributed to the separation of the groups, it was verified that the grouping of control plants together with those treated for 0 and 5 days (group I) was separated mainly by RWC and LWP variables. However, the plants that remained under stress for 10 and 20 days and were clustered with groups II and III, were separated by AA, TBARS, total phenols, ABA, *NCED* transcript levels, total anthocyanins, cyanidin and *UFGT* transcript levels. However, the fully-expanded leaves of plants that remained in the stress for 20 days were more influenced by total anthocyanin, cyanidin and *UFGT* expression levels (Fig.10B).

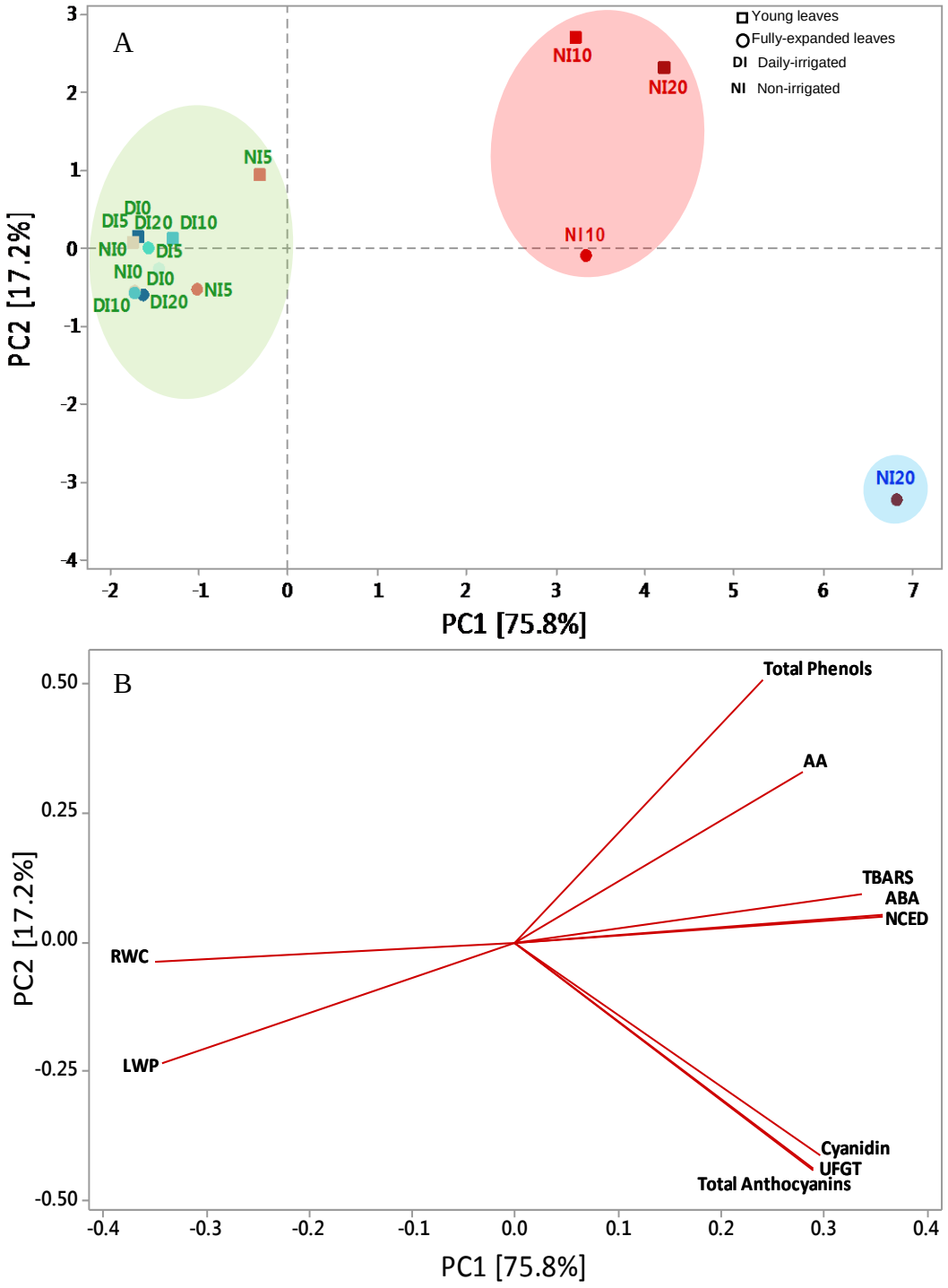

Figure 10. Principal component analysis. (A) Score plot derived data of young (square) and fully-expanded leaves (circle) from *Aristotelia chilensis*, grown under daily-irrigated (C;

blue gradient), and Non-irrigated treatments in different days [0 days (T0), 5 days (T5), 10 days (T10) 20 days (T20); red gradient]. The large circles represent the three clusters formed by the Euclidean distance method. (B) In Loading plot the direction and length of the lines are directly proportional to variables importance in separating groups. PC1, principal component 1; PC2, principal component 2. Abbreviations: Relative water content (RWC), leaf water potential (LWP), antioxidant activity (AA), abscisic acid (ABA), Lipid peroxidation (TBARS), 9-cis-epoxycarotenoid dioxygenase (NCED), UDP-glucose:flavonoid 3-O-glucosyl transferase (UFGT).

3.4 Discussion

Climate change is predicted to exacerbate water limitation in several areas around the world, affecting crop production. In this sense, several studies have shown the negative effects of drought stress on physiology, metabolism and plant growth in different species such as *Phaseolus vulgaris, Glycine max, Arabidopsis thaliana, Beta maritima, Pistacia lentiscus and Lavatera maritime* (Kramer, 1983; Miyashita et al. 2005; Ohashi et al. 2006; Galmés et al. 2007; Choat et al. 2012; Li et al. 2017). Ψ_w and RWC decrease by 30 to 40% in plants by moderate drought stress (Galmés et al., 2007), which in our conditions was observed at day 10 of water withholding. A larger decrease was observed at severe water stress in *A. chilensis* after 20 days under drought, reaching Ψ_w values between -3 and -5 MPa (Fig. 1, 2A-B). Young leaves of stressed plants showed a higher decrease in Ψ_w compared to fully-expanded leaves. In fact, in agreement with our results, Saito et al. (2007) reported that Ψ_w of young leaves was lower than fully-expanded leaves in *Quercus*

species. This can be attributed to that Ψ_w of young leaves must be lower than fully-expanded leaves to ensure water flow through the plant. However, higher Ψ_w of fully-expanded leaves (less negative) led us to consider that these leaves are more effective at closing stomata for maintaining turgor under drought stress (Patakas et al. 1997). It may be also possible that fully-expanded leaves have a higher capacity to synthesize ABA, which is the most important response mechanism involved in stomatal closure induced by drought stress (Dodd, 2005; Choudhary et al. 2012). In our experiment, ABA significantly increased in stressed plants, including young and fully-expanded leaves, reaching its maximum ABA level on the 20th day (Fig. 3). Such elevated ABA levels have been previously reported in *Brassica napus* and *Vitis vinifera* (Berli et al. 2010; Qaderi et al. 2012). Although, ABA biosynthesis involves many steps, the 9-cis-epoxycarotenoid dioxygenase (NCED) enzyme is considered a key regulatory step during ABA biosynthesis in drought stress, due to the observation that *NCED* expression is induced by drought stress before ABA is accumulated (Finkelstein, 2013). In agreement with previous studies (Zhang et al. 2009; Karppinen et al. 2013), *NCED1* expression was increased significantly in NI plants, concomitant with ABA concentration starting on day 10 of the experiment, when Ψ_w and RWC decreased from moderate to severe drought stress (Fig 3, 8A). In addition, *NCED1* expression and ABA concentration were positively correlated (r = 0.98) in our study (Fig. 9). *NCED* overexpression in transgenic *Arabidopsis* increased ABA levels, promoting downstream ABA-inducible genes, and increasing drought tolerance (Luchi et al. 2001). ABA modulates target gene expression by the ABA-responsive element (ABRE) binding protein/ABRE binding factor (ABRE/ABF) transcription factors (Singh and Laxmi, 2015). Thus, Yoshida et al. (2010) reported growth inhibition, and downregulation of LEA

class genes, which are proteins widely recognized to play crucial roles in drought stress tolerance, in *Arabidopsis* and *Oryza sativa* mutants deficient in AREB/ABF transcription factors (*abre1, abre2, and abf3*). Their results suggest that ABA plays an important role in drought stress tolerance activated by ABRE/ABF transcription factors. In our studies, fully-expanded leaves always had a slightly significant ($P \leq 0.05$) higher ABA concentration than young leaves throughout the experiment in stressed plants. Similar to our results, Chen et al. (2013) and Zdunek and Lips (2001) reported that *Triticum aestivum* and *Pisum sativum* fully-expanded leaves had 30% higher ABA concentrations than young leaves when plants were subjected to drought stress. Therefore, we can suggest that higher *NCED1* expression and the subsequent higher ABA concentration seems to contribute to drought stress tolerance, maintaining cell turgor (less negative Ψ_w) mainly in fully-expanded leaves.

When plants are subjected to drought stress, increases of reactive oxygen species (ROS) in different cellular compartments such as chloroplasts, peroxisomes, and mitochondria occur (Cruz de Carvalho, 2008; You and Chan, 2015). These ROS are highly reactive and cause damage to DNA, proteins, carbohydrates, and lipids, which results in oxidative stress (Gill and Tuteja, 2010). The LP was assayed as an index of oxidative stress in our experiment. The LP showed a significant increase in stressed plants including young and fully-expanded leaves (Fig. 4). However, at day 5, young leaves of drought stressed plants increased 60% their LP compared to fully-expanded leaves, which did not change their LP at that time (Fig. 4), indicating that young leaves were earlier affected by drought stress. Both leaf types increased their LP levels about 50% on the 20th day of drought stress compared to control plants. In agreement with our results, Cechin et al (2010) reported higher LP levels (about 30%) in young leaves compared to fully-expanded leaves of

Helianthus annuus subjected to drought stress during 6 days. In a previous study, higher LP levels in fully-expanded leaves could be attributed to the higher amount of chloroplasts compared to young leaves (Lepedus et al. 2011), suggesting that they are the main organelle generating ROS under drought stress, and LP levels are thus leaf age-dependent. Taken together, these findings suggest that fully-expanded leaves of stressed *A. chilensis* plants have a strong antioxidant mechanism to tolerate drought stress for longer time and to maintain lipid peroxidation at the same level as young leaves in our experiment. *A. chilensis* plants showed higher AA in young leaves starting on day 10 of drought stress, while total phenols increased in young and fully-expanded leaves from the 10th day of the experiment (Fig. 5B). Our finding did not differ from other reports, where higher AA and total phenols under drought stress have been reported previously in several species (Martins et al. 2016; Gharibi et al. 2016; Puente-Garza et al. 2017). However, total phenols seemed to be ineffective alone to alleviate LP produced by drought stress in fully-expanded leaves. Among phenolic compounds, anthocyanins are considered antioxidant compounds, which can donate electrons or generate protons, scavenging ROS (Zhang and Tsao, 2016). Thus, it has been reported that anthocyanin have a greater capacity to increase tolerance to abiotic stresses, including drought stress (Fini et al., 2012; Yuan et al., 2012; Nakabayashi et al. 2014; Li et al. 2017; Naing et al. 2017). In our study, TA increased significantly in stressed plants in response to drought stress (Fig. 6). Surprisingly, TA content was higher in fully-expanded leaves at the 20th day of drought stress compared to control plant leaves of the same age, while tri-hydroxylated anthocyanin such as malvidin and delphinidin were detected under severe drought stress at the end of the experiment. The *UFGT* gene encodes a key enzyme in anthocyanin biosynthesis (Luengo-Escobar et al. 2017) and both

Castellarin et al. (2007a) and André et al. (2009) showed that *UFGT* expression was increased under drought stress, resulting in increased total anthocyanins. In fact, in our study, a positive correlation between *UFGT* expression and total anthocyanin (r = 0.97) was found (Fig. 9). *UFGT* is highly modulated by transcriptional regulation via transcription factors (Nguyen et al. 2017). The myeloblastosis viral oncogene homolog (MYB) transcription factors are the best known key component regulating anthocyanin biosynthesis, which binds to the promoters of anthocyanin structural genes (Xu et al. 2017). In this sense, Nakabayashi et al. (2014) reported that *Arabidopsis* plants overexpressing MYB12 and MYB75 transcription factors, both involved on anthocyanin biosynthesis, over-accumulated anthocyanins in drought stressed plants. This accumulation was key to plant survival, suggesting that anthocyanin biosynthesis is highly controlled at the transcriptional level, enhancing drought stress tolerance. Our findings suggest that drought stress induces higher accumulation of anthocyanins levels due to up-regulation of the anthocyanin biosynthetic pathway, triggering tri-hydroxylated anthocyanins biosynthesis in fully-expended leaves, which have been shown to have significant antioxidant activity.

