UNIVERSIDAD DE LA FRONTERA

Facultad de Ingeniería y Ciencias

Doctorado en Ciencias de Recursos Naturales

EXOGENOUS METHYL JASMONATE APPLICATION EFFECTS ON PHYSIOLOGICAL AND BIOCHEMICAL FEATURES OF *Vaccinium corymbosum* CULTIVARS WITH CONTRASTING AL-RESISTANCE UNDER ALUMINUM TOXICITY

DOCTORAL THESIS IN FULFILLMENT OF THE REQUERIMENTS FOR THE DEGREE DOCTOR OF SCIENCES IN NATURAL RESOURCES

ELIZABETH MARIA INES ULLOA INOSTROZA

TEMUCO-CHILE

2017

Exogenous methyl jasmonate application effects on physiological and biochemical features of *Vaccinium corymbosum* cultivars with contrasting Al-resistance under aluminum toxicity

Esta tesis fue realizada bajo la supervisión de la directora de Tesis Dra. Miren Rita Alberdi Lag, perteneciente al Departamento de Ciencias Química y Recursos Naturales de la Universidad de La Frontera y es presentada para su revisión por los miembros de la comisión examinadora.

Elizabeth Maria Ines Ulloa Inostroza

……
Dra. Graciela Palma Cifuentes
Director (s) programa
Doctorado en Ciencias de Recursos Naturales

……
Dra. Miren Alberdi Lag
Profesor Guía

……
Dra. Marjorie Reyes Díaz
Profesor Co-guía

……
Dr. Juan Carlos Parra Aravena
Director Académico de Postgrado
Universidad de La Frontera

……
Dra. Maria De la luz Mora
Evaluador interno

……
Dra. Liliana Cardemil
Evaluador externo

……
Dr. Manuel Pinto
Evaluador externo

Cuando examino mis métodos de pensamiento, llego a la conclusión de que el don de la fantasía me ha significado más que mi talento para absorber el conocimiento positivo.

Albert Einstein

Acknowledgements

I would like to dedicate this thesis with my deepest gratitude to my family, Eric, Pilar, Elsie, Paula, Guillermo and Amanda for their unconditional love and support. The words cannot express how grateful I am by the beautiful life that God has given me and by continuous encouragement of my loved ones. I would not be where I am today without the support from my loving family. Special acknowledgements for my friends; Edith Cantero, Graciela Muñoz, Fabiola Fernandez, Katherine Isla, Carmen Julia Ortega, Paulina Huenuqueo, Macarena Corifuta, Emilia Mariangel and Schwaner-Rios (Pola and Pablo) family for their encouragement and inspiration during the toughest of times.

I would also like to thank my advisor the Dra. Miren Alberdi and Dra. Marjorie Reyes for their continuous support, dedication, and teaching in the development of this thesis, so too by time dedication on this project and friendship during these years of hard work… Thank you!!!. I thank both for welcoming me with open arms into their laboratory and believing in me as Scientist and supporting each crazy idea. Thanks by your guidance, by developed confidence in my ability to communicate and defend my research. For professionals and friends who made possible the develop of this thesis, thanks for trusting me and support, especially to Graciela Muñoz for all those hours of hard work and friendship. I would also like to thank my colleagues and lab members Cristian Meriño, Mariela Mora, and David Alarcón, whose companionship and support greatly improved my post-graduate experience, especially "The Danis" Daniela Peña and Daniela Tapia for their friendship and support. Similarly, I thanks to Rayen Millaleo, Marco Campos, Manuel Chacón, Patricio Barra, and Raphael Cuevas, who participated in this training process of the PhD program. Additionally, I extent my thanks to the thesis committee by their valuable suggestions and feedbacks during my advances, which were essential in the shaping and direction of my research.

A deep gratitude to University of Western Ontario in Canada, Department Biology (London-Canada), over all to the special working group of Dr. Norman Hüner. I deeply thank Dr. Hüner for giving me the opportunity to take part of his excellent workgroup and to Dr. Alex Ivanov and Dr. Leonid Kurepin, who always help me in my scientific

formation.

I thank to fellowship PhD Nº21110919 and FONDECYT Projects Nº 1120917 that support of this work, both of Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) of the Government of Chile, as well as the projects n° 2013 DI 13-2017, 2015 DI 15-2015, and 2016 DI 16-2011 from the Dirección de Investigación at the Universidad de La Frontera, Temuco, Chile. I also thank the financial support of the Doctorado en Ciencias en Recursos Naturales program and Scientific and Technological Bioresource Nucleus (BIOREN) to financial support to participate at different national and international scientific conferences, and also make possible much of this research.

SUMMARY

Vaccinium corymbosum L. (Highbush blueberry) is a plant species native from North America adapted to the edaphoclimatic conditions of the southern central region of Chile, where it grows on acid soils (Andisols), being a constraint for its optimal growth the Al-toxicity (Al^{3+}) typical of these soils. Plants exposed to toxic Al manifested a decline of root cell elongation, resulting in a reduced root system, which limits water and nutrient absorption. This also affects other plant functions, such as the photosynthetic performance (decrease in the photochemical efficiency of PSII and CO_2 assimilation) due to an increase of reactive oxygen species (ROS) induced by the Al-toxicity, which alters functionality of the cell membranes inducing lipid peroxidation (LP) and a loss of cellular compartmentalization. Furthermore, under Al-toxic conditions plants can integrate diverse responses to decrease the damage; among them are phytohormones as the jasmonates (JAS) and its derivatives such as methyl jasmonate (MeJA). It has been reported that MeJA application stimulates the antioxidant response in some agronomic and fruit species under different stress conditions; water stress, salinity, toxic metals (TM), insects, and mushrooms. Nowadays, no studies are found dealing with the interaction of toxic Al and MeJA in *Vaccinium corymbosum* (blueberry) cultivars. We consider of utmost importance to study the exogenous MeJA effects on physiological and biochemical responses as a possible inductor of resistance under Al-toxic in blueberry. Because of this it was *hypothesized that the exogenous application of MeJA to V. corymbosum cultivars with contrasting Al-resistance under exposure to Al-toxicity enhances enzymatic and non-enzymatic antioxidant mechanisms, photosynthetic performance decreasing the Al toxicity in both cultivars, being more evident in Al-sensitive cultivar of blueberry*. Therefore, the *aim* of this PhD thesis was *to study the effects of MeJA application on physiological and biochemical features of V. corymbosum cultivars with contrasting Al-resistance under aluminum toxicity*, through the following specific aims: (I) To determine the application time of MeJA able to diminish the Al-toxicity in blueberry cultivars subjected to Al toxicity, (II) To determine the exogenous MeJA dose application able to decrease the Al toxicity of blueberry cultivars subjected to Al treatments, and (III) To evaluate the enzymatic and non-enzymatic antioxidant mechanisms and photosynthetic performance of blueberry cultivars subjected to toxic dose of Al using the MeJA concentrations able to

decrease the Al-toxicity. To answer these goals three studies compiled in chapters were performed: i) Toxic metals and jasmonates: biological implication in plants (Chapter II), ii) Low doses of exogenous MeJA applied simultaneously with toxic aluminum improves the antioxidant performance of *V. corymbosum* (Chapter III), and iii) Protective effect of methyl jasmonate on photosynthetic performance and its association with antioxidants in two blueberry cultivars with contrasting Al-resistance exposed to Al-toxic (Chapter IV). Thus, according to the main results in this thesis, it is concluded that: the lower MeJA dose (5 μM) applied simultaneously with Al^{3+} in blueberry cultivars with different Al-resistance decreased the Al-toxic effect in leaves and roots. Therefore, a lower Al-concentration and a reduction of oxidative damage by the stimulation of the antioxidant response, which limit the chloroplasts exposition to the Al-toxicity in the leaves, favoring a normal photosynthetic performance in the blueberry cultivars by MeJA application under Al-toxicity, were observed.

CONTENTS

Figure index

CHAPTER II. Toxic metals and jasmonates: biological implication in plants

CHAPTER III. Low doses of exogenous methyl jasmonate applied simultaneously with toxic aluminum improve the antioxidant performance of *Vaccinium corymbosum*

Table Index

significant differences between MeJA application times for the same treatment and cultivar. Asterik (*) shows significant differences between cultivars at the same treatment and time of MeJA application ($P < 0.05$)

CHAPTER IV. Protective effect of MeJA on photosynthetic performance and its association with antioxidants in two blueberry cultivars with contrasting Al-resistance exposed to Al-toxic.

Table 1. Chlorophyll *a+b*, chlorophyll *a/b*, and carotenoids concentration (μg g^{-1} FW) in two blueberry cultivars with contrasting Al-resistance and the MeJA application leaves and under Al-toxicity for different timesanalyze. Values represent the average or means of 3 replicates ± S.E. and doses in 5 μM of MeJA and 100 μM Al. Different lowercase letters show statistically significant differences among the treatments at each time of MeJA application (0 or 24 or 48) in the same cultivar. Different capital letters show significant differences between time analyses for the same treatment and cultivar. Asterisk (*) shows significant differences between cultivars at the same treatment and time of analyses ($P < 0.05$). The start of experiment (0 time) for each parameter did not show statistically significant differences with the control at each time (24 and 48 h) and therefore they were not indicated in the table. 80

CHAPTER I

General Introduction

1. General Introduction

1.1. Acid soils and aluminum phytotoxicity in plants

Acid soils cover 30-40% arable land and more than 70% potential arable land (Von Uexkuell and Mutert, 1995). In these soils pH is lower as 5.5, due to an increment of H^+ ions concentration when basic cations (Ca^{2+}, Mg^{2+}, K^+ and Na^+) are leached from soils (Delhaize and Ryan, 1995). Soil acidity can be intensified due to certain farming practices such as application of ammonium-based N fertilizers (Hede et al. 2001; Zheng, 2010). In Chile, acid soils cover an important part of the national territory. There are more than 5.1 million hectares of volcanic ash soils, originated from intense vulcanological activity. They cover about 50% of soils in the south central region (Besoain, 1985), being Andisols one of the most important soil type. These soils are characterized by high acidity (pH 4.5 to 5.2), high concentration of organic matter (OM) (Mora et al. 2002), presence of amorphous clays (allophane or imogolite) (Mora and Demanet, 1999), high water retention capacity, high anionic absorption capacity, low P and N availability. As consequence of soil acidity, toxic forms of aluminum (Al^{3+}) increased their availability, limiting growth and productivity of plants (Mora et al. 2004; 2005; 2006; Meriño-Gergichevich et al. 2010).

In plants, the Al^{3+} restricts primarily root growth due to an increase of its concentration in roots, which disrupt the functions of the plasma membrane and cell walls (Kopittke et al. 2008; Horst et al. 2010). The root uptake of Al^{3+} from soil occurs by two-pathways: root apoplast (cell wall and the adjacent cell membrane) and root symplast (cell membrane and intra-cell sites) (Ma et al. 2004; Horst et al. 2010; Sujkowska-Rybkowska and Borucki, 2016). The Al^{3+} flow moves through the apoplastic and symplastic root pathways to the Casparian strip, where all Al^{3+} is transported by symplastic pathway to the root xylem and mobilized to leaves (see Chapter V Fig. 1 in this thesis) (Rengel, 1996; Kochian et al. 2015).

The accumulation of Al^{3+} in plant cells, exacerbates the normal metabolic production of reactive oxygen species (ROS), like as superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\cdot}$), hydroperoxyl radical ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2) (see Chapter V Fig. 1 in this thesis), alkoxy radical ($RO^{\cdot}$), peroxy radical ($ROO^{\cdot}$), and singlet oxygen ($^1O_2$) (Mitter

2012; Silva et al. 2013). Increase of ROS causes oxidative stress and lipid peroxidation (LP) of cell membranes, cellular organelles and organic molecules, which can result in cell death (Yamamoto et al. 2002; Guo et al. 2007; Ma et al. 2007). These alterations are due to strong Al^{3+} ion interaction with lipid components of the plasma membrane, causing its rigidification and loss of fluidity (Panda et al. 2009). In wheat (*Triticum aestivum* L.) cultivars with contrasting Al resistance (Al-sensitive and Al-tolerant) exposed under 30 μM Al for 24 h causes an exponential increase in the LP and H_2O_2, and $O_2^{\bullet-}$ contents, as has been reported by Xu et al. (2010). Similarly, in highbush blueberry (*Vaccinium corymbosum* L.) subjected to Al-stress for 48h an increase in the LP and H_2O_2 was found (Reyes-Díaz et al. 2009; Ulloa-Inostroza et al. 2016).

Plants have evolved mechanisms to counteract Al^{3+} damage (Kochian, 1995; Piñeros, 2005; Ryan et al. 2010).Two mechanisms have been proposed to resist Al-toxicity, depending on the site of Al^{3+} detoxification: external exclusion and internal tolerance (Kochian, 1995; Taylor, 1991). The exclusion mechanism avoids toxic Al entering into the symplasm by its retention in the apoplast (Piñeros, 2005; Inostroza-Blancheteau et al. 2012; Ryan et al. 2010), whereas in the internal tolerance mechanism toxic Al is immobilized, compartmentalized, or detoxified in the cell symplasm. Both mechanisms utilize antioxidant responses and organic acids to decrease Al^{3+} effects on apoplast and simplast (Rengel, 1996; Kochian et al. 2015). The former involves enzymatic and non-enzymatic compounds (Mossor-Pietraszewska, 2001). Between the enzymatic compounds, superoxide dismutase (SOD; EC. 1.15.1.1) and catalase (CAT; EC: 1.11.1.6) catalyze reactions to get rid of the ROS forming non-toxic compounds (Gill and Tuteja, 2010). The SOD catalyzes dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2, followed by the CAT that catalyzes the dismutation of two molecules of H_2O_2 into water and oxygen (see Chapter V Fig. 1 in this thesis) (Bhaduri and Fulekar, 2012). In roots of wheat, it is reported an increase of oxidative damage concomitant with enhanced SOD and CAT activity during 24 h of Al exposition (Xu et al. 2010). In the case of non-enzymatic antioxidants, the phenolic compounds (PCs), which are molecules of low molecular weight, have an important role in chelating Al in the apoplast and simplast of cells (Kochian et al. 2015). The PCs have hydroxyl or carboxyl groups, which are good H-donating for scavenging ROS, breaking the cycle of generation of new radicals (see Chapter V Fig. 1 in this thesis) (Ofei-Manu et al. 2001). In some plants an

increase of Al^{3+} concentration enhanced the PCs such as: chlorogenic and ellagic acids in blueberry (Manquián et al. 2013), and total phenols in buckwheat (*Fagopyrum esculentum* Mill.) (Smirnov et al. 2015). Furthermore, organic acids (citric, oxalic, and malic) have a role in detoxifying Al according to the stability constants of the Al-organic acids complexes (Kochian et al. 2015), which participation in these responses are well documented in barley (*Hordeum vulgare*) (Ma et al. 2016), and pummelo (*Citrus grandis*) (Yang et al. 2012).

Physiological processes such as photosynthetic performance (photochemical efficiency of PSII and CO_2 assimilation) are negatively affected by the Al-toxicity (Zhang et al. 2007; Li et al. 2012; Reyes-Díaz et al. 2009; 2010). Photosynthetic performance can be disturbed because of the reduction in chlorophyll and other light absorbing pigments, which can reduce light energy absorption and thereby the efficiency of photosystem II (PSII) and photosystem I (PSI). Photochemical parameters as maximum quantum yield of the PSII (*Fv/Fm*), effective quantum yield (ΦPSII), electron transport rate (ETR), were reduced under Al^{3+} as found in mandarin (*Citrus reshni*) (Chen et al 2005), blueberry (Reyes-Díaz et al. 2009), and wheat (Valle et al 2009), among others. In addition photosynthetic rate (CO_2 assimilation), intercellular CO_2 concentration, stomatal conductance, and transpiration rate are negatively affected by Al^{3+} as observed in leaves of wheat (Ohki, 1985), maize (*Zea mays* L.) (Mihailovic et al. 2008), barley (Ali et al. 2011), and cacao (*Theobroma cacao* L.) (Ribeiro et al. 2013).

In the last decades, an important hormonal role in the reduction of oxidative stress induced Al^{3+} has been described in plants. Jasmonates (JAS), including jasmonic acid (JA) and the volatile methyl jasmonate (MeJA) have been linked with the decrease of the some stresses as salinity (Ismail et al. 2012), ultraviolet radiation (Larronde et al. 2003) in some plants, depending on the dose and time of MeJA application. The JAS are active derivatives of linolenic and linoleic acids (unsaturated fatty acids) released by the action of lipases from membrane phospholipids of the cycle cyclopentanone (Creelman and Mullet, 1995). JAS are able to activate or regulate the secondary metabolism, increasing the antioxidant defense to counteract the oxidative stress induced by Al^{3+} (Xue et al. 2008; Ulloa-Inostroza et al. 2016). The exposure to either Al toxicity or exogenous MeJA application accumulated higher scopolamine and hyoscyamin (secondary metabolites) in *Brugmansia x*

candida plants (Spollansky, 2000). In *Cassia tora* plants exposed to Al toxicity and MeJA application an upregulation of gene expression of lipoxygenases (LOX, EC 1.13.11.12), and L-phenylalanine ammonialyase (PAL, EC 4.3.1.5) was shown (Xue et al. 2008). Later, Roselló et al. (2015) observed increases of JA concentrations in roots of an Al-tolerant variety of rice (*Oryza sativa*) exposed at 500 μM Al for 48 h. Despite the importance of this group of phytohormones in the reduction of oxidative damage and activation of antioxidant defenses in plants under toxic Al, very little studies about this topic exist. More abundant, but contradictory, are the studies showing effects of these phytohormones on plants subjected to other toxic metals, such as Cd, Pb, Cu, and Cs (see Chapter IV in this thesis). Surprisingly, no studies exist considering the importance of the simultaneous or deferred application of MeJA doses and Al-toxic in the plant response.

1.2. Highbush blueberry (*Vaccinium corymbosum*) in Chile and Al^{3+}

In the last two decades an important crop, *V. corymbosum* L. (highbush blueberry) was introduced in our country. Blueberry is a native plant from North America, belonging to the Ericaeae family, adapted to the edaphoclimatic conditions of the southern central region of Chile (Ireland and Wilk, 2006). Currently, there are productive orchards from Coquimbo (29° 20´ S) to Los Lagos (44° 14´ S) regions, covering 14.544 planted hectares with a production of 87.110 tons exported during 2015 with returns of about thousands U.S.$ 509.195 FOB (Muñoz, 2016). This crop has a great impact worldwide and also in our country, because of its beneficial effects on human health and nutrition, due to its richness in antioxidant compounds (in fruits, leaves and roots) (Ribera et al. 2010, Meriño-Gergichevich et al. 2015). Among the antioxidant compounds, anthocyanins are highly concentrated in blueberry fruits compared with those cultivated in the northern hemisphere (Ribera et al. 2010).

Although this plant species required acid conditions for its growth (Haynes and Swift, 1985; Apse and Karklins, 2013), the presence of Al^{3+} in the acid Andisols, constitutes one of the major limiting factor for its growth and productivity (Reyes-Díaz et al. 2009, Inostroza-Blancheteau et al. 2011). Thus, the aim of this research is to study the effect of

exogenous application of MeJA in *V. corymbosum* cultivars with contrasting Al-resistance cultivated under Al-toxic on physiological (photosynthetic performance) and biochemical features (oxidative damage and antioxidant responses). In addition, the MeJA time application and dose able to counteract the deleterious Al effects in blueberry cultivars will be performed.

1.3 Hypotheses of this thesis

Hypothesis: Exogenous application of MeJA to *V. corymbosum* cultivars with contrasting Al-resistance under exposure to Al-toxicity enhance enzymatic and non-enzymatic antioxidant mechanisms, photosynthetic performance decreasing the Al toxicity in the contrasting cultivars, being more evident in the Al-sensitive cultivar of blueberry.

1.4 General aim:

To study the effects of MeJA application on physiological and biochemical features of *V. corymbosum* cultivars with contrasting Al-resistance under aluminum toxicity.

1.4.1 Specific aims:

I. To determine the application time of MeJA able to diminish the Al-toxicity in blueberry cultivars subjected to Al toxicity.

II. To determine the exogenous MeJA dose application able to decrease Al toxicity of blueberry cultivars subjected to Al toxicity.

(III) To evaluate the enzymatic and non-enzymatic antioxidant mechanisms and photosynthetic performance of blueberry cultivars subjected to toxic Al using the MeJA concentrations able to decrease the Al-toxicity. To answer these goals three studies compiled in chapters were performed:

Reference

Ali S, Zeng F, Qiu L, Zhang G (2011) The effect of chromium and aluminum on growth, root morphology, photosynthetic parameters and transpiration of the two barley cultivars. Biol Plantarum 55:291-296

Apse J, and Karklins A. (2013) Modification of Soil Properties for Blueberry (*Vaccinium Corymbosum* L.) Cultivation. Proc Latv Univ Agr 30:1-10.

Besoain E (1985) Mineralogía de arcillas de suelos. In: IICA, ed. Costa Rica;

Bhaduri AM, Fulekar MH (2012) Antioxidant enzyme responses of plants to heavy metal stress. Rev Environ Sci Biotechnol 11:55-69.

Chen L-S, Qi Y-P, Liu X-H (2005) Effects of aluminum on light energy utilization and photoprotective systems in citrus leaves. Annals of Botany 96:35-41

Creelman RA, Mullet JE (1995) Jasmonic acid distribution and action in plants: Regulation during development and response to biotic and abiotic stress. Proc Natl Acad Sci USA 92:4114-4119

Delhaize E, Ryan P (1995) Aluminum toxicity and tolerance in plants. Plat Physiol 107:315-321.

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48: 909–930

Guo TR, Zhang GP, Zhang YH (2007) Physiological changes in barley plants under combined toxicity of aluminum, copper and cadmium. Colloids Surf B 57:182-188

Hede, AR, Skovmand B, López-Cesati J (2001) Acid soil and aluminum toxicity, In: M.P. Reynolds, J.J. Ortiz-Monasterio, and A. Mchab (eds.). Application of physiology in wheat breeding. International Maize and Wheat Improvement Center p. 172 - 182.

Horst WJ, Wang Y, Eticha D (2010) The role of the root apoplast in aluminium-induced

inhibition of root elongation and in aluminium resistance of plants: a review. Ann Bot. 106:185-197.

Inostroza-Blancheteau C, Reyes-Díaz M, Aquea F, Nunes-Nesi A, Alberdi M, Arce-Johnson P (2011) Biochemical and molecular changes in response to aluminium-stress in highbush blueberry (*Vaccinium corymbosum* L). Plant Physiol Biochem 49:1005-1012

Inostroza-Blancheteau C, Rengel Z, Alberd M, Mora M, Aquea F, Arce-Johnson P, Reyes-Díaz M (2012) Molecular and physiological strategies to increase aluminum resistance in plants. Mol Biol Rep 39:2069-2079

Ireland G, Wilk, P (2006) Blueberry production in northern NSW. Primefact 195. 10 Oct. 2008. <http://www.dpi.nsw.gov.au/__data/assets/pdf_file/0005/90356/Blueberry-production-in-northernNSW.pdf>.