Most of the reports on anthocyanin changes with water potential have considered ABA as the primary signal involved on anthocyanin biosynthesis regulation under drought stress (Gagné et al. 2011; Kondo et al. 2014; Murcia et al. 2017; González-Villagra et al. 2017). Thus, when ABA increases under drought stress, it potentially regulates the activation of anthocyanin synthesis at the cytoplasmic level. In fact, Shen et al. (2014) reported genetic evidence where the ABA-induced MYBA activates the promoters of anthocyanin biosynthesis structural genes, suggesting that this MYB plays an important role in ABA-induced anthocyanin biosynthesis. In the same sense, González-Villagra et al.

(2017) proposed a molecular model for ABA and miRNA156 involving on the induction of anthocyanin biosynthesis under drought stress. This proposed model shows that ABA binds to the ABA receptor and induces upregulation of microRNA156, which in turns induces greater levels of anthocyanin gene expression, and thereby higher anthocyanin levels, indicating that this could be an important strategy to tolerate drought stress. In fact, Shen et al. (2014) reported a direct relation between ABA and anthocyanin biosynthesis in *Prunus avium* fruit, where the suppression of NCED1 decreases the transcript of biosynthetic anthocyanin genes and transcription factor PacMYBA, these results in the observed decrease in anthocyanin levels. In summary, our study revealed that *A. chilensis* plants showed an antioxidant mechanism to cope with drought stress. Plants subjected to drought stress; mainly fully-expanded leaves of stressed *A. chilensis* had higher ABA concentrations, total anthocyanin levels, and tri-hydroxylated anthocyanins, which together might contribute to the maintenance of lipid peroxidation. These results are also supported by a measured higher level of *NCED* and *UFGT* expression, which allowed *A. chilensis* to increase anthocyanin biosynthesis, thus contributing to drought stress tolerance.

3.5 Conclusions

These results provide a more comprehensive analysis of the mechanisms that underlie how *A. chilensis* is able to cope with drought stress and shows that between young and fully-expanded leaves different sensitivity to this stress is due to the leave's differing ability to synthesize specific anthocyanins with antioxidant activity minimizes the effects of oxidative stress. In addition, this ability to synthesize different and higher amount of anthocyanins could be related to higher *NCED1* and *MYB* expression and ABA levels,

enhancing drought stress tolerance. Further studies are required to clarify the specific ABA signaling mechanism involved on anthocyanin biosynthesis in relation to tolerating drought stress.

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CHAPTER 4

"Abscisic acid (ABA) is involved in phenolic compounds biosynthesis, mainly anthocyanins, in leaves of *Aristotelia chilensis* plants (Mol.) subjected to drought stress"

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Abscisic acid (ABA) is involved in phenolic compounds biosynthesis, mainly anthocyanins, in leaves of *Aristotelia chilensis* plants (Mol.) subjected to drought stress

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Abstract

Abscisic acid (ABA) acts to regulate the physiological and biochemical mechanisms required to tolerate drought stress, which is generally considered the most severe abiotic stress. Because of this, it has been postulated that ABA might be involved in regulation of the biosynthesis of plant phenolic compounds, especially anthocyanins that accumulate in plants subjected to drought stress; however, the evidence for this postulate remains elusive. Therefore, to approach this issue, we studied whether ABA is involved in the accumulation of phenolic compounds, especially anthocyanin biosynthesis, using drought stressed *Aristotelia chilensis* plants, an endemic berry in Chile. Our approach was to use fluridone, an ABA biosynthesis inhibitor, and then subsequent ABA applications to young and fully-expanded leaves of drought stressed *A. chilensis* plants. At different times (24, 48 and 72 h) of the experiment, plants were harvested and leaves were separately collected to determine the biochemical status. We observed that fluridone treatments significantly decreased ABA concentrations and total anthocyanin (TA) concentrations in *A. chilensis* stressed plants, including both young and fully-expanded leaves. TA concentrations following fluridone treatment were reduced around 5-fold, reaching control plant levels only after 24 h. ABA application strongly restored ABA levels as well as TA concentrations in *A. chilensis* stressed plant at the 48 h point of the experiment. We also observed that TA concentrations followed the same pattern as ABA concentrations in the ABA treated plants. qRT-PCR revealed that *AcUFGT* gene expression decreased in fully-expanded leaves of stressed *A. chilensis* plants treated with fluridone, while a subsequent ABA application increased *AcUFGT* expression. Taken together, our results suggest that ABA is involved in the regulation of anthocyanin biosynthesis under drought stress.

4.1 Introduction

Plant growth and crop productivity are affected negatively by drought stress, which is considered the most severe of all abiotic stresses (Osakabe et al. 2014). Plants activate plant defense mechanisms to cope with drought stress by preventing water loss and also counteracting oxidative stress. Abscisic acid (ABA) is considered the key plant hormone which regulates the physiological and biochemical mechanisms enabling plants to tolerate drought stress (Finkelstein, 2013) and some authors have postulated that ABA might play an important role regulating the accumulation of phenolic compounds, including anthocyanins, that is observed in drought stressed plants (Jiang and Joyce; 2003; Deluc et al. 2009; Bucchetti et al. 2011). For example, Nagira et al. (2006) showed that osmotic stress in *Torenia fournieri* plants elevated endogenous ABA levels before anthocyanin biosynthesis induction and suggested that changes in the endogenous ABA concentration might play an important role modulating anthocyanin biosynthesis induction under drought stress. González-Villagra et al. (2017) proposed a model to explain how ABA could be involved in anthocyanin biosynthesis through the regulation by a microRNA (microRNA156) which acts to increase the expression of anthocyanin biosynthesis genes. Other authors have also suggested that different factors might influence anthocyanin concentrations more than endogenous ABA (Gagné et al. 2011; Kondo et al. 2014). How changes are reported can affect the interpretations as well since Antolín et al. (2006) reported that ABA and anthocyanin concentrations based on fresh weight increased in *Vitis vinifera* cv. Tempranillo fruits under drought stress but there was no difference in anthocyanin content on a per berry basis. Drought stress induces higher anthocyanin concentration due to an up-regulation of key anthocyanin pathway genes such as

dihydroflavonol 4-reductase (DFR), *UDP-glucose:flavonoid 3-O-glucosyl transferase* (*UFGT*) and transcription factors such as Myeloblastosis A1 (MybA1) and Myeloblastosis 5A (Myb5A) (André et al. 2009; Borsani et al. 2010; Castellarin et al. 2007; Santesteban et al. 2011). In fact, the relationship between *UFGT* expression and anthocyanins has been demonstrated by correlation analysis, showing a high positive correlation ($r \geq 0.95$; $p \leq 0.05$), indicating that the anthocyanin concentration is increased due at least in part to up-regulation of *UFGT* expression (Castellarin et al. 2007b). There are, however, only a few reports regarding changes in endogenous ABA levels that link such changes with anthocyanin biosynthesis induction. Therefore, the role of ABA in regulation of anthocyanin concentrations under drought stress is still unresolved. In addition, the induction mechanisms resulting in higher anthocyanin concentration has not yet been elucidated (Ferrandino and Lovisolo, 2013: Petrussa et al. 2013; Murcia et al. 2017). Previously, it was reported that different leaf ages, comparing young to fully-expanded leaves, show a distinct response relative to their ability to synthesize anthocyanin as well as ABA in response to stress (Gould et al. 2000; Hughes et al. 2007). Thus, leaf age appears to be a confounding factor involved in understanding the functional role of ABA regulation of anthocyanin biosynthesis. Understanding the induction mechanisms responsible for higher anthocyanin concentrations under drought stress might represent a powerful practical tool to manage and modify anthocyanin concentration in plant organs for agricultural and human health advantages. In this regard, it is important to know whether ABA is responsible for the increase of phenolic compounds including specifically anthocyanin biosynthesis under drought stress.

Aristotelia chilensis (Mol.), also known as maqui, is an endemic berry in Chile belonging to the Elaeocarpaceae family. It is an evergreen tree, distributed from Illapel (Coquimbo Region) to Chiloé (Los Lagos Region) (Hoffman *et al*., 2005). *A. chilensis* is a pioneer species, colonizing and growing on stressed and disturbed environments, thus making it an interesting model for studying plants with a well evolved abiotic stress resistance mechanism (Fredes et al. 2014). This endemic species has also been of great interest for farmers and consumers because of the antioxidant properties related to its high anthocyanin concentration. Currently, commercial crops *A. chilensis* are being established, promoting the development of morpho-phenological and physiological, and genetic diversity studies to establish agronomic parameters and to develop strategies of selection and breeding (Fredes et al. 2014; Vogel et al. 2014). Thus, in this study, we investigated ABA regulation of phenolic compound biosynthesis, mainly anthocyanins, in young and fully-expanded leaves of drought stressed *A. chilensis* plants.

4.2 Materials and methods

4.2.1 Plant material and treatments

Micropropagated in-vitro Maqui plants (*Aristotelia chilensis*) donated by BestPlant Co. (Curico, Chile) were used in this study. One-year-old plants were transplanted to 2 L pots with Andisol soil and acclimated in a greenhouse (temperature: 25±3 °C; photoperiod: light 16/8 h dark; humidity: 60-70%; and a mean photosynthetic active radiation (PAR) at midday of 300 µmol m^{-2} s^{-1}) for two weeks. Plants were then divided into two groups (20 plants for each group); daily irrigated (DI) and non-irrigated (NI). The DI plants were

irrigated at field capacity; meanwhile, NI plants were exposed to water withholding to initiate drought stress. The experiment was carried out for 20 days. When NI plants were stressed (the 20th day of drought stress, based on previous results), 100 µM Fluridone (Sigma, St. Louis, MO, USA) was homogenously applied by spraying on leaves. After 24 h, in some cases, leaves were sprayed with a solution of 100 µM abscisic acid (Sigma). Both solutions were dissolved in ultrapure water containing 0.05% (v/v) of Tween 20, which was used as the surfactant wetting agent. Control solutions contained ultrapure water with only Tween 20. At different times (24, 48 and 72 hours) of the experiment, plants were harvested and leaves were collected at two different positions from the plants, representing different leaf ages: young leaves, from the middle to top; and fully-expanded leaves, from middle to basal leaves, for physiological and biochemical analysis. Leaves were frozen separately in liquid nitrogen and stored at -80 ºC to determine biochemical characteristics.

4.2.2 ABA concentration

Endogenous ABA was quantified by the isotope dilution method, essentially as described by Liu et al. (2012) for auxin analysis, using NH_2 resin solid phase extraction (SPE) TopTip minicolumns. After methylation by diazomethane, the samples were then injected into a gas chromatograph (GC) coupled to a single quadrupole mass spectrometer (MS) (GC-MS, Agilent 6890N GC System with an Agilent 7683 Automatic Liquid Sampler and an Agilent 5973 MS; column, temperatures, carrier gas and other analysis conditions were exactly as described in Liu et al. 2012) and the samples were analysed using selected ion monitoring (SIM) with Agilent Chemstation software. Deuterated-abscisic acid ([$^2H_6$]ABA)

was used as internal standard (Liu et al. 2012), and it was synthesized according to Dobrev et al. (2005) yielding a product with no detectable unlabeled ABA and a major predominate ion at *m/z* 194 for the [$^2H_4$]ABA isotopomer. Endogenous ABA concentration was thus determined from the ion abundance at the base peak of each compound: the *m/z* value of 190 for plant ABA, and the *m/z* value of 194 for [$^2H_4$]ABA using the isotope dilution equation which accounts for the isotopomer distribution in the internal standard (Liu et al. 2012).

4.2.3 Phenolic compound analyses by HPLC-photodiode array detection

Phenolic acids and flavonols were analyzed in leaves of DI and NI plants using a high performance liquid chromatograph (HPLC; Jasco LC-Net II/ADC) with a Kromasil reverse-phase (RP)-18 column (250 x 4.6 mm i.d) equipped with a photodiode array detector (DAD) (Jasco MD 2015 Plus) (Ruhland and Day, 2000). The phenolic acids chlorogenic, caffeic, ferulic, gallic, and *p*-coumaric acid, and the flavonols quercetin, myricetin, kaempferol and rutin were used as standards (Sigma). These compounds were dissolved in methanol for the preparation of calibration curves. Absorbance was detected at 320 nm. Acidified water (phosphoric acid 10%) (A) and 100% acetonitrile (B) was used as the mobile phase. The eluent gradient was: 0-9 min of 100% A, 9.1-19.9 min of 81% A, and 19% B, 20-15 min of 100% B.

4.2.4 Total and profile of anthocyanin

Total anthocyanins (TA) were determined using the pH differential method (Chang et al. 2002). To determine TA, absorbance was measured spectrophotometrically at 530 and 675

nm (UV/VIS Unico SpectroQuest 2800). TA was expressed as mg of cyaniding-3-O-glucoside equivalent (C3G) per gram of dry weight. The anthocyanin profile was based on anthocyanidin determination using the protocol described by Ribera et al. (2010), where delphinidin, malvidin, petunidin, cyanidin and peonidin were used as standards (Sigma). Anthocyanin profiles were obtained by HPLC as described above. The mobile phase was composed of acidified water (acetic acid 10%) (A) and 100% acetonitrile (B) with the following eluent gradient: 0-23.9 min of 90% A - 10% B, 23.9-24.1 min of 80% A – 20% B, 24.1-27 min of 20% A – 80% B, and then 27.1-37 min of 90% A – 10% B.