Ismail A, Riemann M, Nick P (2012) The jasmonate pathway mediates salt tolerance in grapevines. J Exp Bot 63:2127-2139

Kochian, 1995; Kochian L (1995) Cellular mechanisms of aluminum toxicity and resistance in plants. Annu Rev Plant Physiol Plant Mol Biol 46:237-260

Kochian LV, Pineros MA, Liu J, Magalhaes JV (2015) Plant Adaptation to acid soils: The molecular basis for crop aluminum resistance. Annu Rev Plant Biol 66:571-598

Kopittke PM, Blamey FPC, Menzies NW (2008) Toxicities of soluble Al, Cu, and La include ruptures to rhizodermal and root cortical cells of cowpea. Plant Soil 303:217-227

Larronde F, Gaudillière JP, Krisa S, Decendit A, Deffieux G, Mérillon JM (2003) Airborne methyl jasmonate induces stilbene accumulation in leaves and berries of grapevine plants. Am J Enol Vitic 54:60-63

Li Z, Xing F, Xing D (2012) Characterization of target site of aluminum phytotoxicity in photosynthetic electron transport by fluorescence techniques in tobacco leaves. Plant Cell Physiol 53:1295-1309

Ma B, Wan J, Shen ZG (2007) H_2O_2 production and antioxidant responses in seeds and early seedlings of two different rice varieties exposed to aluminum. Plant Growth Regul 52:91-100

Ma Y, Li Ch, Ryan PR, Shabala S, You J, Liu J, Liu Ch, Zhou M (2016) A new allele for aluminium tolerance gene in barley (*Hordeum vulgare* L.). BMC Genomics. doi: 10.1186/s12864-016-2551-3

Manquián K, Zúñiga GE, Barrientos H, Escudey M, Molina M (2013) Effect of aluminum on antioxidant activity and phenolic compounds content in *in vitro* cultured blueberries. Bol Latinoam Caribe Plant Med Aromat 12:603-611

Meriño-Gergichevich C, Alberdi M, Ivanov AG, and Reyes-Díaz M (2010) Al^{3+}-Ca^{2+} Interaction in plants growing in acid soils: Al-phytotoxicity response to calcareous amendments. J Soil Sci Plant Nutr 10:217-243

Meriño-Gergichevich C, Ondrasek G, Zovko M, Šamec D, Alberdi M, Reyes-Díaz M (2015) Comparative study of methodologies to determine the antioxidant capacity of Al-toxified blueberry amended with calcium sulfate. J Soil Sci Plant Nutr 15:965-978.

Mihailovic N, Drazic G, Vucinic Z (2008) Effects of aluminium on photosynthetic performance in Al-sensitive and Al-tolerant maize inbred lines. Photosynthetica 46:476-480

Mora M, Demanet F (1999) Uso de enmiendas calcáreas en suelos acidificados. Frontera Agrícola (Chile) 5:43-58.

Mora ML, Cartes P, Demanet R, Cornforth IS (2002) Effects of lime and gypsum on pasture growth and composition on an acid Andisol in Chile, South America. Commun Soil Sci Plant Anal 33:2069-2081.

Mora M, Alfaro M, Williams PH, Stehr W, Demanet R (2004) Effect of fertilizer input on soil acidification in relation to growth and chemical composition of a pasture and animal production. R C SueloNutr Veg 4:29-40

Mora ML, Demanet R, Vistoso E, Gallardo F (2005) Influence of sulfate concentration in mineral solution on ryegrass grown at different pH and aluminum levels. J. Plant Nutr. 28:1–16.

Mora ML, Alfaro MA, Jarvis SC, Demanet R, Cartes P (2006) Soil aluminum availability in Andisols of Southern Chile and its effect on forage production and animal metabolism. Soil Use Manage. 22:95-101.

Mossor-Pietraszewska T (2001) Effect of aluminium on plant growth and metabolism. Acta Biochim Pol 3;673- 686

Muñoz (2016) Boletín frutícola Marzo-2016. Publicación de la Oficina de Estudios y Políticas Agrarias (ODEPA) del Ministerio de Agricultura, Gobierno de Chile

Ofei-Manu P, Wagatsuma T, Ishikawa S, Tawaraya K (2001) The plasma membrane strength of the root-tip cells and root phenolic-compounds are correlated with Al tolerance in several common woody plants. Soil Sci. Plant Nutr. 47:359-375

Piñeros MA, Shaff JE, Manslank S, Carvalho A, Vera M, Kochian LV (2005) Aluminum resistance in maize cannot be solely explained by root organic exudation: A comparative physiological study. Plant Physiol 137:231-241

Rengel Z **(**1996) Uptake of aluminum by plant cells. New Phytol 134:389-406

Reyes-Díaz M, Alberdi M, Mora ML (2009) Short-term aluminum stress differentially affects the photochemical efficiency of photosystem II in Highbush Blueberry genotypes. J Amer Soc Hort Sci 134:14-21

Reyes-Díaz M, Inostroza-Blancheteau C, Millaleo R, Cruces E, Wulff-Zottele C, Alberdi M, Mora ML (2010) Long-term aluminum exposure effects on physiological and biochemical features of Highbush Blueberry cultivars. J Amer Soc Hort Sci 135: 212-222

Ribera AE, Reyes-Díaz M, Alberdi M, Zuniga GE, Mora ML (2010) Antioxidant compounds in skin and pulp of fruits change among genotypes and maturity stages in

Highbush blueberry (*Vaccinium corymbosum* L.) grown in southern Chile. J Soil Sci Plant Nutr 10:509-536

Ryan PR, Tyerman SD, Sasaki T, Furuichi T, Yamamoto Y, Zhang WH (2011) The identification of aluminium-resistance genes provides opportunities for enhancing crop production on acid soils. J Exp Bot 62:9-20.

Ribeiro MAQ, Almeida AAFD, Mielke MS, Gomes FP, Pires MV, Baligar VC (2013) Aluminum effects on growth, photosynthesis, and mineral nutrition of cacao genotypes. J Plant Nutr 36:1161-1179

Roselló M, Poschenrieder C, Gunsé B, Barceló J, Llugany M (2015) Differential activation of genes related to aluminium tolerance in two contrasting rice cultivars. J Inorg Biochem 152:160–166

Silva JOC, Paiva EAS, Modolo LV, Clésia Cristina Nascentes CC, Costa Françaa MG, (2013) Removal of root apices enables study of direct toxic effects of aluminum on rice (*Oryza sativa L.)* leaf cells. Envrion Exp Bot 95:41-49

Spollansky TC, Pitta-Alvarez SI, Giulietti AM (2000) Effect of jasmonic acid and aluminum on production of tropane alkaloids in hairy root cultures of *Brugmansia candida*. Electron J Biotechn 3:72-75

Sujkowska-Rybkowska M, Borucki W 2016 Pectins esterification in the apoplast of aluminum-treated pea root nodules. J Plant Physiol 184:1-7

Taylor GJ (1991) Current views of the aluminum stress response: The physiological basis of tolerance. Curr Top Plant Biochem Physiol 10:57-93

Ulloa-Inostroza EM, Alberdi M, Meriño-Gergichevich C, Reyes-Díaz M (2016) Low doses of exogenous methyl jasmonate applied simultaneously with toxic aluminum improves the antioxidant performance of *Vaccinium corymbosum.* Plant Soil. doi: 10.1007/s11104-016-2985-z

Valle SR, Carrasco J, Pinochet D, Calderini DF (2009) Grain yield, above-ground and root biomass of Al-tolerant and Alsensitive wheat cultivars under different soil aluminum concentrations at field conditions. Plant Soil 318:299-310.

Von Uexküll HR, Mutert E (1995) Global extent, development and economic impact of acid soils. In: Date RA, Grundon NJ, Raymet GE, Probert ME, editors. Plant–Soil Interactions at Low pH: Principles and Management. Dordrecht, The Netherlands: Kluwer Academic Publishers. 5-19 pp

Xue YJ, Ling T, Yang ZM (2008) Aluminum-induced cell wall peroxidase activity and lignin synthesis are differentially regulated by jasmonate and nitric oxide. J Agric Food Chem 56:9676-9684

Xu XM, Lin H, Maple J, Björkblom B, Alves G, Larsen JP, Møller SG (2010) The *Arabidopsis* DJ-1a protein confers stress protection through cytosolic SOD activation. J Cell Sci 123;1644-1651

Yamamoto Y, Kobayashi Y, Devi SR, Rikiishi S, Matsumoto H (2002) Aluminum toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. Plant Physiol 128:63-72

Zhang X-B, Liu P, Yang YS, Xu GD (2007) Effect of Al in soil on photosynthesis and related morphological and physiological characteristics of two soybean genotypes. Botanical Studies 48:435-444

CHAPTER II

Toxic metals and jasmonates: biological implication in plants.

(In preparation)

Toxic metals and jasmonates: biological implication in plants.

2.1 Abstract

The industrialization and urbanization as well as the human activities have generated increasing toxic metals (TM) in soils available for plants. The TM in soil alter the normal plant functions, causing serious damages (with exception the metallophytes), such as membrane lipid peroxidation (LP), photosynthesis reduction, alterations in nutrient transport, cellular compartmentalization damage, among others. In addition, in response to these damages, important signaling molecules to counteract this condition are found. Jasmonic acid (JA) and methyl jasmonate (MeJA; methyl ester of JA), likewise known as jasmonates (Jas), are molecules synthesized by plants in response to stress conditions through the reception of the stimuli and activation of defense signals. Studies with TM and Jas in plants have reported contradictory results. Some researchers showed that exogenous MeJA or JA application in plants growing under TM stress conditions promote an increase in reactive oxygen species (ROS) production, inducing biological membrane damages and cell death. On the other hand, other reports consider that the exogenous MeJA or JA applications are able to reduce the negative effects of TM, ameliorating the decrease of biomass, carbohydrates, proteins and antioxidant contents (e.g. ascorbic acid and glutathione), besides a decrease in LP. This review provides insights about the recent advances in research on TM and Jas (JA or MeJA) and their interactions as well as the involved biochemical, physiological, and molecular feature.

Keywords: Abiotic stress; Aluminum; Cadmium; Copper; Jasmonic acid; Lead; Methyl jasmonate.

2.2. Introduction

The plants are exposed to many environmental stresses, such as high radiation (Ritter and Koller, 1994), water stress (Álvarez et al. 2012), salinity (Ashraf M. 2004), and toxic metals (TM) (Maksymiec and Krupa, 2002a; Maksymiec et al. 2007), affecting negatively

their growth, development and productivity (Bidar et al. 2007; Cui et al. 2005; Granero and Domingo, 2005). The TM is augmented by human activities as industrialization and urbanization, which have deteriorated ecosystems (Weck and Clijsters, 1996). To cope this stress, plants activate mechanisms to counteract its negative effects. However, there are species adapted to metalliferous substrate named metallophytes and those which are outstanding from metallophytes habitats corresponding to non-metalophytes. Metallophyte species have morphological, physiological, biochemical and molecular mechanisms to counteract TM excess, accumulating in their structure (Bidar et al. 2007; Gardea-Torresdey et al. 2005). Non-metallophytes species did not accumulate TM in their tissues. It is accepted that under TM effect the first activated mechanisms are biochemical, among them the activation of antioxidant systems (enzymatic and nom-enzymatic), which mitigate the increase of reactive oxygen species (ROS) and lipid peroxidation (LP) of membranes (Beltrano et al. 1998; Hung and Kao, 1996; Maksymiec, 1998; Maksymiec and Krupa, 2002b; Maslenkova et al. 1990; Ozawa et al. 2000; Xiang and Oliver 1998). Concerning the effects of TM stresses on plants, the stimulation in the gene expression and metabolic pathways that prevent the toxic effect of the TM has also been reported (Hossain M.A et al. 2012). In this concern, phytohormones like jasmonates (Jas) and their methyl ester (MeJA) could ameliorate the negative effects of TM (Maksymiec and Krupa, 2006; Piotrowska-Niczyporuka et al. 2009; 2012). However, studies about the effect of JA or MeJA on TM have demonstrated contradictory effects on plants. Authors have established a synergistic negative effect between TM and JA or MeJA (Maksymiec et al. 2005; Maksymiec and Krupa, 2006; Piotrowska-Niczyporuka et al. 2012), whereas others concluded that plant defense mechanisms against TM are activated by Jas, depending on the TM, MeJA doses and plant species (Maksymiec and Krupa, 2002a; Wang et al. 2008; Piotrowska-Niczyporuka et al. 2009). In *Wolffia arrhiza* plants subjected to toxic lead (Pb) concentrations, and high JA concentrations (100 µM), a decrease in chlorophyll a and carotenoids pigments was found, whereas at low concentrations of JA (0.1 µM), a decrease in the oxidative damage exerted by TM in the cellular compartments was observed (Piotrowska-Niczyporuka et al. 2009). Based on the contradictory results we will perform a review provides insights about the recent advances in research on TM and Jas (JA or MeJA) and their interactions as well as the involved biochemical, physiological, and

molecular feature.