4.2.5 Total RNA isolation and cDNA synthesis

Total RNA was isolated as described by Jaakola et al. (2001). RNA concentrations were measured spectrophotometrically using a Spectral Scanning Multimode Reader Varioskan Flash µDrop™ Plate (Thermo Scientific, Wilmington, USA). Likewise, RNA purity was determined using the A260/A280 and A260/A230 absorbance ratios. RNA quality was also evaluated visually following gel electrophoresis of the denatured RNA. First-strand cDNA was synthesized from 2 µg of total RNA from *A. chilensis* leaves, which was reverse-transcribed by M-MLV (Promega, MA, USA) following the manufacturer`s recommendations. To remove genomic DNA, the cDNA was cleaned according to Jaakola et al. (2004) using a DNA gel extraction kit (Millipore Corporation, Bedford, MA, USA).

4.2.6 Real-time quantitative PCR (qRT-PCR) analysis

Quantitative real-time (qRT-PCR) reactions were conducted in order to determine the expression patters of *AcUFGT* in *A. chilensis* leaves. All qRT-PCR reactions were performed using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, Santa

Clara, California) in an ABI 7300 Real-Time PCR system (Applied Biosystems, Foster City CA, USA) using the procedure described by Inostroza-Blancheteau et al. (2014). *Elongation Factor 1 alpha (EF1a)* sequences of *Vitis vininfera*, *Populus euphratica*, and *Prunus persica* were obtained from Genbank®. Sequence alignments were done using the Clustal Omega program (www.ebi.ac.uk) and primers were design using AmplifX 1.7.0. Transcripts were sequenced and confirmed in Genbank®. Finally, specific primers were designed based on the sequences in AmplifX1.7.0. *AcUFGT* primers were kindly providing by Dr. Victor Polanco from Universidad Mayor, Chile. The specific primers used in this study are shown in Table 1, which amplified 180 bp fragments. *EF1a* is a stably expressed gene that was used as the internal control. All the experiments were performed using three biological replicates. Cycling conditions were 95 ºC for 10 min, followed by 40 cycles at 94 ºC for 30 s, 60 ºC for 30 s, and 72 ºC for 30 s. Gene expression data (Ct values) were employed to quantify relative gene expression using the comparative $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001).

Table 1 Primer sequences used for qRT-PCR analysis

Gene	Forward primer (5` to 3`)	Reverse primer (5` to 3`)
UFGT	TTC CAG GAA TGT CTC AAG TA	CAA AGG AGT TTA TGA AGA CT
EF1A	CTC CTG GGC ATC GTG ACT TT	CCA AGG GTG AAA GCA AGC AA

4.2.7 Experimental design and data analysis

A completely randomized design was used with three replicates for each treatment and time. The results are expressed as mean and standard error of the mean (±SE) for each

treatment. All data passed the normality and equal variance Kolmogorov-Smirnov tests. Means were analyzed using a two-way ANOVA. The Tukey multiple comparison test at $P \leq 0.05$ was used. Sigma Stat 3.5 (SYSTAT Software Inc.) was used to performed the statistical analysis.

4.3 Results

4.3.1 ABA concentrations in response to fluridone and ABA applications in drought stressed *A. chilensis* plants

We previously determined that by the 20th day of the experiment without water *A. chilensis* experienced severe drought stress as evidenced by the highest ABA and TA levels in the stressed plants at this time. In addition, we observed significant differences between young and fully-expanded leaves of stressed plants in both these parameters. Thus, in order to better understand the role of ABA in regulating anthocyanin biosynthesis, we applied a fluridone solution (an inhibitor of phytoene desaturase, which inhibits biosynthesis of carotenoids). Treatment with the ABA inhibitor fluridone was followed by ABA application (after 24 h) on the leaves of plants subjected to severe drought stress. As expected, treatment with the fluridone solution reduced ABA concentrations in young leaves of stressed *A. chilensis* plants as well as in the fully-expanded leaves (Fig. 1). The ABA concentrations in both young and fully-expanded leaves was reduced about 75% in the stressed plants by fluridone application with respect to stressed plants without application of fluridone at the 24 h time point of the experiment (Fig. 1). When ABA was applied to young and fully-expanded leaves of *A. chilensis* plants, endogenous ABA

concentrations were increased in all treatments, including the fluridone treatment (Fig. 1 A and C). The ABA concentration also increased significantly, about 10-fold, in all treatments with respect to treated plants without ABA application at the 48 h time point of the experiment (Fig. 1). After 48 h, plant leaves without ABA application remained relatively constant their ABA levels during the experiment (Fig. 1 B and D). In all treatments with ABA applications, ABA concentrations decreased at the 72 h time point of the experiment (Fig 1 A and C).

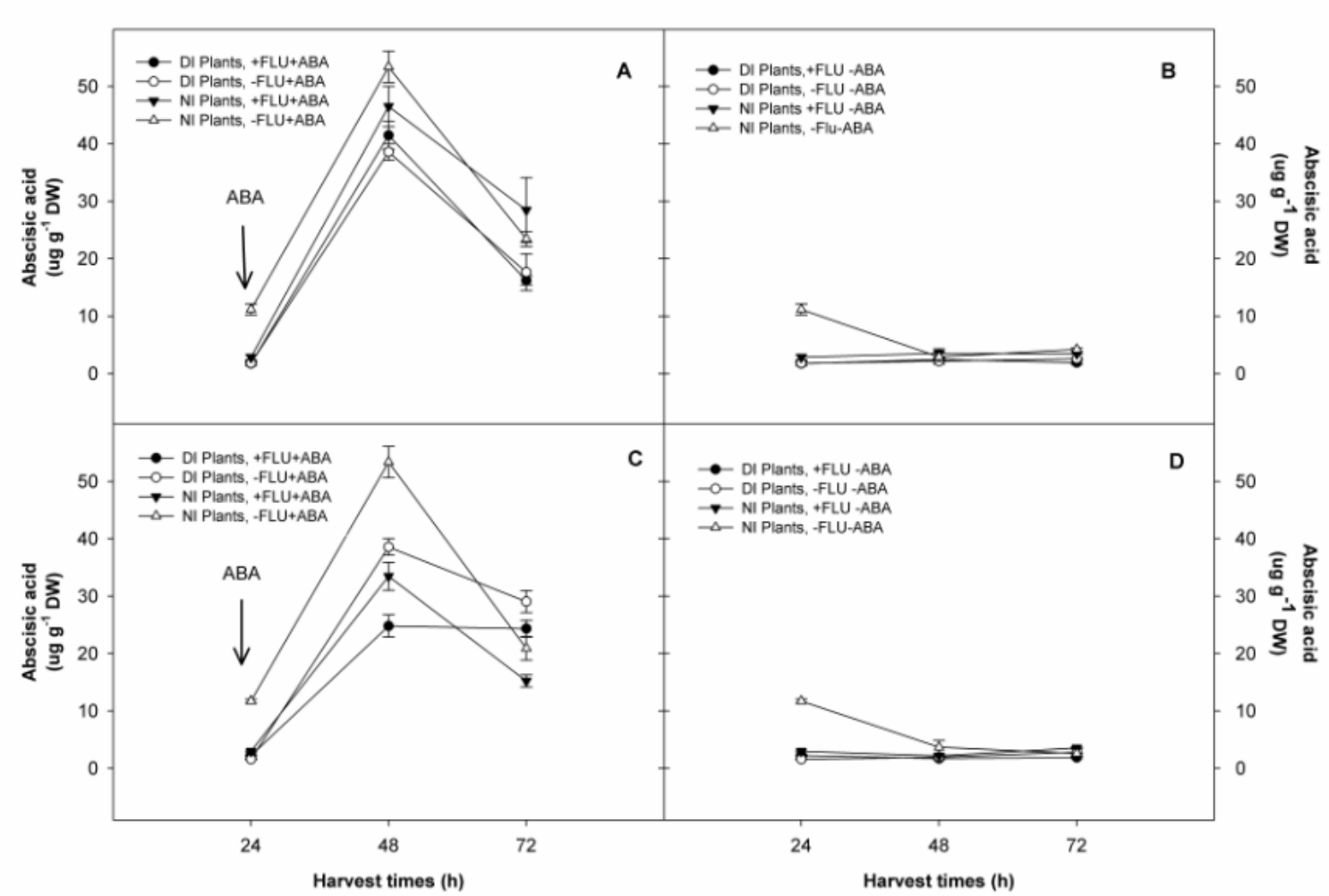

Figure 1. Endogenous abscisic acid (ABA) concentration changes in response to two different water treatments and with or without fluridone solution application and with or without a subsequent ABA solution application. *Aristotelia chilensis* plants were either Daily

Irrigated (DI) or Non-Irrigated (NI). A) Young leaves with ABA application; B) Young leaves without ABA application; C) Fully-expanded leaves with ABA application; and D) Fully-expanded leaves without ABA application. Values represent means ± SE (n=3).

4.3.2 Phenolic compound levels in response to ABA inhibitor and ABA applications in *A. chilensis* plants under drought stress.

Flavonoids and phenolic acids were analyzed in young and fully-expanded leaves of *A. chilensis* treated plants (Fig. 2 and 3). Thus, young leaves of DI plants had higher endogenous pools of phenolic compounds compared to young leaves of NI plants throughout the experiment (Fig 2). Pools of phenolic compounds (PPC) decreased significantly in young leaves of DI plants treated with fluridone at 24 h (Fig. 2); whereas, at 72 h PPC doubled in the same leaf type of DI plants treated with ABA compared to young leaves without ABA application (Fig. 2). In contrast, DI plants without fluridone treatment maintained PPC in the young leaves throughout the experiment (Fig. 2). Young leaves of stressed plants treated with fluridone increased their PPC at 24 h, decreasing to about 30% after 24 h with ABA application as compared to stressed plants without exogenous ABA (Fig. 2). Fully-expanded leaves did not change significantly in their PPC content in DI plants throughout the experiment (Fig. 3). However, fully-expanded leaves of stressed plants had a slight increase (20%) of PPC with fluridone application throughout the experiment independent of ABA application (Fig. 3). The HPLC-DAD analyses revealed that rutin was the most abundant among flavonoids in young and fully-expanded leaves of *A. chilensis*. Surprisingly, rutin increased significantly in both leaf types of drought stressed *A. chilensis* plants (40 and 30%, in young and fully-expanded leaves, respectively) treated with fluridone and without ABA application compared to plants without fluridone during the experiment (Fig. 2 and 3). In addition,

after ABA application, rutin levels decreased significantly (about 40-50%) in both type leaves of DI plants and reached values similar to those of drought stressed plants without fluridone application (Fig 2 and 3). Without ABA application, rutin did not change during the time in both type leaves of DI plants independent of fluridone treatment (Fig. 2 and 3). Quercetin levels had a similar behaviour as the rutin levels throughout the experiment (Fig. 2 and 3). Among the phenolic compounds, coumaric acid and ferulic acid levels in both young and fully-expanded leaves were 20% higher without ABA application with fluridone treated stressed plants compared to stressed-plants without fluridone and ABA applications (Fig. 2 and 3).

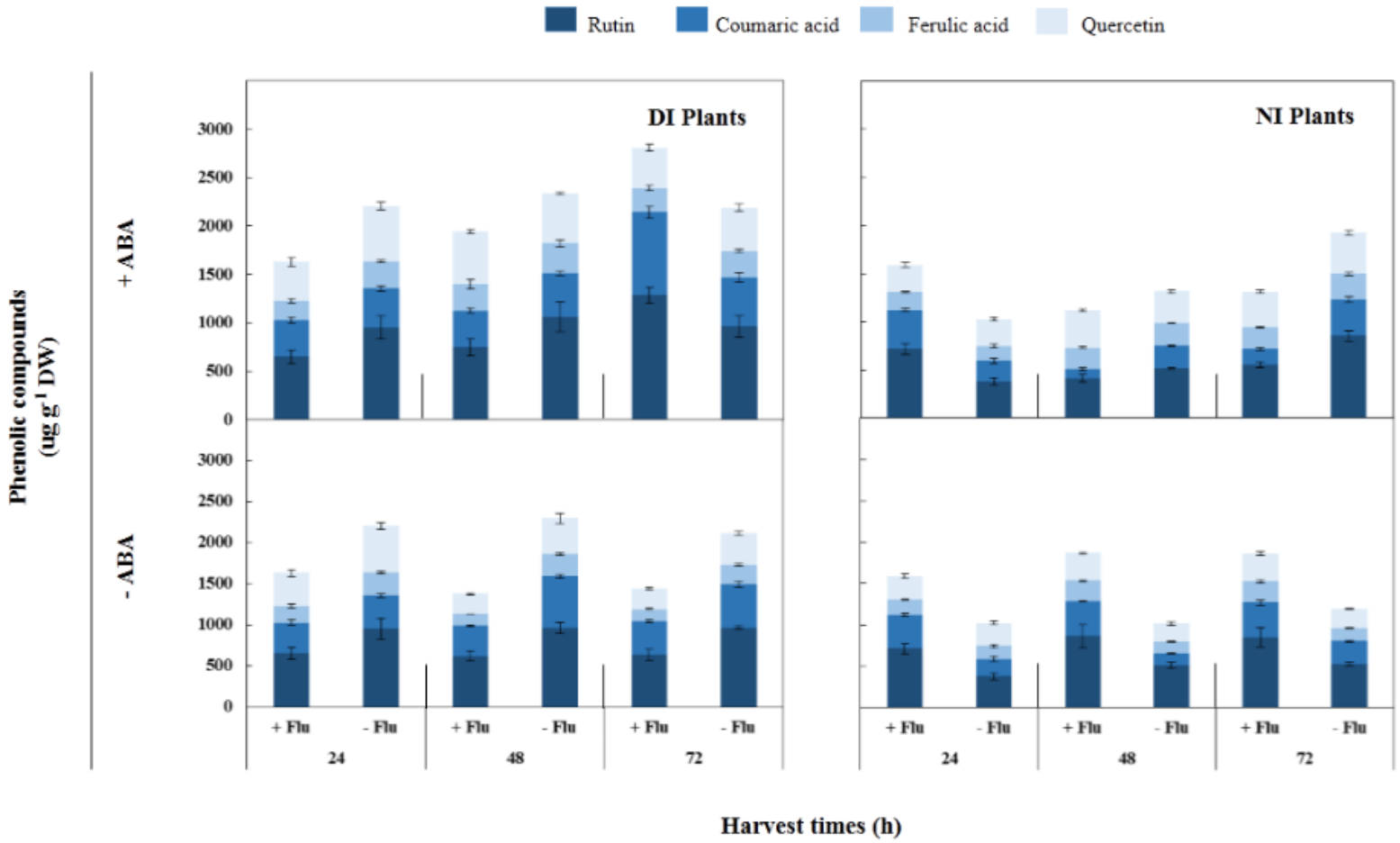

Figure 2. Phenolic compounds in young leaves in response to two different water treatments with fluridone solution application, and a subsequently ABA solution application. Data on the left graph set shows daily irrigated (DI) plants and on the right data for non-irrigated (NI) *Aristotelia chilensis* plants. Values represent means ±SE (n=3).

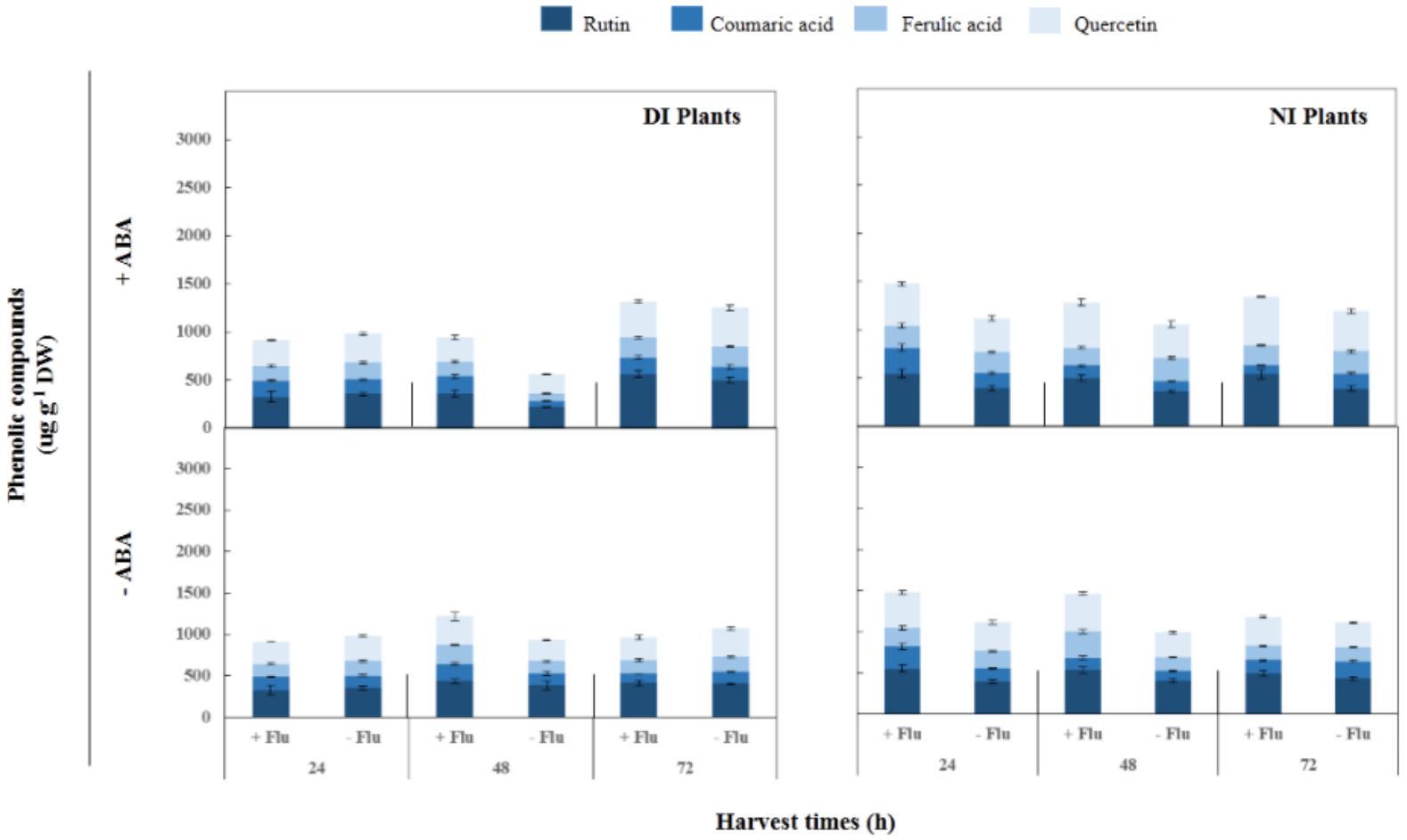

Figure 3. Phenolic compounds in fully-expanded leaves in response to two different water treatments with fluridone solution application, and a subsequent ABA solution application. Data on the left graph set shows daily irrigated (DI) plants and on the right data for non-irrigated (NI) *Aristotelia chilensis* plants. Values represent means ±SE (n=3).

4.3.3 Profiles and total levels of anthocyanins in response to fluridone and ABA application under drought stress

TA levels decreased about 5-fold in fully-expanded leaves of stressed plants treated with fluridone compared to stressed plants not fluridone treated at 24 h (Fig 4 A and B). In contrast, young leaves of stressed *A. chilensis* plants treated with fluridone did not change their TA levels significantly at 24 h (Annex 1). Surprisingly, exogenous ABA strongly reversed the effects of fluridone on TA concentrations in young and fully-expanded leaves of *A. chilensis* stressed plants at 48 h (Fig 4 and annex 1). The TA concentration decreased in stressed *A. chilensis* plants treated with ABA at the end of the experiment (72 h), following the same pattern as ABA concentration in ABA treated stressed plants (Fig 4 A). TA levels were not different in DI *A. chilensis* plants not treated with ABA (Fig 4 B). When young and fully-expanded leaves were analyzed by HPLC-DAD to obtain the anthocyanidin profile, delphinidin, cyanidin, and malvidin were found to be present in fully-expanded leaves of the stressed plants during all the experiment (Table 2). Delphinidin was slightly decreased with fluridone treatment and with ABA application increasing 15-fold with respect to stressed plants treated without fluridone at 48 and 72 h. Petunidin was detected in fully-expanded leaves of drought stressed *A. chilensis* stressed plants and decreased 30% with fluridone treatment, however ABA application reversed the decrease at 48 and 72 h (Table 2). Also, ABA treatment increased malvidin levels in fully-expanded leaves of drought stressed *A. chilensis* at 48 and 72 h. By contrast, only cyanidin was detected in young leaves of control and stressed plants treated with fluridone and ABA applications at 24 and 48 h (Table S1).

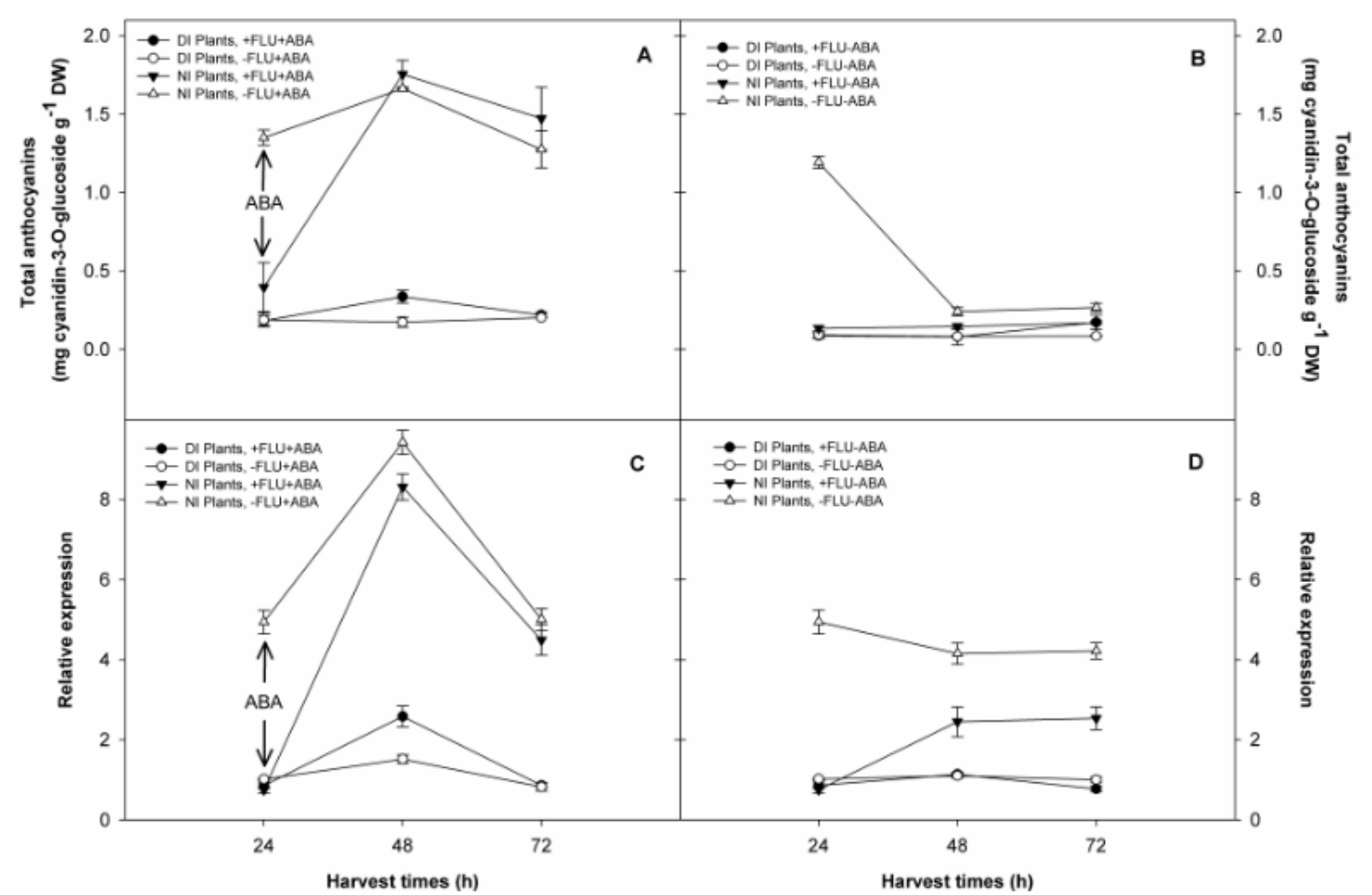

Figure 4. Total levels of anthocyanins (A and B) and relative expression of *AcUFGT* (C and D) in fully-expanded leaves in response to two different water treatments with fluridone solution application, and a subsequent ABA solution application. A and C) Fully-expanded leaves with ABA application and B and D) Fully-expanded leaves without ABA application. *Aristotelia chilensis* plants were either Daily Irrigated (DI) or Non-Irrigated (NI). Values represent means ±SE (n=3).

Table 2 Anthocyanidins (µg g^{-1} DW) in fully-expanded leaves of *A. chilensis*. ND = No Detected. Values represent the means of three samples ±SD. (P<0.05). Different lowercase letters show statistically significant differences among the treatments for the same water irrigation regime and time. Different capital letters show significant differences between water irrigation regime for the same time and treatment.