2.3. Toxic metal in plants

The TM are defined as chemicals whose elemental forms have a density equal to or greater than 6 g cm^{-3} or an atomic number greater than 20 (excluding alkali and alkaline earth metals), presenting values less than 0.1% and usually less than 0.01% in the earth's crust (Gardea-Torresdey et al. 2005; Hossain M.A et al. 2012). Currently, TM has been classified into two types: I) TM with known biological functions: arsenic (As), boron (B), cobalt (Co), chromium (Cr), copper (Cu), molybdenum (Mo), manganese (Mn), nickel (Ni), selenium (Se) y zinc (Zn) and II) TM with unknown biological functions: barium (Ba), cadmium (Cd), mercury (Hg), lead (Pb), antimony (Sb), and bismuth (Bi) (Lasat, 2002). Although aluminum (Al) is not strictly a TM, in this review it is included among the TM, because its effects generate toxic functional toxic alterations in plants (Krtková et al. 2012; Reyes-Díaz et al. 2009; 2010). The root is the primary target of TM in plants, depending on soil characteristics (soil components, physico-chemical properties, pH, temperature, and humidity) (Hamon et al. 1995; Atanassova, 2004). A good alternative to decrease toxic effect of metals in soil is the incorporation of plant species able to change the rhizosphere pH through the root exudates, which can alter the redox state of rhizosphere zone (Pérez de Mora et al. 2006; Yang et al. 2010). The exudation organic compounds of low molecular weight as: malate, citrate and oxalate can change the chemical state of metals (Larsen et al. 1998; Ma et al. 2000; Maiti et al. 2004; Mora et al. 2009). These organic compounds form stable complexes with toxic ions (e.g. Al) in the soil solution, changing the TM bioavailability. This constitute the first TM avoiding effects named exclusion mechanisms (external tolerance) (Fig. 1) (Bao et al. 2011; Dong et al. 2007; Malinowski et al. 2004; Manara, 2012; Qin et al. 2007).

Then, when TM is inside of cell roots, the first stress response is the increase of ROS production, causing oxidative stress (OS) and LP of membranes is induced (Mithöfer et al. 2004; Sytar et al. 2013; Zhang et al. 2007). Under these conditions, the antioxidant

mechanisms are activated both enzymatic (superoxide dismutase (SOD), ascorbate peroxidase (APX), CAT (catalase), among others) and non-enzymatic (ascorbic acid, α-tocopherol, flavonoids, carotenoids, among others) to minimize damage in organic molecules (Cakmak and Horst, 1991; Ghanati et al. 2005; Mora at al. 2009; Reyes-Díaz et al. 2009; 2010; Shao et al. 2008).

Inside the cell, as internal mechanism tolerance, TM can be immobilized by binding them to polysaccharides, glycoproteins and specific proteins as phytochelatins or carboxylic acids (malate, citrate, oxalate, succinate, among others) and in this form is translated from the cytosol to vacuole achieving their inactivation and accumulation (Manara, 2012; Wojas et al. 2010). When TM are not stored within the root cells, they can be translocated as free or complex ions with organic acids via xylem, due to the favorable water potential from the roots to the leaves (Longnercker y Robson, 1993; Manara, 2012; Maywald and Weigel, 1997). When these mechanisms fail (external and/or internal mechanisms), the TM cause disastrous damage at cell level, adversely affecting the photosynthetic apparatus performance (Alaoui-Sossé et al. 2004, Atanasova et al. 2004; Maksymiec, 1998; Weck and Clijsters, 1996), by releasing components as proteins, lipids and thylakoid membranes that disturb photosynthetic activity of the PSI and PSII, inhibiting electron transport decreasing the efficiency of the CO_2 assimilation (Dhir et al. 2011; Giotta et al. 2006; Maksymiec 1998; Millaleo et al. 2013; Vinit-Dunand et al. 2002; Wang et al. 2009). Moreover, TM can cause damage to DNA (Shao et al. 2008), protein and enzyme functions as well as membrane permeability that contribute to the loss of cell compartmentalization and thereafter cell lysis. These alterations induce morphological changes that have been extensively described as the root reduction, expressed with shorter and thicker roots because of metal contact with the membrane (Rauser W, 1999). As a consequence, there are limitations in the uptake of water and nutrients, a decrease in biomass and a reduction in the development of the whole plant (roots, stem, and leaves), noticeable through leaf chlorosis and necrosis (Table 1). Preeti and Tripathi, (2011) in *Albizia procera*, concluded that there is a direct relationship between the concentration of toxic metals (As, Cd, Pb) and the morphological and biochemical responses in plants, which are related to adaptive changes that affect the functioning of the plants during or after stress. Thus, there is

consensus that exclusion and inclusion TM mechanisms differ in the site of TM detoxification, but these mechanisms are not fully elucidated (Kochian, 1995; Memon and Schröder, 2009; Taylor, 1991; Vardar and Ünal, 2007).

2.4. Jas and TM in plants

Chemically, Jas are linolenic acid (LA)-derived cyclopentanone-based compound with wide distribution in the plant kingdom (Murofushi et al. 1999). The biosynthetic pathway of Jas starts from unsaturated linolenic (18:3) fatty acid located in membrane organelles (Creelman and Mullet, 1995; 1997; Schaller and Stintzi, 2009; Staswick, 2008), and structurally they have a cyclopentane ring substituted at C2 and C3. The cell compartments involved in the synthesis of JA and MeJA synthesized from oxidation of the membrane and then becoming OPDA (acid 12-oxo-phytodienoic) are shown in Fig. 2. Three step reductions (including lipoxidation, cyclisation and β-oxidation) are catabolized by carboxyl methyltransferase (JMT) to form volatile MeJA. Due to its volatility and diffusive ability through membranes, MeJA is an important signaling molecule related to intra and inter-plant communications (Farmer, 2003).

In the last 15 years, studies associating JA and MeJA with the effect exerted by TM as Cd, Cu and Pb have been reported (Keramat et al. 2009; Maksymiec and Krupa, 2002; 2006; Maksymiec et al. 2007c; Maksymiec et al. 2011; Piotrowska-Niczyporuka et al. 2009). These researches are based on the fact that JA and MeJA have a primordial role in plant defense and are components of major signaling pathways to respond to stress conditions in plants (Repka et al. 2004; Schaller and Stintzi, 2009; Wang et al. 2008).

The exogenous application of MeJA or JA in plants growing under TM stress promotes an increase in the OS as H_2O_2, superoxide radicals, LP, decreasing root growth (Maksymiec, 2011; Maksymiec and Krupa, 2002; 2006; 2007b), enhancing progressively damage in plants (Heath, 2000; Maksymiec and Krupa 2002; 2006; 2007b; Maksymiec et al. 2007c; Maksymiec et al. 2011; Piotrowska-Niczyporuka et al. 2009). The JA potentiates Cu and Cd toxicity, due to an increase in the metals absorption and ROS generation (Maksymiec

W. 2007a). Piotrowska-Niczyporuka et al. (2012) reported the influence of exogenous application of phytohormones (auxin, cytokinin, gibberellin and JA) upon growth and metabolism of green microalga *Chlorella vulgaris* (Chlorophyceae) under TM (Cd, Pb and Cu) condition. In this report also a decrease in chlorophylls, carotenoids, soluble proteins, Asc, and GSH concentration as well as the antioxidant enzyme activity has been observed. Maksymiec et al (2005) observed that TM stress (Cu or Cd) was a potential abiotic elicitor for triggering JA accumulation in mature leaves of *A. thaliana* and in young and the oldest *Phaseolus coccineus* L. plant leaves. In these plants, the accumulation of Jas showed a biphasic character dynamics, with a rapid increase of JA levels occurring after 7 h in *A. thaliana* and 14 h in *P. coccineus*, decreasing thereafter. Therefore, these authors have concluded that JA is connected with the toxic action mechanism of both TM in plants, differentially reacting to exogenous JA, having variable dynamics depending on the plants studied as well as their leaf growth stage.

Despite all of the above, exogenous MeJA applications are also able to reduce the toxic effects of some stresses, such as TM (Chen et al. 2014; Keramat et al. 2009; Piotrowska-Niczyporuka et al. 2009). Based on results obtained by Piotrowska-Niczyporuka et al. (2009) in the aquatic *Wolffia arrhiza* plant subjected to high JA concentrations (100 µM), an increase of the toxicity to Pb was found, showing a decrease in chlorophyll, LP, carotenoids and fresh weight, protein content. Whereas at lower concentration (0.1 µM), an inhibition of Pb accumulation, an increase the activities of the enzymes CAT, Asc peroxidase, NADH peroxidase and non-enzymatic Asc and GSH were observe, reducing oxidative damage and activating the mechanisms of response to OS. In the same way, Keramat et al. (2009) concluded that the application of MeJA (0.01 and 0.1 mM) on *Glycine max* L. plants could cause alleviation of Cd 500 µM damages by the reduction of malondialdehyde (MDA) and H_2O_2 content, increasing the activity of SOD, Asc peroxidase and CAT in plants treated with MeJA and Cd. Similar results was obtained by Yan et al. (2013) in *Capsicum frutescens* var. fasciculatum seedlings with Cd and MeJA, where the lower dose of MeJA (0.1 μmol^{-1}) was most effective to counteract the Cd toxic effect compared with high doses of MeJA (10 and 1000 μmol^{-1}), decreasing of total dry weight

(g) and CAT activity, increasing parameters as H_2O_2 and LP (MDA concentration) at 7 days.

Despite this, we believe that different activation mechanisms to counteract the metal accumulation can act in leaves and roots. There are different tolerance mechanisms to decrease the metal damage in plants, as the activation of: chelation (intra and extra cellular), antioxidant system (enzymatic and nom enzymatic), phytochelatine and metalloprotein (Hossain et al. 2012; Keramat et al. 2009; Ma et al. 2000; Maiti et al. 2004; Piotrowska-Niczyporuka et al. 2009). Chen et al. (2014) observed that *Kandelia obovata* seedlings alleviate the oxidative damage by Cd (200 μmol L^{-1}) due to positive changes in enzymatic response in leaves with MeJA application (0.1 to 1 μmol L^{-1}); however a decrease in Cd concentration of root was observed, but an increase in the Cd concentration (two-times) in leaves was found.