Harvest times (h)	Fluridone (µM)	ABA (µM)	Irrigation treatment	Delphinidin (µg g^{-1} DW)	Cyanidin (µg g^{-1} DW)	Petunidin (µg g^{-1} DW)	Malvidin (µg g^{-1} DW)
24	0		DI	ND	4.5±0.3B	ND	ND
	0		NI	125.0±23.2	145.5±19.5A	ND	184.6±29.8
	100		DI	ND	ND	ND	ND
	100		NI	ND	ND	ND	ND
48	0	0	DI	ND	3.2±0.2Ba	ND	ND
	0	0	NI	26.6±6.2c	26.1±3.4Acd	5.7±0.3b	27.9±2.1cd
	0	100	DI	ND	7.4±1.1Ba	ND	7.2±0.3Bb
	0	100	NI	56.7±0.0b	111.8±7.1Ab	ND	89.1±16.7Ab
	100	0	DI	ND	16.0±2.2Aa	ND	ND
	100	0	NI	21.8±5.7c	28.5±2.3Ac	3.8±0.5b	40.6±5.6c
	100	100	DI	ND	8.6±0.8Ba	ND	23.3±0.6Ba
	100	100	NI	348.7±12.5[a]	237.2±15.7Aa	9.7±1.2a	300.0±4.5Aa
72	0	0	DI	ND	6.3±1.0Ba	ND	ND
	0	0	NI	94.1±21.9a	45.0±3.5Acd	ND	53.0±3.6cd
	0	100	DI	ND	ND	ND	ND
	0	100	NI	114.1±18.7a	138.8±18.7a	3.7±0.6a	263.3±27.5a
	100	0	DI	ND	ND	ND	ND
	100	0	NI	87.4±12.2a	47.0±8.9c	2.3±0.4b	70.7±4.8bc
	100	100	DI	ND	7.6±1.6Ba	ND	23.3±0.8B
	100	100	NI	98.5±15.3a	87.0±5.1Ab	3.8±0.4a	114.4±29.7Ab

4.3.4 Gene expression analysis of the response to fluridone and ABA treatment by drought stressed *A. chilensis*

The expression of *AcUFGT* was analyzed using qRT-PCR (Fig. 4 C, D). Fluridone treatment reduced about 5-fold *AcUFGT* expression in fully-expanded leaves of stressed *A. chilensis* plants compared to drought stressed plants not treated with fluridone at 24 h (Fig. 4 C and D). *AcUFGT* expression significantly increased with ABA application (Fig. 4 C). In particular, stressed *A. chilensis* plants treated with ABA showed up-regulation of *AcUFGT* expression by 8-fold with respect to stressed plants not treated with ABA at 48 h (Fig 4 C). By 72 h, however, all treatments showed a strong reduction in *AcUFGT* expression relative to 48 h. *AcUFGT* expression levels did not change in fully-expanded leaves of fluridone treated plants not also treated with ABA between 48 and 72 h (Fig 4 D). Control plants (those not treated with fluridone nor ABA) remained unaltered in their *AcUFGT* expression levels throughout the experiment (Fig. 4 D).

4.4 Discussion

This study shows that ABA is involved in the regulation of anthocyanin biosynthesis in *A. chilensis* subjected to drought stress. We used two basic approaches, treatment with ABA itself to increase endogenous ABA levels and treatment with fluridone which is an inhibitor of phytoene desaturase, an enzyme of the carotenoid biosynthesis pathway, which reduces ABA biosynthesis through reduction of the levels of xanthophyll ABA precursors (Yoshioka et al. 1998; Seo and Koshiba, 2002, Nisar et al. 2015). Fluridone treatment, as expected, reduced ABA levels in both young and fully-expanded leaves of drought stressed *A. chilensis* plants, and exogenous ABA increased significantly ABA levels in all ABA treated plants. ABA treatment was effective at recovering TA levels after previous fluridone treatments. Fluridone treatments also increased the pool of phenolic compounds (PPC;

determined by the sum of individual phenolic compounds), and exogenous ABA reduced it. Jian and Joyce (2003) showed that ABA treated *Fragaria x ananassa* fruits showed increased TA levels compared with untreated fruits. Differing reports have shown both that PPC increases in plants subjected to drought stress, while, others showed that these compounds decreased (Petridis et al. 2012; Khoyerdi et al. 2016). Therefore, PPC responses under drought stress is not consistent for all plants and tissues. Shen et al. (2014) demonstrated that MYBA, a transcription factor involved on anthocyanin biosynthesis, increased in expression levels in ABA treated *Prunus avium* fruit suggesting that higher ABA levels associated with environmental stress might play an important role in the anthocyanin biosynthesis. Likewise, transcription factors, including a number of MYBs, which are involved in regulating *UFGT* expression and anthocyanin biosynthesis, have ABA-response elements (ABRE) (Ambawat et al. 2013, Lim et al. 2016). These ABRE increase MYBs expression, and as a consequence *UFGT* expression and anthocyanin biosynthesis. Therefore, from our data we suggest that when the ABA biosynthesis inhibitor was applied to plants, there would likely be less transcription factor MYBs, increasing anthocyanin precursors of phenylpropanoid pathway, and inhibiting anthocyanin biosynthesis. In contrast, when ABA was applied to plants, increases MYBs expression, and thus *UFGT* expression, triggered anthocyanin biosynthesis.

Our results showed that fully-expanded leaves have a greater ability to synthesize higher amounts of anthocyanin, and different anthocyanidins compared to young leaves. We therefore analyzed *AcUFGT* expression in fully-expanded leaves of *A. chilensis* (Fig. 4 C and D). The expression of *AcUFGT* was affected differently by fluridone and ABA treatments. We found a strong down-regulation of *AcUFGT* expression in stressed plants treated with fluridone while exogenous ABA recovered *AcUFGT* expression levels, which coincided with the highest TA levels (Fig 4 C). Previous studies showed that *UFGT* expression was induced by drought stress and exogenous ABA in several

species including *Vitis vinifera*, *Vaccinium corymbosum*, *Vitis rotundifolia* and *Malus sieversii* (Jeong et al. 2004; Castellarin et al. 2007a; Castellarin et al. 2007b; André et al. 2009; Koyama et al. 2010; Zifkin et al. 2012; Sun et al. 2017). Jia et al. (2017) showed that *UFGT* expression increased 5-fold in *Vitis vinifera* treated with ABA. It has been suggested that ABA might contribute to plant drought stress tolerance in past by inducing an increase of anthocyanins, which then help the plants to cope with abiotic stress induced antioxidants by scavenging reactive oxygen species (Jiang and Joyce; 2003 Deluc et al. 2009; Bucchetti et al. 2011; Agati et al. 2012; Nakabayashi et al. 2014; Sperdouli and Moustakas, 2014). This basic hypothesis is supported by the observed anthocyanin biosynthesis increases and distribution. Anthocyanin biosynthesis occurs in different cell compartments, with efficient transport systems, when plants are exposed to abiotic stresses (Polster et al. 2006; Zhao and Dixon, 2009; Agati et al. 2012; Agati et al. 2013; Kovinich et al. 2015; Li et al. 2017). In fully-expanded leaves of stressed plants not only was cyanidin detected but also petunidin, which was not detected in young leaves (Table 2 and annex 2, respectively). In addition, it is known that antioxidant activity is dependent on anthocyanin structure, being higher in anthocyanins with more hydroxyl groups attached to their structure, as is the case of petunidin (Kahkonen and Heinonen, 2003). Thus, it is likely a consequence of drought stressed plants that synthesize ABA rapidly, also increase the biosynthesis of anthocyanins with higher antioxidant activity, like petunidin, to cope with oxidative stress.

We observed that ABA application has an effect at short-time points due to the higher ABA levels rapidly increasing *AcUFGT* expression and TA levels. Over longer times however, ABA levels decreased in all treatments resulting in lower *AcUFGT* expression and decreasing TA levels. Hung et al. (2007) reported TA levels increasing after 24-36 h ABA application, and then TA levels decreased due to ABA homeostatic mechanisms which reduced the higher ABA levels resulting from

application. According to Seiler et al. (2011), ABA homeostasis is maintained in the face of artificially higher ABA levels by reduction by two possible mechanisms, ABA catabolism and ABA inactivation. The main route to ABA catabolism is converting it to phaseic acid, while ABA inactivation is mainly conjugating it to form the glucose ester (Xu et al. 2002; Kushiro et al. 2004; Lee et al. 2006). Our studies demonstrated that higher ABA levels promoted higher *AcUFGT* expression, triggering anthocyanin biosynthesis with strong antioxidant activity, mainly in fully-expanded leaves of drought stressed plants and that reduction in ABA had essentially the opposite effect.

4.5 Conclusions

Our experiments allowed us to demonstrate that ABA regulated aspects of anthocyanin biosynthesis under drought stress. Furthermore, fluridone was an effective ABA inhibitor in *A. chilensis* stressed plant including young and fully-expanded leaves, and also demonstrated that ABA application was able to recover both endogenous ABA concentrations in fluridone treated plants as well as increase total anthocyanin and also inducing a different anthocyanin profile. In addition, we showed that high total anthocyanins are due at least in part to higher *AcUFGT* expression. However, it will be necessary in future studies to further explore the molecular mechanisms for ABA downstream processes leading to induction of anthocyanin biosynthesis under drought stress. A better understanding of these processes with allow management and modification of anthocyanin concentrations in plant organs thereby increasing plant tolerance to drought stress.

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Supporting Information

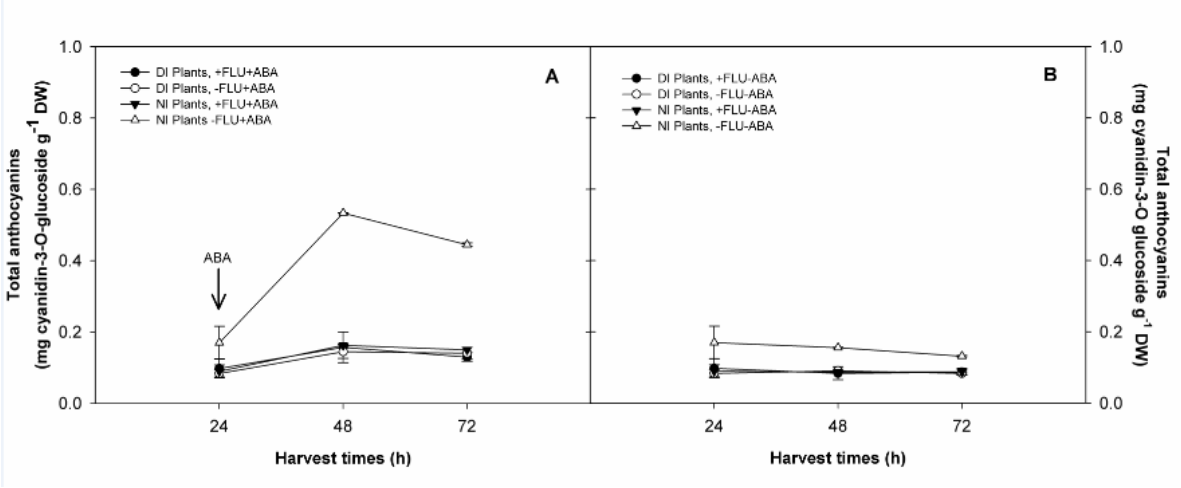

Fig. S1. Changes in total anthocyanins in young leaves in response to two different water treatments with or without fluridone solution application and with or without a subsequently ABA solution application. A) Young leaves with ABA application and B) Young leaves without ABA application. *Aristotelia chilensis* plants were either Daily Irrigated (DI) or Non-Irrigated (NI). Values represent means ±SE (n=3).

Table S1. Levels of anthocyanidins (µg g^{-1} DW) in young leaves of *A. chilensis*. ND = No Detected. Values represents the means of 3 ±SD. ($P<0.05$). Different lowercase letters show statistically significant differences among the treatments for the same water irrigation and time. Different capital letters show significant differences between water irrigation for the same time and treatment.

Harvest times (h)	Fluridone (µM)	ABA (µM)	Irrigation treatment	Delphinidin (µg g^{-1} DW)	Cyanidin (µg g^{-1} DW)	Malvidin (µg g^{-1} DW)
24	0	-	DI	ND	3.0±0.7B	ND
	0	-	NI	65.2±1.2	66.6±1.8A	65.4±0.6
	100	-	DI	ND	ND	ND
	100	-	NI	ND	ND	ND
48	0	0	DI	ND	3.6±0.2Ba	ND
	0	0	NI	ND	63.4±1.8Ab	ND
	0	100	DI	ND	2.8±0.7Ba	ND
	0	100	NI	87.5±2.0	78.4±1.4Aa	ND
	100	0	DI	ND	ND	ND
	100	0	NI	ND	ND	ND
	100	100	DI	ND	ND	ND
	100	100	NI	ND	ND	ND
72	0	0	DI	ND	ND	ND
	0	0	NI	ND	ND	ND
	0	100	DI	ND	ND	ND
	0	100	NI	ND	ND	ND
	100	0	DI	ND	ND	ND
	100	0	NI	ND	ND	ND
	100	100	DI	ND	ND	ND
	100	100	NI	ND	ND	ND

Chapter 5

General discussion and conclusions

5.1 General discussion

As mentioned in the introduction, drought is the main stress factor to plants, decreasing plant water status, plant growth and crop yields. Our findings reflected this situation, where Ψ_w and RWC were severely affected by drought stress at the end of the experiment; meanwhile, ABA levels significantly increased in young and fully-expanded leaves at the same time (Chapter 3; Fig. 1, 2 A-B). According to Galmés et al. (2007), Ψ_w, RWC, and plant growth decrease by 30-40% in plants by moderate drought stress, meanwhile, Ψ_w, RWC, and plant growth largely decrease under severe drought stress, which in our experiment was observed at day 20 of water withholding. We performed a previous experiment to evaluate severity and recovery of *A. chilensis* plants exposed to drought stress (annex 4). This experiment allowed us determine that *A. chilensis* is able to recovery after a severe drought stress. Negative effects of drought stress have been reported in different species such as *Arabidopsis thaliana, Phaseolus vulgaris, Glycine max* and *Beta maritima* (Ohashi et al. 2006; Galmés et al. 2007; Choat et al. 2012; Li et al. 2017). A reduced plant growth in plants subjected to drought stress could be attributed to stomatal closure, and thereby reduced CO_2 levels, since, drought stressed plants increase ABA levels reducing stomatal aperture, and thus preventing water loss, which it is a physiological mechanism to cope drought stress (Pinheiro and Chavez, 2010; Finkelstein, 2013; Flexas et al. 2014; Basu et al. 2016). It has been also proposed that a reduction in photosynthesis and plant growth might be due to loss of ATP content, which starts to decrease with moderate water stress (Tezara et al. 1999; Flexas and Medrano, 1999; Lawlor and Gornic, 2002). Consequently, plant growth can be reduced by stomatal and metabolic limitations.