We believe that the contradictory reports (positive and negative JA or MeJA effects) are mainly due to alterations in the signal pathways in cell that can affect the normal conduct of wall and cell activity in plants. These contradictory responses could explain due to that under TM stress conditions the plants respond best to low JA or MeJA doses than to high doses, influencing directly the metabolism and growth of plants (Yan et al. 2013). This can be explained different dose applied in herbaceous and woody plants under TM (Table 1). Where, high Jas are applied in woody plants with other stress. Thus studies woody species *Kandelia obovata* subjected to Cd toxicity showed a significant decreased LP at lower MeJA (0.1-10 μM) dose, but Cd concentration increased compared to the Cd treatment alone.

Based on this evidence, we think that is extremely important to carry out detailed studies of dose-response according to species. Basically, we postulate the that contradictory responses are by the following alterations: (a) in concentration of chelate elements in wall cell and in the symplast as antioxidant compounds, can be related with phenolic compounds that retain TM and leave less available and variation in enzymatic response according to agree tolerance to TM of the specie, (b) changes in the behavior of seconds messenger in cell, where Jas initiate cascade of its second messengers that able to modulate antioxidant

response and organic acid under TM stress, and (c) the exudation organic acid that chelation TM and decrease the toxicity to plants.

2.5. Conclusions

In general, the relationship studies between TM and JA or MeJA showed discrepant results, attributable to the doses of JA or MeJA application. While high JA or MeJA doses application to plants under TM conditions can induce a saturation of receptors in the cell membrane, inactivation of TM transporters, or exudation of organic acids which let less available TM, inhibiting the responses against plant stress. Low MeJA doses determine a protective antioxidant effect to counteract the stress. It appears that, it is necessary to perform a screening with different doses of exogenous MeJA or JA to determine the dose able to provoke the better responses (antioxidant capacity, antioxidant enzymes, and metal accumulation, so on) to counteract the toxic effect by TM. Furthermore, the type, levels, sites of damage and time exposition of plant to TM will be considered because differences exist in the level of damage that they can exert in a single plant. MeJA application seems to be a very interesting alternative for handling toxicity by TM, but further studies are necessary.

Acknowledgments: We thank hearty to FONDECYT Projects Nº 1120917 that support of this work and fellowship PhD Nº21110919, both of Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) of the Government of Chile.

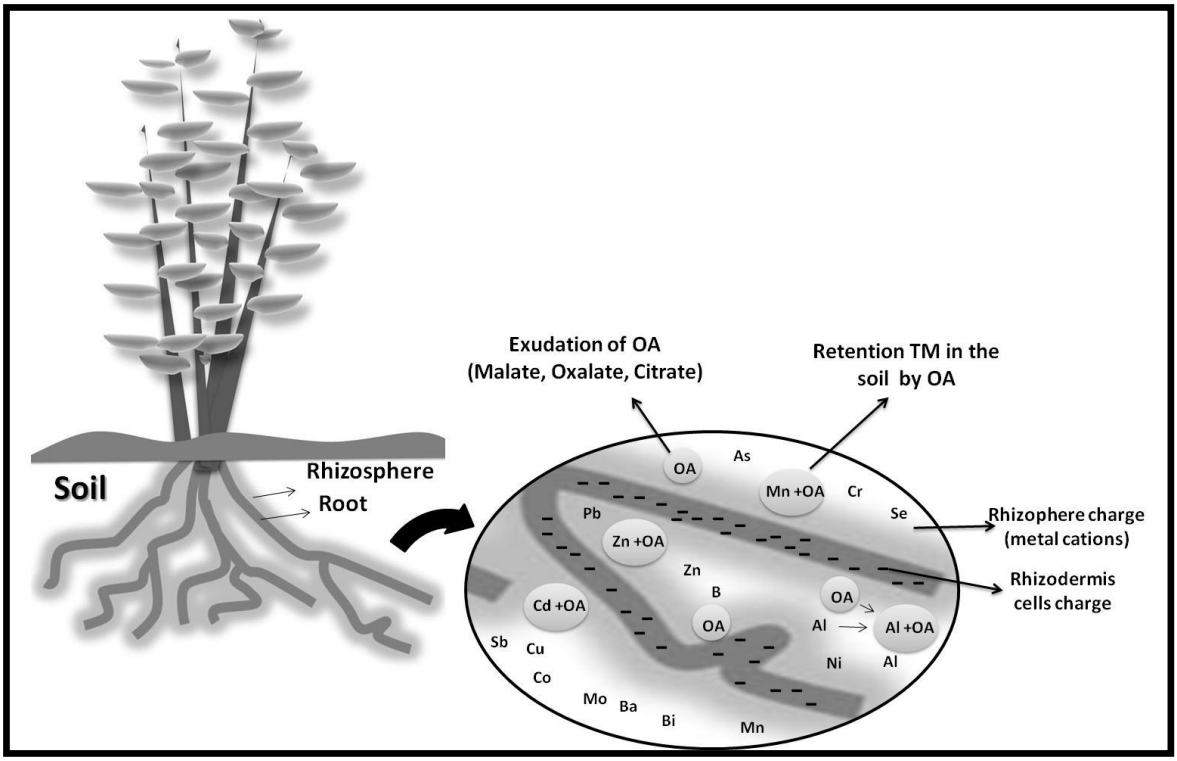

Fig. 1 Mechanisms of exclusion (external tolerance) involved in the mobilization and retention of TM in the soil. Soil acts as a sink and source of TM. The interaction between the TM of the soil and plant stimulates the organic acids secretion to immobilize TM in the soil and prevent the introduction into the plant. Without this mechanism the plants would be severely affected by the absorption of TM, since differences in electric charge between the carboxyl groups of the cells of the root surface and electric charges TM facilitates the entry of the TM in plants (Delgadillo-López et al. 2011).

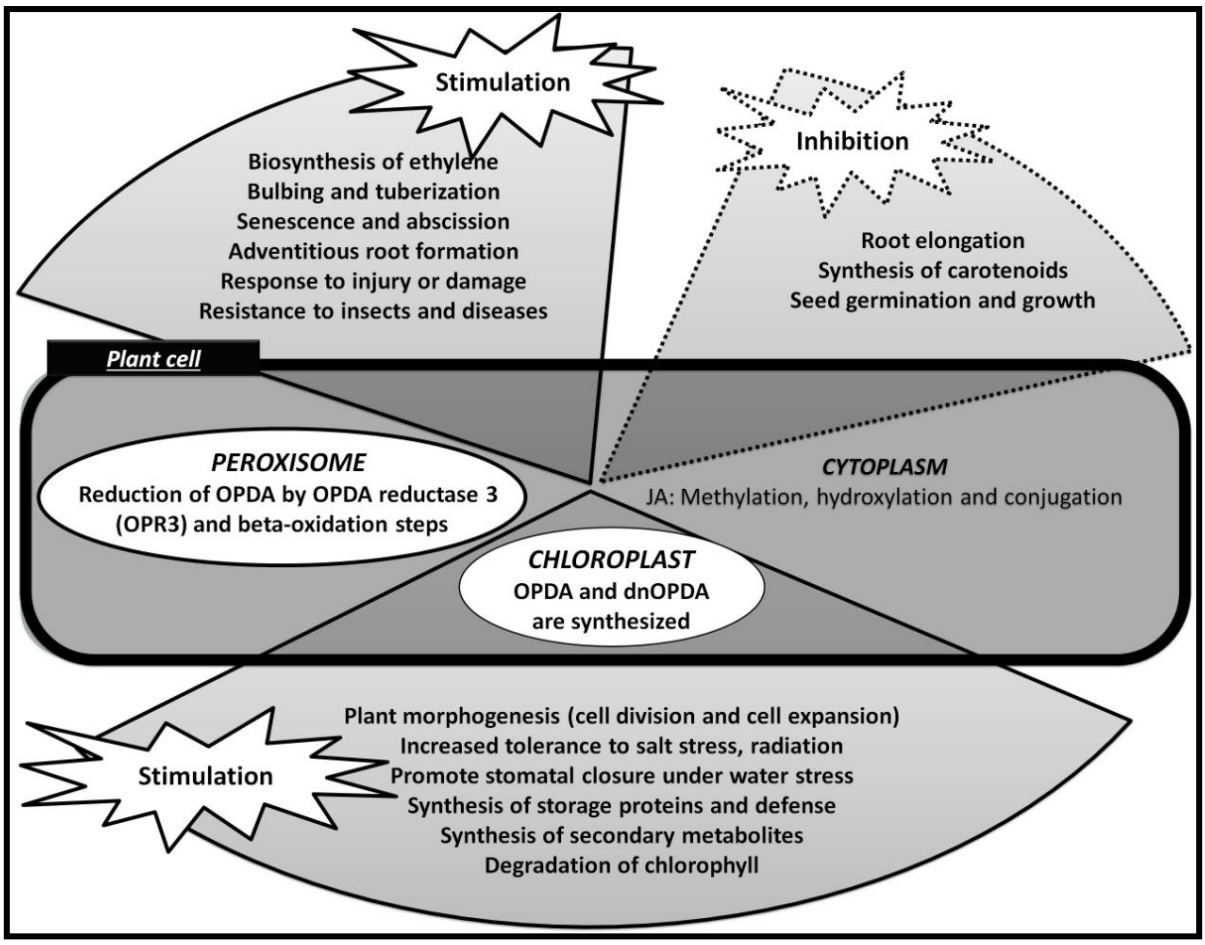

Fig. 2 Schematic representation of cell compartments involved in the synthesis of JA and MeJA and their stimulatory and inhibitory effects on plants.

References.

Alaoui-Sossé B, Genet P, Vinit-Dunand F, Toussaint M, Epron D, Badot PM (2004) Effect of copper on growth in cucumber plants (*Cucumis sativus*) and its relationships with carbohydrate accumulation and changes in ion contents. Plant Sci 166:1213-1218

Álvarez S, Gómez-Bellot MJ, Castillo M, Bañón S, Sánchez-Blanco MJ (2012) Osmotic and saline effect on growth, water relations, and ion uptake and translocation in *Phlomis purpurea* plants. Environmental and Experimental Botany Volume 78:138–145

Ashraf M (2004) Some important physiological selection criteria for salt tolerance in plants. Flora - Morphology, Distribution, Functional Ecology of Plants 199:361-376

Atanasova L, Pissarska MG, Popov GS, Georgiev GI (2004) Growth and endogenous cytokinins of juniper shoots as affected by high metal concentrations. Biol Plant 48:157-159

Bao J, Liu W, Liu L, Jin Y, Dai J, Ran X, Zhang Z, Tsuda S (2011) Perfluorinated Compounds in the Environment and the Blood of Residents Living near Fluorochemical Plants in Fuxin, China. Environmental Science and Technology 45:8075-80

Beltrano J, Ronco MG, Montaldi ER, Carborne A (1998) Senescence of flag leaves and ears of wheat hastened by methyl jasmonate. Journal of Plant Growth Regulation 17:53-57

Bidar G, Garçon G, Pruvot C, Dewaele D, Cazier, F, Douay F, Shirali P (2007) Behavior of *Trifolium repens* and *Lolium perenne* growing in a heavy metal contaminated field: Plant metal concentration and phytotoxicity. Environmental Pollution 147:546–553

Cakmak I, Horst WJ. 1991. Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). Physiol Plant 83 (1991), pp. 463–468.

Chen J, Yan Z, Li X. (2014). Effect of methyl jasmonate on cadmium uptake and antioxidative capacity in *Kandelia obovata* seedlings under cadmium stress. Ecotoxicology

and Environmental Safely 104:349-256

Creelman RA, Mullet JE (1995) Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. Proc Natl Acad Sci USA 92:4114–4119

Creelman RA, Mullet J (1997) Oligosaccharins, brassinolides, and jasmonates: nontraditional regulators of plant growth, development, and gene expression. Plant Cell 9:1211-23

Cui J, Bahrami AK, Pringle EG, Hernandez-Guzman G, Bender CL, Pierce NE, Ausubel FM (2005) Pseudomonas syringae manipulates systemic plant defenses against pathogens and herbivores. Proceedings of the National Academy of Sciences of the USA 102:1791-1796

Delgadillo-López AE, González-Ramírez CA, Priego-García F, Villagómez-Ibarra JR, Acevedo-Sandoval O (2011) Fitorremediación, una alternativa para eliminar la contaminación. Tropical and Subtropical Agroecosystems 14:597-612

Dhir B, Sharmilab P, Saradhib P, Sharmab S, Kumarc R, Mehtad D (2011) Heavy metal induced physiological alterations in *Salvinia natans*. Ecotoxicology and Environmental Safety. 74:1678–1684

Dong J, Mao W, Zhang G, Wu F, Cai Y (2007) Root excretion and plant tolerance to cadmium toxicity- a review. Plant soil environ 53:193-200

Farmer EE, Alméras E, Krishnamurthy V (2003) Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Curr Opin Plant Biol 6:372-8

Gardea-Torresdey JL, Peralta-Videa JR, De la Rosa G, Parsons JG (2005) Phytoremediation of heavy metals and study of the metal coordination by X-ray absorption spectroscopy. Coord Chem Rev 249:1797-810

Ghanati F, Morita A, Yokota H (2005) Effects of aluminum on the growth of tea plant and

activation of antioxidant system. Plant Soil 276:133-141

Giotta L, Agostiano A, Italiano F, Milano F, Trotta M (2006) Heavy metal ion influence on the photosynthetic growth of *Rhodobacter sphaeroides*. Chemosphere 62:1490-1499

Granero S, Domingo JL (2005) Levels of metals in soils of Alcalá de Henares, Spain: Human health risks. Environment International 28:159-164

Hamon RE, Lorenz SE, Holm PE, Christensen TH, McGrath SP (1995) Changes in trace metal species and other components of the rhizosphere during growth of radish. *Plant Cell Environ* 18:749-756

Heath MC (2000) Hypersensitive response-related death. Plant Mol Biol 44:321-334

Hossain MA, Munemasa S, Nakamura Y, Morib IC, Murata Y (2012) K252a-sensitive protein kinases but not okadaic acid-sensitive protein phosphatases regulate methyl jasmonate-induced cytosolic Ca^{2+} oscillation in guard cells of *Arabidopsis thaliana*. Journal of Plant Physiology 168:1901-1908

Hung KT, Kao CH (1996) Promotive effect of jasmonates on the senescence of detached maize leaves. Plant Growth Regul 19:77-83

Keramat B, Manouchehri K, Javad M (2009) Effects of methyl jasmonate in regulating cadmium induced oxidative stress in soybean plant (*Glycine max* L.). African Journal of Microbiology Research 3:240-244

Kochian L (1995) Cellular mechanisms of aluminum toxicity and resistance in plants. Annu Rev Plant Physiol Plant Mol Biol 46:237-260

Krtková J, Havelková L, Křepelová A, Fišer R, Vosolsobě S, Novotná Z, Martinec J, Schwarzerová K (2012) Loss of membrane fluidity and endocytosis inhibition are involved in rapid aluminum-induced root growth cessation in *Arabidopsis thaliana*. Plant Physiology and Biochemistry 60:88-97

Larsen E, Hansen M, and Gossler W (1998) Speciation and health risk considerations of arsenic in the edible mushroom *Laccaria amethystina* collected from contaminated and uncontaminated locations. Appl Organometallic Chem 12:285-291

Lasat M (2002) Phytoextraction of toxic metal: a review of biological mechanisms J Environ Qual 31:109-120

Longnecker N, and Robson A (1993) Distribution and transport of zinc in plants. In A.D. Robson (ed.) Zinc in soils and plants. Kluwer Academic Publishers, Boston. p. 9-92

Ma Z, Wood CW, Bransby DI (2000) Soil management impacts on soil carbon sequestration by switchgrass. Biomass and Bioenergy 18:469–477

Maiti RK, Hernandes piñero JL, Gonzalez Oreja JA, Lopez Santiago D (2004) Plant based bioremediation and mecanisms of heavy metal tolerance of plants: A review. Proc Indian natn Sci acad 70:1-12

Maksymiec W (1997) Effect of copper on cellular processes in higher plants. Photosynthetica 34:321-342

Maksymiec W (2007c) Signaling responses in plants to heavy metal stress. Acta Physiol Plant 29:177-187

Maksymiec W, Baszyński T (1996a) Chlorophyll fluorescence in primary leaves of excess Cu-treated runner bean plants depends on their growth stages and the duration of Cu-action. J. Plant Physiol 149:196-200

Maksymiec W, Baszyński T (1996b) Different susceptibility of runner bean plants to excess copper as a function of the growth stage of primary leaves. - J. Plant Physiol 149:217-221

Maksymiec W, Baszyński T (1998) The role of Ca ions in changes induced by excess Cu^2 in bean plants. Acta Physiol Plant 20:411-417

Maksymiec W, Baszyński T (1999a) Are calcium ions and calcium channels involved in the mechanisms of Cu^2 toxicity in bean plants? The influence of leaf age. Photosynthetica 36:267-278

Maksymiec W, Baszyński T (1999b) The role of Cu^2 ions in modulating changes induced in bean plants by an excess of Cu^2 ions. Chlorophyll fluorescence measurements. Physiol Plant 105:562-568

Maksymiec W, Bednara J, Baszyński T (1995) Responses of runner plants to excess copper as a function of plant growth stages: effects on morphology and structure of primary leaves and their chloroplast ultrastructure. Photosynthetica 31:427-435.

Maksymiec W, Krupa Z (1999) The *in vivo* effect of methyl jasmonate on the level of Cu-toxicity towards bean plants. Folia Histochem Cytobiol 37:63

Maksymiec W, Krupa Z (2002a) Jasmonic acid and heavy metals in Arabidopsis thaliana-a similar physiological responses to both stressors? J Plant Physiol 159:509-515

MaksymiecW, Krupa Z (2002b) The in vivo and in vitro influence of methyl jasmonate on oxidative processes in *Arabidopsis thaliana* leaves. Acta Physiol Plant 24:351-357

Maksymiec W, Krupa Z (2006) The effects of short term exposition to Cd, excess Cu ions and jasmonate on oxidative stress appearing in *Arabidopsis thaliana*. - Environ exp Bot 57:187-194

Maksymiec W, Krupa Z (2007) Effects of methyl jasmonate and excess copper on root and leaf growth. Biol Plant 51:322-326

Maksymiec W, Wianowska D, Dawidowicz AL, Radkiewicz, Mardarowicz M, Krupa Z (2005) The level of jasmonic acid in *Arabidopsis thaliana* and *Phaseolus coccineus* plants under heavy metal stress. J Plant Physiol 162:1338-1346

Maksymiec W, Wójcik M, Krupa Z (2007) Variation in oxidative stress and photochemical activity in *Arabidopsis thaliana* leaves subjected to cadmium and excess copper in the

presence or absence of jasmonate and ascorbate. Chemosphere 66:421-427

Maksymiec W (2011) Effects of jasmonate and some other signalling factors on bean and onion growth during the initial phase of cadmium action. Biologia plantarum 55:112-118

Malinowski D, Zuo H, Belesky D, Alloush A (2004) Evidence for copper binding by extracellular root exudates of tall fescue but not perennial ryegrass infected with Neotyphodium spp. endophytes. Plant and Soil 267:1-12

Manara A (2012) Plant Responses to Heavy Metal Toxicity. Chapter II. A. Furini (ed.), Plants and Heavy Metals, SpringerBriefs in Biometals. doi: 10.1007/978-94-007-4441-7_2

Maslenkova L (1990) Oxygen-evolving activity of thylakoids from barley plants cultivated on different concentrations of jasmonic acid. Plant Physiology 93:1316-1320

Maywald F, Weigel HJ (1997) Biochemistry and molecular biology of heavy metal accumulation in higher plants. Landbauforschung Volkenrode 47:103-126

Memon AR, Schroder P (2009) Implications of metal accumulation mechanisms to phytoremediation. Environ Sci Pollut R 16:162-175

Mithöfer A, Schulze B, Boland W (2004) Biotic and heavy metal stress response in plants: evidence for common signals. FEBS Letters 566:1-5

Mora ML, Alfaro MA, Jarvis SC, Demanet R, Cartes P (2006) Soil aluminum availability in Andisols of Southern Chile and its effect on forage production and animal metabolism. Soil Use Manage. 22:95-101.

Mora M, Rosas A, Ribera A, Rengel R (2009) Differential tolerance to Mn toxicity in perennial ryegrass genotypes: involvement of antioxidative enzymes and root exudation of carboxylates. Plant Soil 320:79-89.

Murofushi N, Yamane H, Sakagami Y, Imaseki H, Kamiya Y, lwamura H, Hirai N, Tsuji H, Yokota T, Ueda J (1999) Plant Hormones. In: Comprehensive Natural Products

Chemistry (Editor in Chief: Sir Derek Barton, Koji Nakanishi, Executive Editor: Otto Meth-Cohn), Vol. 8, Natural Products, Pheromones, Plant Hormones, and Aspects of Ecology (Volume Editor: Kenji Mori), Elsevier, Amsterdam, pp. 19-136.

Ozawa R, Arimura G, Takabayashi J, Shimoda T, Nihioka T (2000) Involvement of jasmonate and salicylaterelated signaling pathways for the production of specific herbivore-induced volatiles in plants. Plant and CellPhysiology 41:391-398

Pérez de Mora A, Madejón E, Burgos P, Cabrera F (2006) Trace element availability and plant growth in a mine-spill contaminated soil under assisted natural remediation I. Soils Sci Total Environ 363:28-37

Piotrowska A, Bajguz A, Godlewska-Żyłkiewicz B, Czerpak R, Kamińska M (2009) Jasmonic acid as modulator of lead toxicity in aquatic plant *Wolffia arrhiza* (Lemnaceae). Environ Exp Bot 66:507-513.

Piotrowska-Niczyporuka A, Bajguza A, Zambrzyckab E, Godlewska-Żyłkiewiczb B (2012) Phytohormones as regulators of heavy metal biosorption and toxicity in green alga *Chlorella vulgaris* (Chlorophyceae). Plant Physiology and Biochemistry 52:52-65

Preeti P (2011) Effect of heavy metals on morphological and biochemical characteristics of *Albizia procera* (Roxb.) Benth. seedlings. International Journal of Environmental Science 1:1009

Qin R, Hirano Y, Brunner I (2007) Exudation of organic acid anions from poplar roots after exposure to Al, Cu and Zn. Tree Physiology 27:313-320

Rauser W (1999) Structure and Function of Metal Chelators Produced by Plants. Cell Biochemistry and Biophysics 31:19-28.