Plants subjected to drought stress produce higher levels of reactive oxygen species (ROS) in different cellular compartments, which results in protein damage, DNA damage, and lipid peroxidation (Yazici et al 2007). Actual evidence shows that ROS are not only involved in damage and growth

impairment, but also signalling as secondary messengers (Mitller et al. 2011; Hideg et al. 2013), as we showed in our proposed molecular model in Chapter 2 (Fig. 3). Young and fully-expanded of *A. chilensis* plants exposed to drought stress showed similar lipid peroxidation levels at the end of the experiment, being significantly higher in stressed plants compared to control plants (Chapter 3, Fig. 4). In contrast, previous studies have shown that fully-expanded leaves have higher lipid peroxidation, which could be attributed to the higher amount of chloroplasts compared to young leaves (Foyer and Noctor, 2005; Lepedus et al. 2011), indicating that chloroplasts are the main organelle generating ROS under drought stress. A possible explication to maintain lipid peroxidation in fully-expanded leaves at the same level as young leaves might be that fully-expanded leaves of *A. chilensis* have a strong antioxidant mechanism to tolerate drought stress. In this sense, our results indicated that young leaves of stressed *A. chilensis* plants showed higher PPC levels (determined by the sum of individual phenolic compounds), meanwhile, fully-expanded leaves of *A. chilensis* plants increased total anthocyanins from the 10^{th} day of drought stress (Chapter 3, Fig. 5, 6). These results agree with other reports, where higher total phenols have been reported in several species subjected to drought stress such as *Salvia officinalis* and *Agave salmiana* (Martins et al. 2016; Gharibi et al. 2016; Puente-Garza et al. 2017). Among phenolic compounds, anthocyanins are considered as plant secondary metabolites with greater antioxidant activity, due to higher hydroxyl groups number attached to their structure, which scavenge ROS, increasing tolerance to abiotic stresses (Nakabayashi et al. 2014; Zhang and Tsao, 2016; Naing et al. 2017). Our results of higher total anthocyanins agree with Nakabayashi et al. (2014), where they showed that drought stress increased total anthocyanins. In addition, these authors showed that overexpression of anthocyanin biosynthetic genes, and thereby higher anthocyanin amount mitigates the accumulation of ROS. André et al. (2009) and Castellarin et al. (2007) reported that tri-hydroxylated anthocyanins such as delphinidin, petunidin, and malvidin

were higher in drought stressed *Solanum tuberosum* and *Vitis vinifera* plants compared to well-watered treatments, while the content of di-hydroxylated anthocyanins such as cyanidin and peonidin, was similar for both treatments, suggesting that plants subjected to drought stress increase tri-hydroxylated anthocyanin biosynthesis due to their greater antioxidant power in order to cope with drought stress. In fact, we detected cyanidin in control and stressed plants throughout the experiment; meanwhile, delphinidin was detected in drought stressed plants at day 20. Interestingly, we detected three different tri-hydroxylated anthocyanidins in fully-expanded leaves compared to young leaves, where we detected only one (Chapter 4, Table 2 and S2). General phenylpropanoid pathway consists of two main branches, where F3'H and F3'5'H are the enzymes catalyzing di-hydroxylated and tri-hydroxylated anthocyanin biosynthesis, respectively (Winkel-Shirley 2006; Boudet 2007). Thus, we suggests that *F3'5'H* gene could be highly expressed in our drought stressed plants triggering tri-hydroxylated anthocyanin biosynthesis. Therefore, these tri-hydroxylated anthocyanins help to increase the defense mechanism against ROS, tolerating drought stress.

The *9-cis-epoxycarotenoid dioxygenase 1* (*NCED1*) gene encodes an important enzyme in the ABA biosynthetic pathway. In our study, *NCED1* gene expression was affected by drought stress, increasing its expression in drought stressed plants, concomitant with ABA concentration (positively and significantly correlated, $r = 0.98$, $P < 0.05$; Chapter 3, Fig. 9). This has been also reported in previous studies with *Vaccinium myrtillus* and *Vitis vinifera* subjected to drought stress (Zhang et al. 2009; Karppinen et al. 2013). On the other hand, fluridone treatments reduced *NCED1* expression and ABA levels in young and fully-expanded leaves of drought stress *A. chilensis* plants at the 24 h of the experiment (Chapter 4, Fig. 1, 4), indicating that *NCED1* gene is the key regulatory step in ABA biosynthesis pathway, as proposed by Finkelstein (2013). Likewise, total anthocyanins were reduced in both leaf types in plants subjected to drought stress by fluridone treatments. However, exogenous

ABA increased significantly about 10-fold ABA and anthocyanin levels in all ABA treated plants (Chapter 4, Fig. 1 A and C). As in our study, ABA treatment was effective at recovering TA levels after previous fluridone treatments in *Fragaria x ananassa* fruits (Jian and Joyce 2003). We found that fluridone strongly decreased anthocyanin biosynthesis, whilst ABA application recovered anthocyanin synthesis by triggering *AcUFGT* expression in drought stresses plants, which was increased in fully-expanded leaves (Fig. 4 C and D). *UFGT* expression analyses have shown that drought stress and exogenous ABA promotes their expression in several species such as *Vitis vinifera*, *Vaccinium corymbosum*, *Vitis rotundifolia* and *Malus sieversii* (Jeong et al. 2004; Castellarin et al. 2007a; Castellarin et al. 2007b; André et al. 2009; Koyama et al. 2010; Zifkin et al. 2012; Sun et al. 2017). According to Singh and Laxmi (2015) ABA modulates target gene expression by the ABA-responsive element (ABRE) binding protein/ABRE binding factor (ABRE/ABF) transcription factors. It has been reported that MYBs, which are transcription factors that activate or represses anthocyanin biosynthesis structural genes, contains several stress-related *cis*-elements in the promoter sequence such as ABRE (Shen et al. 2017). Among these transcription factors MYBA1 is a fundamental component on anthocyanin biosynthesis, since it activates *UFGT* expression (Kobayashi et al. 2002; Walker et al. 2007). Cui et al. (2017) showed that drought stress up-regulated *MYBA1* and thereby *UFGT* expression triggering anthocyanin biosynthesis. Therefore, we suggested that a high expression of *MYBA1* could be involved on high *UFGT* expression observed in our study, triggering anthocyanin biosynthesis in our drought stressed plants. In our finding, young leaves showed high PPC and low anthocyanin levels compared to fully expanded leaves. As we mentioned above, transcription factors can also represses structural genes of anthocyanin biosynthesis. Thus, Salvatierra et al. (2013) reported a transcription factor, MYB1, repressing anthocyanin biosynthesis in *Fragaria chiloensis* (white Chilean strawberry). They showed that down-regulation of *MYB1* resulted an up-

regulation of anthocyanin biosynthesis, meanwhile, control treatments (with normal *MYB1* expression) showed higher phenol and flavonoid levels. Therefore, we suggest that young leaves of *A. chilensis* stressed plants could have repress anthocyanin biosynthesis at *MYB* and/or *UFGT* levels. Hung et al. (2007) reported that TA levels increase after 24-36 h ABA application, and then TA levels decreased due to ABA homeostatic mechanisms, which reduced the higher ABA levels resulting from application. These agreed with our results, where we found a decrease in ABA and total anthocyanins after 48 h of ABA application. According to Seiler et al. (2011), ABA homeostasis is maintained in the face of artificially higher ABA levels by reduction by two possible mechanisms: ABA catabolism and ABA inactivation. Some authors have suggested that different factors might have a higher influence on anthocyanin concentrations than endogenous ABA (Gagné et al. 2011; Kondo et al. 2014). However, we suggest that this evidence demonstrate the direct relationship between ABA and anthocyanin biosynthesis in drought stressed plants. Thus, ABA contribute to plant drought stress tolerance by inducing an increase of anthocyanins, which then help the plants to cope abiotic stress, inducing antioxidants by scavenging reactive oxygen species. At molecular level, we have proposed a model, which explain how ABA could be involved in anthocyanin biosynthesis through the regulation of a microRNA (156), which increases the expression of anthocyanin biosynthesis genes (Chapter 2, published as González-Villagra et al. 2017). This thesis contributes to understanding of molecular mechanism where ABA regulates anthocyanin biosynthesis. As we mentioned above *A. chilensis* is an endemic berry in Chile that produces leaves and fruits rich in anthocyanins and natural antioxidants (Sanchez et al. 2016). Anthocyanins, natural antioxidants, and their pharmacology properties of *A. chilensis* have been of great interest for farmers and consumers leading to the elaboration of products derived from this species. Thus, this thesis might be a great

contribution to increase anthocyanin levels in *A. chilensis*, and also promote tri-hydroxylated anthocyanin biosynthesis, which have great antioxidant power.

Finally, we can indicate that the hypothesis of this thesis was validated according to the main results in this study. In summary, fluridone inhibited *NCED* expression and their concomitant ABA biosynthesis, which in turns inhibited *UFGT* expression and anthocyanin biosynthesis. However, ABA application recovered *NCED* expression, ABA biosynthesis, *UFGT* expression and anthocyanin biosynthesis. Thus, a basic model including the main responses to drought stress was elaborated (Chapter 5, Fig. 1).

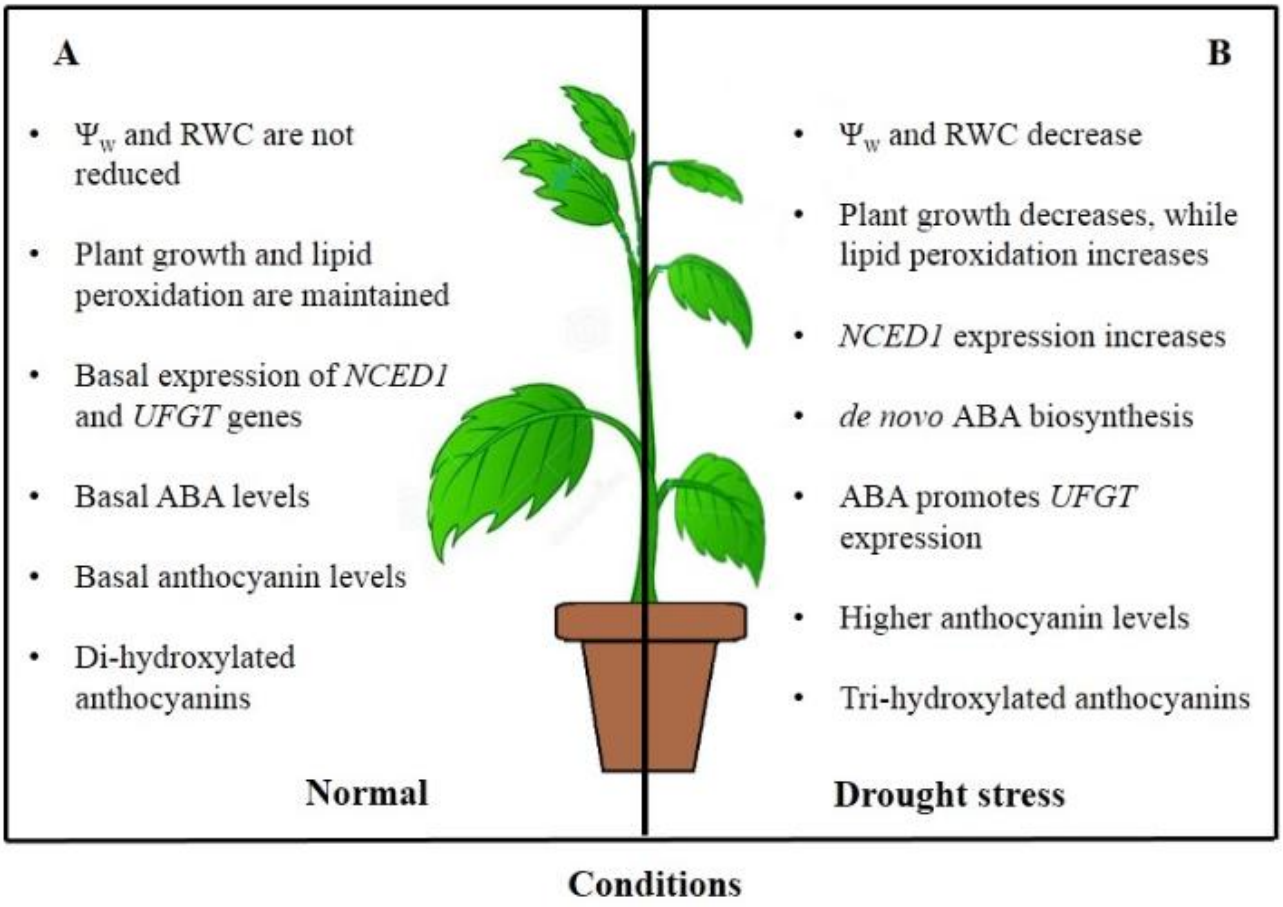

Fig 1. Proposed model describing the main responses in *A. chilensis* plants subjected to drought stress. A) Normal conditions; B) Drought stress. Under drought stress, Ψ_w and RWC decrease, plant growth is reduced and lipid peroxidation increases, while, *NCED1* expression increases, triggering ABA biosynthesis. This higher ABA levels promotes anthocyanin biosynthesis by *UFGT* expression. Under normal conditions (without drought stress), Ψ_w and RWC are not reduced, lipid peroxidation and plant growth are maintaining, *NCED1* and ABA levels are basal, and anthocyanin biosynthesis is not increased, maintaining basal levels.