Repka V, Fischerová I, Šilhárová K (2004) Methyl jasmonate is a potent elicitor of multiple defense responses in grapevine leaves and cell-suspension cultures. Biol Plant 48:273-83

Reyes-Díaz M, Alberdi M, Mora ML (2009) Short-term aluminum stress differentially

affects the photochemical efficiency of photosystem II in highbush blueberry genotypes. J Amer Soc Hort Sci 134:14-21

Reyes-Díaz M, Inostroza-Blancheteau C, Millaleo R, Cruces E, Wulff-Zottele C, Alberdi M, Mora ML (2010) Long-term aluminum exposure effects on physiological and biochemical features of Highbush Blueberry cultivars. J Amer Soc Hort Sci 135:212-222

Ritter S, Koller D (1994) Movements of the Trifoliate Leaf of Bean (*Phaseolus vulgaris L.*) during a Simulated Day, and their Consequences for Solar-Tracking Fidelity and Interception of Solar Radiation. Journal of Plant Physiology 143:64-71

Schaller A, Stintzi A (2009) Enzymes in jasmonate biosynthesis. Structure, function, regulation. Phytochemistry 70:1532-1538

Shao HB, Chu LY, Shao MA, Jaleel CA, Mi HM (2008) Higher plant antioxidants and redox signaling under environmental stresses. C R Biologies 331:433-441

Staswick PE (2008) JAZing up jasmonate signaling. Trends in Plant Science 13:66-71

Sytar O, Kumar A, Latowski D, Kuczynska P, Strzałka K, Prasad MNV (2013) Heavy metal-induced oxidative damage, defense reactions, and detoxification mechanisms in plants. Acta Physiol Plant 35:985-999

Taylor G (1991) Current views of the aluminum stress response: the physiological basis of tolerance. Curr Top Plant Biochem Physiol 10:57-93

Vardar F, and Ünal M (2007) Aluminum toxicity and resistance in higher plants. Advances in Molecular Biology 1:1-12

Vinit-Dunand F, Epron D, Alaoui-Sossé B, Badot PM (2002) Effects of copper on growth and on photosynthesis of mature and expanding leaves in cucumber plants. Plant Sci 163:53-58

Wang J (2009) Monogalactosyldiacylglycerol deficiency affects jasmonic acid biosynthesis

and defense responses to insect herbivores in *Nicotiana tobacum*. Plant Science 176:279-285

Wang S, Bowman L, Ding M (2008) Methyl jasmonate enhances antioxidant activity and flavonoid content in blackberries (*Rubus sp*.) and promotes antiproliferation of human cancer cells. Food Chemistry 107:1261-1269

Weck J, Clijsters M (1996) Oxidative damage and defense mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amounts of copper. Physiol Plant 96:506-512

Wojas S, Ruszczyńska A, Bulska Ewa, Clemens S, Antosiewicz DM (2010) The role of subcellular distribution of cadmium and phytochelatins in the generation of distinct phenotypes of AtPCS1- and CePCS3-expressing tobacco. Journal of Plant Physiology 167:981-988

Xiang C, Oliver DJ (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. Plant Cell 10:1539-1550

Yan Z, Chen J, Li X (2013) Methyl jasmonate as modulator of Cd toxicity in *Capsicum frutescens* var. fasciculatum seedlings. Ecotoxicology and Environmental Safety 98:203-209

Yang S X, Liao B, Li J T, Guo T, Shu W S (2010) Acidification, heavy metal mobility and nutrient accumulation in the soil–plant system of a revegetated acid mine wasteland. Chemosphere 80:852-859

Zhang F, Wanga Y, Loua Z, Donga J (2007) Effect of heavy metal stress on antioxidative enzymes and lipid peroxidation in leaves and roots of two mangrove plant seedlings (*Kandelia candel* and *Bruguiera gymnorrhiza*). Chemosphere 67:44-50

CHAPTER III

Low doses of exogenous methyl jasmonate applied simultaneously with toxic aluminum improve the antioxidant performance of Vaccinium corymbosum

(Published in Plant and Soil)

Low doses of exogenous methyl jasmonate applied simultaneously with toxic aluminum improve the antioxidant performance of V*accinium corymbosum*

3.1 Abstract

The effect of different MeJA doses applied prior to or simultaneously with a toxic Al dose on *Vaccinium corymbosum* cultivars with contrasting Al resistance, was studied to determing changes in the biochemical and physiological properties of the plants. Legacy (Al-resistant) and Bluegold (Al-sensitive) plants were treated with and without toxic Al under controlled conditions: a) without Al and MeJA, b) 100µM Al, c) 100µM Al+5µM MeJA, d) 100µM Al+10µM MeJA and e) 100µM Al+50µM MeJA. MeJA was applied to leaves 24h prior to or simultaneously with Al in nutrient solution. After 48h, Al-concentration, lipid peroxidation (LP), H_2O_2, antioxidant activity, total phenols, total flavonoids, phenolic compounds and superoxide dismutase activity (SOD) of plant organs were analyzed. Al-concentrations increased with Al-treatment in both cultivars, being Al, LP and H_2O_2 concentrations reduced with low dose of simultaneous MeJA application. Higher MeJA doses induced more oxidative damage than the lowest. Legacy increased mainly non-enzymatic compounds, whereas Bluegold increased SOD activity to counteract Al^{3+}. Low MeJA doses applied simultaneously with Al^{3+}, increased Al-resistance in Legacy by increasing phenolic compounds, while Bluegold reduced oxidative damage through increment of SOD activity, suggesting a diminution of its Al-sensitivity. Higher MeJA doses could be potentially toxic. Studies are needed to determine the molecular mechanisms involved in the protective MeJA effect against Al-toxicity.

Keywords: Al-resistant; Al-sensitive; Blueberry; Jasmonates.

3.2 Introduction

Soil acidity (pH<5.5) solubilizes the aluminum (Al) complex to toxic aluminum (Al^{3+}), which represents the most harmful form for plant crops (Delhaize et al. 2012; Kochian et al.

2015). At lower concentrations, Al^{3+} negatively affects physiological, biochemical and morphological processes, depending on the plant species, genotypes and degree of tolerance (Barceló and Poschenrieder 2002). The most evident response to Al toxicity in plants is the overproduction of reactive oxygen species (ROS) with the concomitant increase in lipid peroxidation (LP) in the cell membranes. This induces oxidative stress (OS) in cells and organelles, resulting even in cell death (Yamamoto et al. 2002; Guo et al. 2007; Ma et al. 2007). Plants counteract excess ROS by activating antioxidant systems, including enzymatic mechanisms like superoxide dismutase (SOD), which is the first line of defense to scavenge ROS (Wang et al. 2005). In addition, non-enzymatic antioxidant compounds such as total phenols (TP) may also be increased to counteract Al^{3+}-induced OS (Shao et al. 2008). Under stress conditions, the phenol concentrations increase and this causes the Al^{3+} to have a stronger affinity with phenols than other organic molecules, limiting the Al toxicity (Wang et al. 2015). Another important hormone involved in plant responses to toxic Al is jasmonic acid (JA) (Spollansky et al. 2000). JA and its methyl ester, methyl jasmonate (MeJA), are synthesized from the linoleic and linolenic acids derived from cyclopentanone-based compounds of the jasmonates (JAS) (Creelman and Mullet 1995; 1997; Pauwels et al. 2008; Staswick 2008; Schaller and Stintzi 2009). JA is considered a plant growth regulator and acts as a signal molecule that participates in the regulation of various metabolic pathways. The exposure of plants to toxic metals (TM) stimulates the synthesis and activity of antioxidant metabolites and antioxidant enzymes that can protect plant tissues against stress (Poonam et al. 2013). There is little information about the effects of MeJA application on plants under Al toxicity. Spollansky et al. (2000) and Xue et al. (2008) reported that in *Brugmansia candida* and *Cassia tora* plants exposed to Al toxicity and MeJA application, a high lignin accumulation in the cell wall, oxidative stress, peroxidase and NADH activity were observed in the roots of both species. However, reports on other species in the presence or absence of MeJA application under abiotic stresses such as water stress in strawberries (*Fragaria x ananassa*) (Wang 1999), salinity and radiation in grapevines (*Vitis vinifera*) (Larronde et al. 2003; Ismail et al. 2012), low temperature in peaches (*Prunus persica*) (Menga et al. 2009), and toxic metals (TM) in *Arabidopsis thaliana* (Maksymiec and Krupa 2002; 2007a;b) are more abundant. These studies involve application of MeJA simultaneously with the stressor. Yet there are no

studies that differ in the application time of MeJA and the stress factor and its antioxidant responses in plants. We believe that prior application of MeJA could activate defense mechanisms to counteract stress conditions, preventing its harmful effect. Creelman and Mullet (1995) and Chen et al. (2014) reported that the MeJA effect on plants under toxic metal (TM) stress depends on the intensity of the stress factor as well as the sensitivity of the species or cultivars. In fact, studies performed by Li et al. (2014) indicated that the MeJA treatment significantly enhanced resistance to fungal pathogens in two rice cultivars, but the resistant cultivar maintained a higher level of resistance than the susceptible under the same treatment. In addition, Li et al. (2014) pointed out the importance of the comparative studies between resistant and susceptible cultivars, for a better understanding of the resistance mechanisms in the plants exposed to JAS.

In the aquatic plant *Wolffia arrhiza* treated with a high JA concentration (100 µM) and increased lead (Pb) toxicity, a decrease was found in chlorophyll and carotenoid pigments. Conversely, at low concentrations of JA (0.1 µM), a decrease in the oxidative damage by Pb in this species was observed, accompanied by an increase in biomass, carbohydrates, proteins, antioxidant concentrations (ascorbic acid and glutathione) and a decrease in LP (Piotrowska et al. 2009). A reduction in LP and increased SOD activity was reported in soybean (*Glycine max* L.) plants grown under cadmium (Cd) toxicity (500 µM), by adding a low MeJA dose (0.01 µM) (Keramat et al. 2009). Thus, it appears that a low MeJA dose is more effective at reducing the harmful effects of TM in these species.

Although there are few studies related to MeJA and TM, most are in plants of agricultural interest, and in particular fruit crops (Yoon et al. 2010; Ismail et al. 2012). In the last decade the use of natural stimulant compounds such as MeJA has gained interest due to restrictions in the use of agrochemicals in fruit export. Highbush blueberry (*Vaccinium corymbosum* L.) is a species native to North America, belonging to the Ericaeae family, which in the last two decades has become an important crop for the nutritional properties of its fruits, which are rich in antioxidant activities and anthocyanin concentrations (Castrejón et al. 2008). Studies performed on highbush blueberry leaves under Al^{3+} stress have shown different antioxidant capacities and physiological responses depending on the cultivars and degree of resistance to Al toxicity (Reyes-Díaz et al. 2009; 2010). These reports have

shown different responses by Brigitta, Bluegold and Legacy to Al toxicity, with Bluegold demonstrating a greater Al sensitivity than Legacy and Brigitta. Therefore, the aim of this work was to evaluate the effect of different MeJA doses applied to leaves at different times (prior to or simultaneously with the application of toxic Al) on the antioxidant performance of roots and leaves of *V. corymbosum* cultivars.

3.3 Materials and methods

3.3.1 Plant material

Two-year-old plants of *V. corymbosum* cultivars (Legacy and Bluegold), previously classified by Reyes-Díaz et al. (2009; 2010) as Al-resistant and Al-sensitive, respectively were used in this study. Plants from these cultivars were produced *in vitro* and grown in a substrate of oat shell:sawdust:pine needles at a 1:1:1 proportion. They were provided by Berries San Luis (Quillém, Lautaro, Chile; 38º 29` S, 72º 23` W). Healthy plants of these cultivars with uniform size with a plant height of 35.21± 0.16 cm (from crown to apex), and 17.02± 0.08 cm (from crown to root tips) in roots were selected.

3.3.2 Growth conditions in nutrient solution.

The experiment was carried out in a greenhouse in the Instituto de Agroindustria, Universidad de La Frontera, Temuco, Chile (38°45´S, 72°.40´W). Plants were transferred and grown in a Hoagland nutrient solution [2 mMCa $(NO_3)_2$, 3 mM KNO_3, 1 mM $MgSO_4$, 0.1 mM KH_2PO_4 with micronutrients: 25 µM H_3BO_3, 10 µM $MnSO_4$, 1 mM NH_4NO_3, 0.07 µM $(NH_4)_6Mo_7O_{24}$, 2 µM $ZnSO_4$, 0.4 µM $CuSO_4$, 20 µM FeEDTA] under controlled conditions (temperature 25 ± 0.2 °C, 300µmol m^{-2} s^{-1} photosynthetic photon flux and 70% relative humidity) for seven days for plant conditioning prior to starting the experiment. Solutions were aerated continuously with an aquarium pump and changed twice in the week.