5.2 Conclusions and future directions

Our results showed that fluridone was an effective ABA inhibitor in drought stressed *A. chilensis* plants including young and fully-expanded leaves. Meanwhile, ABA application was able to recover both endogenous ABA concentrations in fluridone treated plants as well as increase total anthocyanin and also inducing a different anthocyanin profile. We showed that *NCED1* triggers ABA biosynthesis, and thus promoting *UFGT* gene expression, and thereby anthocyanin biosynthesis, and their accumulation. Therefore, our study allows us to demonstrate that ABA regulates anthocyanin biosynthesis under drought stress. However, it will be necessary in future studies to further explore the molecular mechanisms for ABA downstream processes leading to induction of anthocyanin biosynthesis under drought stress. A better understanding of these processes will allow us management and modification of anthocyanin concentrations in plant organs thereby increasing plant tolerance to drought stress.

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ANNEXES

Annex 1

- **RNA extraction and cDNA synthesis from *A. chilensis* to molecular studies**

Using this method, we could isolate total RNA successfully. The purity of the total RNA was assessed using the A260/280 and 260/230 ratios given by NanoDrop. Agarose gel electrophoresis (1% w/v) revealed that intact ribosomal RNA bands (28 and 18 S) were clearly visible, indicating that RNA is undegraded (**Fig 1**). Besides, Agarose gel (1% w/v) revealed that cDNA was successfully synthesized (**Fig 2**).

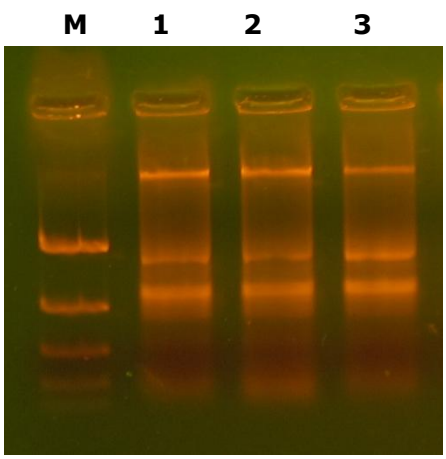


Fig 1. Visualization of total RNA. Lane 1-3: leaf RNA. M:low range ladder (100-2000 bp)

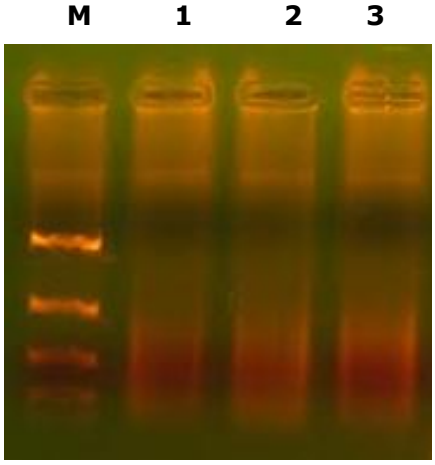


Fig 2. Visualization of cDNA. Lane 1-3: leaf cDNA. M:low range ladder (100-2000 bp)

- **DNA-free cDNA without Dnase treatment (Jakkola et al. 2004)**

Using this method, we could obtain DNA-free cDNA without DNAse treatment.

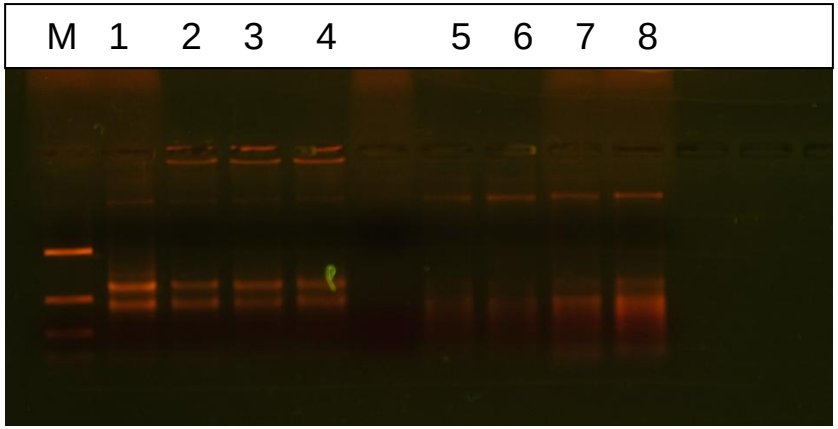


Fig 1. Visualization of total RNA, lane 1-4; and cDNA without DNAasa treatment, lane 5-8. M:low range ladder (100-2000 bp)

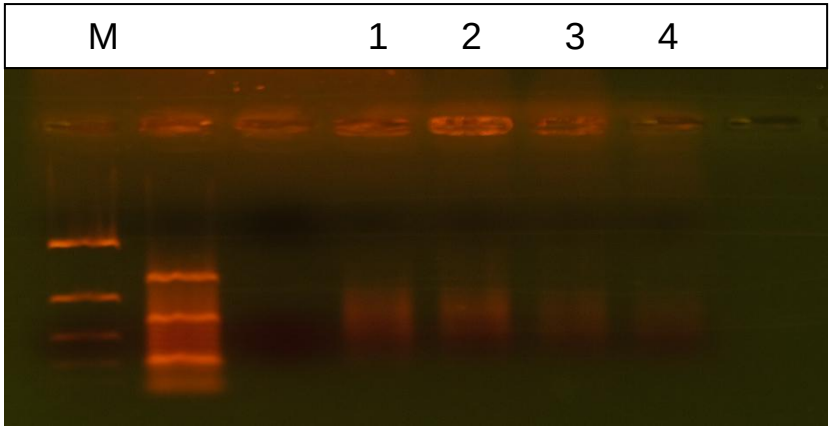


Fig 2. Visualization of cDNA with cDNA cleaning by gel. Lane 1-4: leaf cDNA. M:low range ladder (100-2000 bp)

Annex 2

- *Aristotelia chilensis UDP-glucose: flavonoid 3-O-glucosyltransferase (AcUFGT) gel*

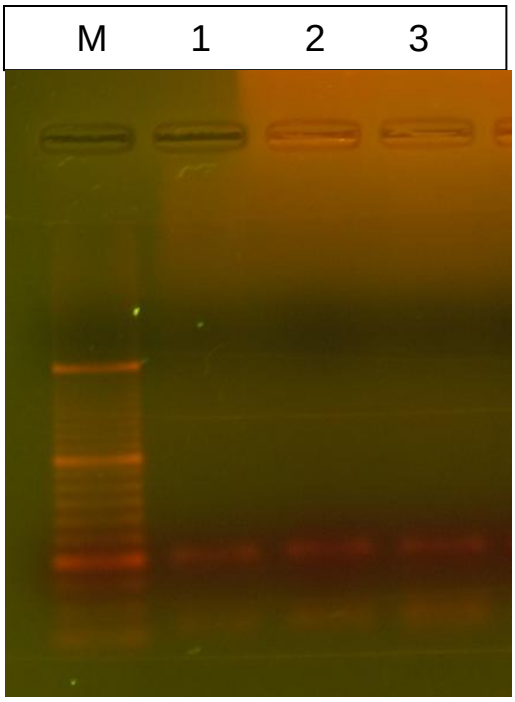


Fig 1. Visualization of AcUFGT PCR product. Lane 1-3: different leaf samples. M:low range ladder (100-2000 bp)

- **Sequencing results**

Forward:
CGACGGAAATCCTGTTGTAGTTTTTGGGACCTGGAATCACTCTTCTCGCGTATGTTACATCAAATG
GGCATAGTGTTACCACAAGCTGCTGCAGTCTTCATAAACTCCTTTGA

Reverse:
GTGGATATTTTGTGACCATTTGATGTAACATACGCGAGAAGAGTGATTCCAGGTTCCCAAAAACA
ATTCCTTCAGGCAAGTCACGTATAAGTACTTGAGACATTCCTGGAAA

- **BLAST results**

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenPept Graphics

Description	Max score	Total score	Query cover	E value	Ident	Accession
UDP-glucose:flavonoid 3-O-glucosyltransferase [Vitis vinifera]	53.9	53.9	83%	8e-08	81%	AEI60387.1
UDP-glucose:flavonoid 3-O-glucosyltransferase [Vitis vinifera]	53.9	53.9	83%	9e-08	81%	AEI60286.1
UDP-glucose:flavonoid 3-O-glucosyltransferase [Vitis vinifera]	54.3	54.3	83%	2e-07	81%	AEI60337.1
UDP-glucose:flavonoid 3-O-glucosyltransferas [Vitis vinifera]	53.9	53.9	83%	2e-07	81%	AEI60396.1

- *Aristotelia chilensis nine-cis-epoxycarotenoid dioxygenase* (*AcNCED*)

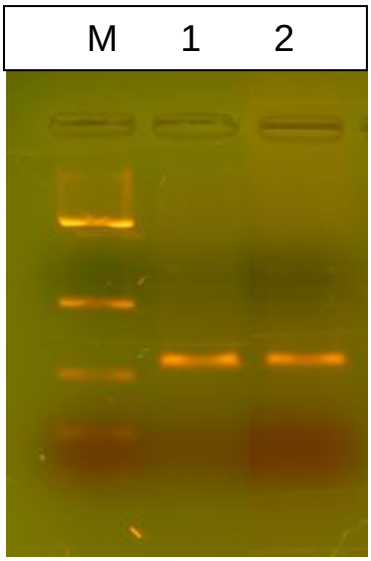


Fig 1. Visualization of AcNCED PCR product. Lane 1-2 different leaf samples. M:low range ladder (100-2000 bp)

- **Sequencing results**

Forward:
TTGGATGTTTTTATCTGAATTAGACTCATTTGAAGACTGGCCAGTCCACTCGCCGCGCCATTCTTT
CCGAGCCTGAACAAGTGAATTTAGAAGCAGGGATGGTGAACAAGAACTTTCTTGGAAGAAAGAC
CCGGTTCGCGTACTTAGCCCTTGCTGAACCGTGGCCTAAAGTGTCAGGTTTTGCCAAAGTTGACA
TCTCAACTGGAGAGGTAAACAAGTACATCTATGGAGACCAAAGGTTTGGTGGTGAGCCTTTGTTT
CTTCCCAGAGACCCCAATTCAGAGATAGAAGATGATGGCTATGTTTTAACTTTTGTTCATGATGA
GAAGGAATGGAAATCAGAGCTGCAA

Reverse:

CAGGGGAAACATAGCCATCATCTTCTATCTCTGAATTGGGGTCTCTGGGAAGAAACAAAGGCTCA
CCACCAAACCTTTGGTCTCCATAGATGTACTTGTTTACCTCTCCAGTTGAGATGTCAACTTTGGCA
AAACCTGACACTTTAGGCCACGGTTCAGCAAGGGCTAAGTACGCGAACCGGGTCTTTCTTCCAAG
AAAGTTCTTGTTCACCATCCCTGCTTCTAAATTCACTTGTTCAGGCTCGGAAAGAATGGCGCGGC
GAGTGGACTGGCCAGTCTTCAAATTGAGTCTAATTTCAGATAAAACACTCTTCAAACTCTCGTCA
CATTCGTTGAAAAATGGAGTCA

- **BLAST results**

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenPept Graphics

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: 9-cis-epoxycarotenoid dioxygenase NCED1, chloroplastic-like [Populus euphratica]	209	209	95%	2e-62	88%	XP_011029543.1
hypothetical protein POPTR_0011s11370g [Populus trichocarpa]	208	208	95%	3e-62	88%	XP_002316871.1

- *Aristotelia chilensis Elongation Factor 1 alpha (AcEF1a)*

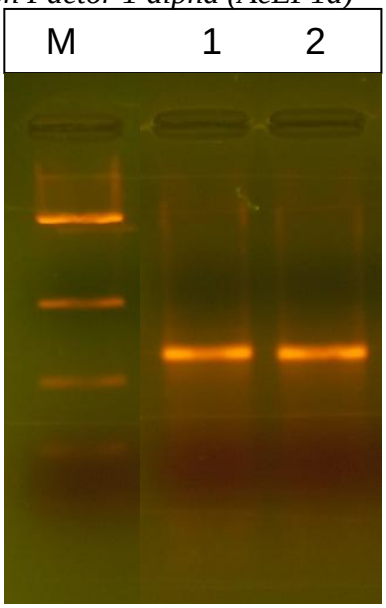
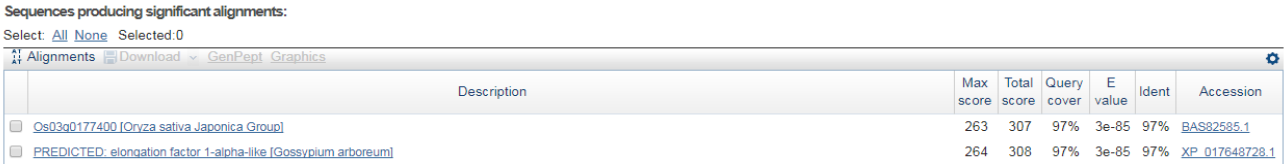


Fig 1. Visualization of EF1a PCR product. Lane 1-3 different leaf samples. M:low range ladder (100-2000 bp)

- **Sequencing results**

Forward:
GCAGCTGACGTGAGCGTGGTATCACCATTGATATTGCCTTGTGGAAGTTCGAGACCACCAAGTAT
CTACTGCACTGTCATTGATGCTCCTGGGCATCGTGACTTTATCAAGAACATGATTACTGGTACCTC
ACAGGCTGACTGTGCTGTCCTCATTATTGACTCCACCACTGGTGGTTTTGAAGCTGGTATCTCCAA
GGATGGCCAGACCCGTGAGCATGCTTTGCTTGCTTTCACCCTTGGTGTCAAGCAGATGATCTGCT
GCTGCAACAAGATGGATGCCACCACCCCCAAGTACTCCAAGGCCAGGTATGAAGAAATTGTGAA
AGAAGTTTCTTCCTACTTGAAGAAGGTCGGTTACAACCCTGACAAAATCCCCTTTGTGCCTATCTC
TGGATTTGAGGGTGACAACATGATTGAGAGGTCTACCAACCTTGATGGTTTACAAGGGACCC

Reverse:
GCATATGATGTCACTCAATCAGAGATAGGCACCAAGTGGGATTTTGTCATGGTTGTAACCCACCT
TCTTCATTAGGAAGAAACTTCCTTCACAATTTCCTCCTACCTAGCCTTGGAATACTTGGGGGTGGT
GGCATCCATCTTGTTGCACAACAAATCATTTGCTTGACACCAAGGGTGAAAACAAGCAAAACATG
CTCACGGGTCTGGCCATCCTTTGAAATACCCACTTCAAAACCACCAGTGGTGGAGTCAATAATGA
GGACAGCACAGTCAGCCTGTGAGGTACCAGTAATCATGTTCTTGATAAAGTCACGATGTCCAGGG
GCATCAATGACAGTGCAGTAGTACTTGGTGGTCTCAAACTTCCACAAGGCAATATCAATGGTAAT
ACCACGCTCACGCTCAGCCTTGAGCTTGTCCAACACCCAGGCAACTTTTAAATGAAAAAAAAATT
TGTCAAAGATGTCTCGTTCCTACGTGAAGAAAGGTTGTGTCCAACGTTCACAATAATCCTATTAG
TTCCCCGATCTGGATTGATAGGTGGAAACAAGGGTTGCAGAGGTCTATTAACCTTGACTGGCAGG
TGGGTTTGAAGT


Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenPept Graphics

Description	Max score	Total score	Query cover	E value	Ident	Accession
Os03g0177400 [Oryza sativa Japonica Group]	263	307	97%	3e-85	97%	BAS82585.1
PREDICTED: elongation factor 1-alpha-like [Gossypium arboreum]	264	308	97%	3e-85	97%	XP_017648728.1

Annex 3

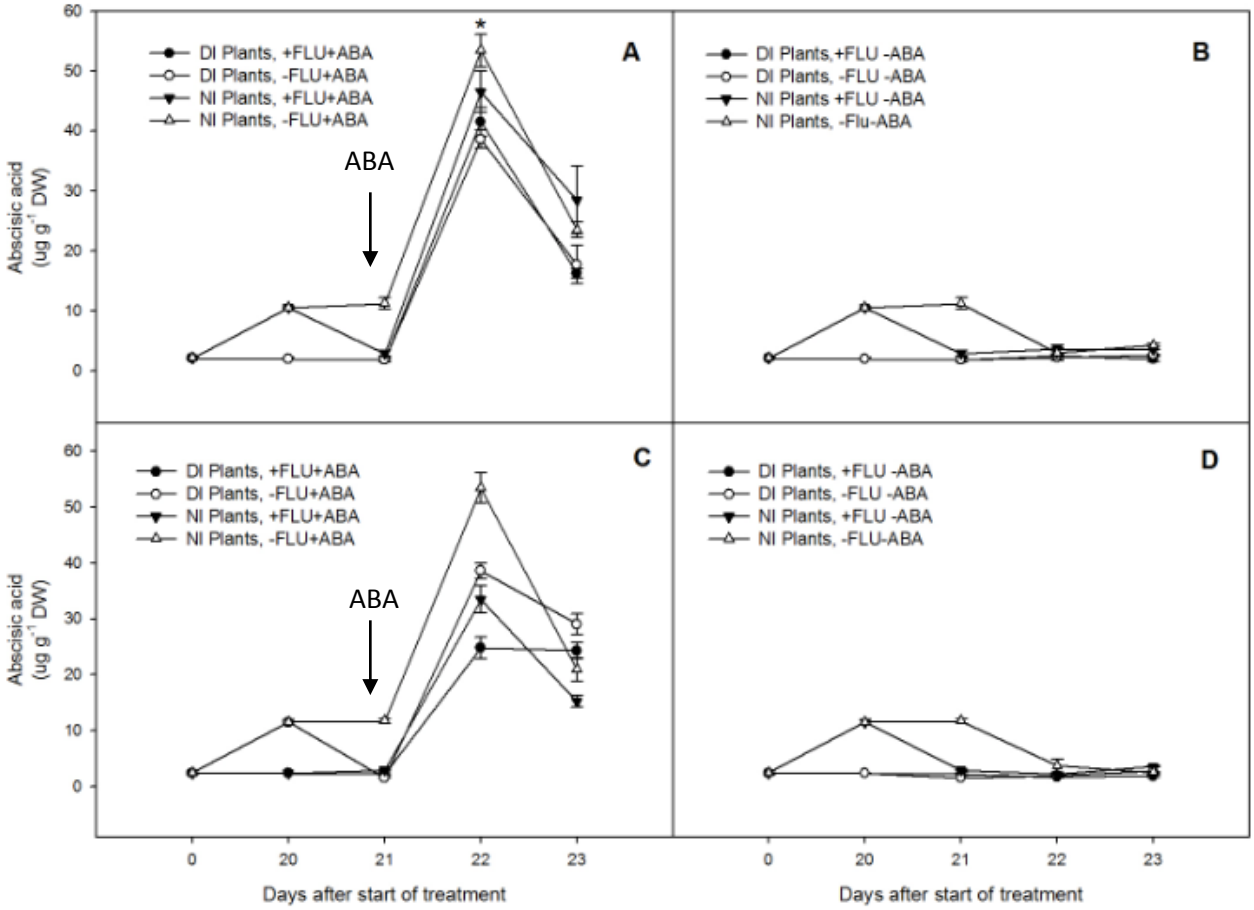


Figure Endogenous abscisic acid (ABA) concentration changes in response to two different water treatments and with or without fluridone solution application and with or without a subsequent ABA solution application. *Aristotelia chilensis* plants were either Daily Irrigated (DI) or Non-Irrigated (NI). A) Young leaves with ABA application; B) Young leaves without ABA application; C) Fully-expanded leaves with ABA application; and D) Fully-expanded leaves without ABA application. Values represent means ± SE (n=3).

Annex 4

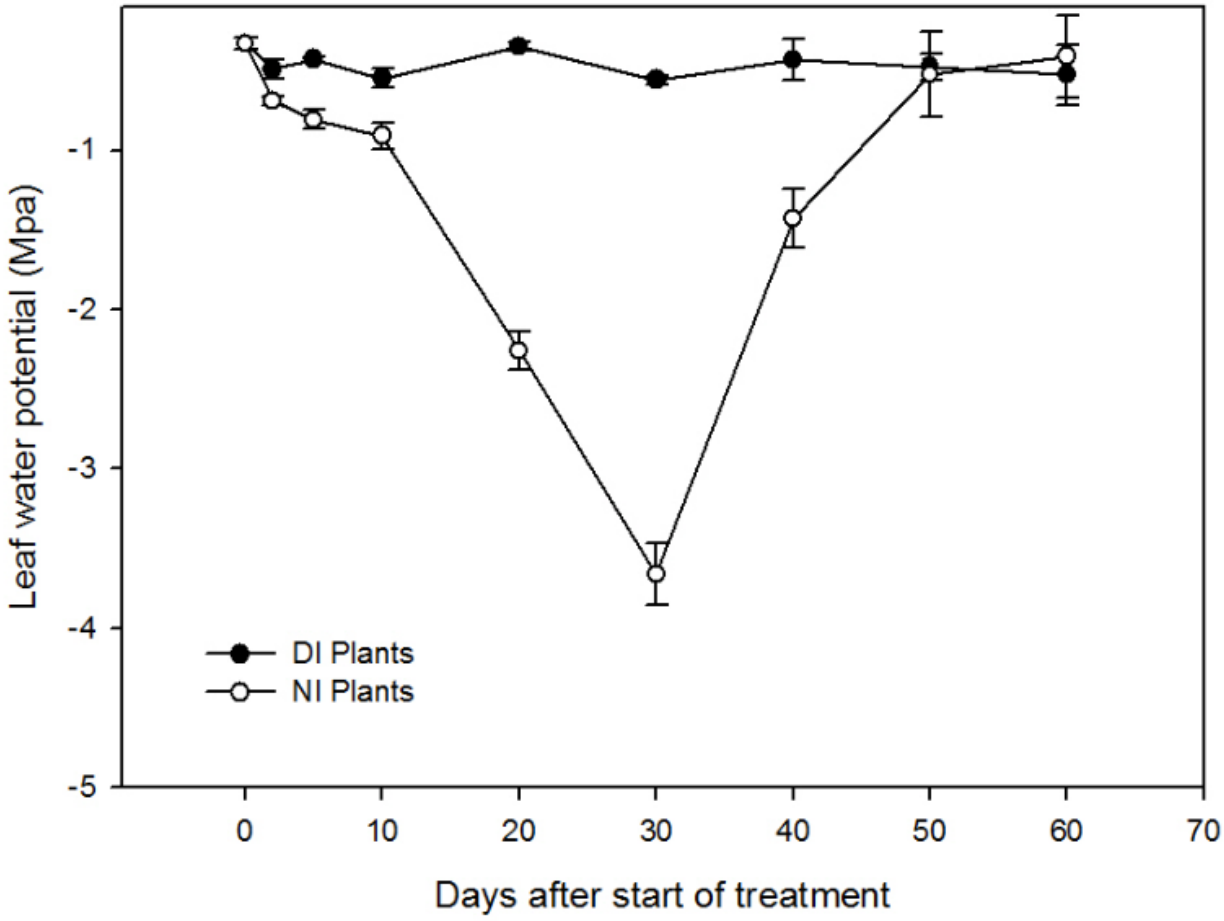


Figure. Leaf water potential of *Aristotelia chilensis* plants grown under two water treatments; Daily-irrigated (DI) and Non-irrigated (NI). DI plants were irrigated daily at field capacity, meanwhile, NI plants were subjected to drought stress. At the 30th day of drought stress, NI plants were irrigated to evaluate plant recovery. All values represent averages of three biological replicates ±SE.

Annex 5

Published paper

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Research article

Age-related mechanism and its relationship with secondary metabolism and abscisic acid in *Aristotelia chilensis* plants subjected to drought stress

Jorge González-Villagra[a], Acácio Rodrigues-Salvador[b], Adriano Nunes-Nesi[b], Jerry D. Cohen[c], Marjorie M. Reyes-Díaz[d,e,*]

Annex 6

Physiologia Plantarum (submitted)

Physiologia Plantarum
An International Journal for Plant Biology

Abscisic acid (ABA) is involved in phenolic compounds biosynthesis, mainly anthocyanins, in leaves of *Aristotelia chilensis* plants (Mol.) subjected to drought stress

Journal:	*Physiologia Plantarum*
Manuscript ID	PPL-2018-00002
Manuscript Type:	Regular manuscript - Ecophysiology, stress and adaptation
Date Submitted by the Author:	03-Jan-2018
Complete List of Authors:	Gonzalez, Jorge; Universidad de La Frontera Cohen, Jerry; University of Minnesota, Department of Horticultural Science and Microbial and Plant Genomics Institute Reyes, Marjorie; Universidad de La Frontera, Ciencias Químicas y Recursos Naturales
Key Words:	fully-expanded leaves, water stress, maqui, phytohormone, UFGT expression