3.3.3 Treatments and experimental design.

The experimental design was completely randomized with three replicates per treatment with a total of 60 plants for the two cultivars. After conditioning as described above, plants were treated with or without toxic Al (as $AlCl_3$), with 26.8 % of Al^{3+}as a free metal determined by Geochem speciation (Shaff et al. 2010). The pH was adjusted at 4.5 for 48 h. The MeJA was homogenously applied by spraying on leaves 24 hours prior to the application of Al^{3+} to the nutrient solution or simultaneously with the Al^{3+} application. The MeJA was dissolved in ultrapure water 1 L (< 1 µS) with 0.05% (v/v) tween 20 for plants with MeJA, whereas in the controls (without MeJA) 0.05% v/v tween 20 dissolved in ultrapure water was applied. Dose coverage was 25 ml per plant calculated as total foliar area of plant. Plants were located in screens to avoid drift of MeJA dilution between treatments. The following treatments were applied: a) without Al and MeJA (Control), b) 100 µM Al (Al), c) 100 µM Al + 5 µM MeJA (Al + 5 MeJA), d) 100 µM Al + 10 µM MeJA (Al + 10 MeJA), and e) 100 µM Al + 50 µM MeJA (Al +50 MeJA). Finally, 48h after adding Al to the nutrient solution, leaves and roots were harvested and stored at -80°C (Revco Elite Series Ultra-Low Temperature, Thermo Scientific™) for biochemical analyses; subsamples were taken and dried for chemical analysis.

3.3.4 Chemical analysis

Aluminum concentration: Samples were dried in a forced air oven (for 48 h at 70 ºC in a Memmert model 410, Schwabach, Germany) until a constant dry weight was reached, and then ground in a mill. Samples were weighed and ashed at 500 ºC (JSMF-30T, electric Muffle Furnace of JSR Research Inc, Korea) for 8 hours and then digested with 2M hydrochloric acid. The Al concentration was determined using a simultaneous multielement atomic absorption spectrophotometer (model 969; UNICAM, Cambridge, UK) as described by Sadzawka et al. (2004).

3.3.5 Biochemical parameters

Lipid peroxidation (LP): It was used as indicator of damage by oxidative stress. A thiobarbituric acid reacting substance (TBARS) assay according to the modified method of Du and Bramlage (1992) was used. The final malondialdehyde (MDA) products were measured at 532, 600 nm and 440 nm. LP is a good criterion for determining Al resistance in plants (Reyes-Díaz et al. 2010); hence, it was used to establish Al-sensitivity or resistance of the evaluated cultivars.

Hydrogen peroxide concentration (H_2O_2): The H_2O_2 concentration was determined according to Loreto and Velikova (2001). The H_2O_2 concentration was measured at 390 nm and expressed as $\mu molg^{-1}$ fresh weight.

Total antioxidant activity (AA): AA was determined in leaves and roots using the DPPH method of Chinnici et al. (2004). The extracts were prepared according to the method used by Reyes-Díaz et al. (2010). Absorbance was measured in a spectrophotometer at 515 nm and expressed in Trolox equivalents (TE).

Total phenols (TP): TP were determined with the Folin-Ciocalteu reagent using the method of Slinkard and Singleton (1977). Absorbance was measured at 765 nm using a UV/VIS spectrophotometer. Results were expressed as milligrams of chlorogenic acid equivalent per gram of fresh weight (mg CAE g^{-1} FW).

Flavonoid compound analyses: Total flavonoids were determined using the method of Cheng and Breen (1991) at an absorbance of 510 nm using a UV/VIS spectrophotometer. Results were expressed as milligrams of rutin equivalent per mg of fresh weight (µg rutin eq. g^{-1} FW). The HPLC analysis was performed as described earlier by Ruhland and Day (2000) with minor modifications, at a flow rate of 1.0 ml min^{-1}. The signals were detected at 320 nm and the data were expressed as milligrams per mg of fresh weight (µg g^{-1} FW) . The mobile phase was: (A) acidified water (phosphoric acid 10%) and (B) 100% acetonitrile, and the eluent gradients were as follows: 0-9 min of 100% A, 9.1-19.9 min of

81% A and 19% B, 20-25 min of 100% B.

Superoxide dismutase (SOD) activity: The SOD was assayed according to Giannopolitis and Ries (1977) by monitoring the superoxide radical-induced nitro blue tetrazolium (NBT) reduction at 560 nm. The enzymatic activity values were standardized for the protein content according to Bradford's method (Bradford 1976).

3.3.6 Statistical analysis

The results were based on 3 replicates. All data passed the normality and equal variance tests according to Kolmogorov-Smirnov. Statistical data analyses were carried out by three-way ANOVA (where factors were: treatment, time of MeJA application, and cultivar). Tukey's test was used to identify means with significant differences ($P \leq 0.05$) using the statistical software SAS v. 8.01.

3.4 Results

The Al concentration in leaves and roots was generally increased under Al application alone in both cultivars, when compared to the control ($P \leq 0.05$; Fig.1A, B, C, and D). Leaves and roots of Bluegold showed 60- and 2.7-fold increase of Al-concentration under Al treatment, respectively than their respective controls ($P \leq 0.05$; Fig. 1C and D). Leaves and roots of Legacy showed 4- and 3.4-fold Al increase under Al treatment, respectively in comparison with their respective controls ($P \leq 0.05$; Fig. 1A and B). In leaves and roots of Legacy, Al-concentration was lower (54% and 85%, respectively) than in leaves and roots of Bluegold ($P \leq 0.05$; Fig. 1A, B, C, and D), The simultaneous application of MeJA and toxic Al showed that Legacy leaves were able to reduce their Al concentration in all MeJA doses, being reduced by 45% at the lowest (5µM) and highest dose of MeJA ($P \leq 0.05$; Fig. 1A). By contrast, when MeJA was applied prior to toxic Al^{3+} only the highest (50 µM) dose of MeJA reduced the Al concentration in leaves (38%) compared to the Al treatment alone ($P \leq 0.05$; Fig. 1A). The Al concentration of Legacy roots showed no statistically significant differences in any of the treatments when MeJA was applied prior to Al^{3+};

however, these concentrations were statistically significantly higher than the control treatment ($P \leq 0.05$; Fig. 1B). The situation was different when MeJA was applied simultaneously with Al^{3+}, where a reduction in Al root concentration (13.5%) at the lower doses of MeJA was found compared to the prior application ($P \leq 0.05$; Fig. 1B). In Bluegold leaves and roots, an increase in Al concentration (60- and 2.7-fold, respectively) was observed in the Al treatment alone compared to the control ($P \leq 0.05$; Fig. 1C and D). All combined treatments were able to decrease the Al concentration of both organs in the two cultivars compared to the Al treatment ($P \leq 0.05$; Fig.1C and D). However, in Bluegold leaves, the Al concentration was more reduced when MeJA was applied simultaneously with Al^{3+} compared to previously applied MeJA ($P \leq 0.05$; Fig. 1C).

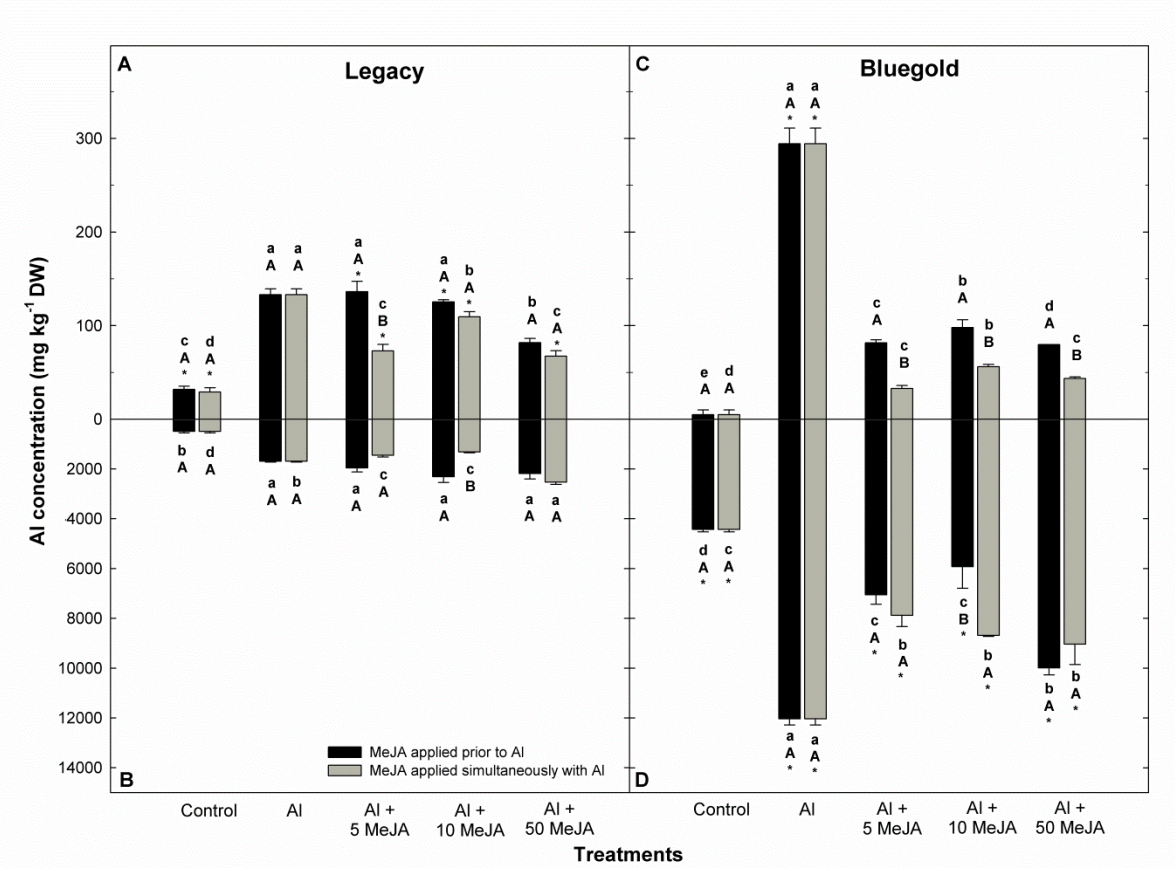

Fig. 1 Aluminum concentration (mg kg^{-1} DW) in leaves (A, C) and roots (B, D) of Legacy and Bluegold cultivars. Values represent the average or means of 3 replicates ± S.E. and doses in µM of MeJA and Al. Different lowercase letters show statistically significant differences among the treatments at each time of MeJA application (prior or

simultaneously) in the same cultivar. Different capital letters show significant differences between MeJA application times for the same treatment and cultivar. Asterisk (*) shows significant differences between cultivars at the same treatment and time of MeJA application ($P \leq 0.05$).

Legacy leaves showed significantly higher LP than those of Bluegold ($P \leq 0.05$; Fig. 2A and C). The simultaneous application of MeJA and Al^{3+} reduced leaf LP in all treatments, compared to Al treatment alone ($P \leq 0.05$; Fig. 2A). When MeJA was applied prior to Al^{3+}, a reduction in LP was observed at the lowest and highest MeJA doses compared to the simultaneously application of MeJA and Al^{3+}, with the highest LP being obtained with the highest MeJA dose (50 µM) ($P \leq 0.05$; Fig. 2A). Legacy roots showed low LP in plants treated with MeJA, regardless of dose and application time, and the LP was lower than in plants treated with Al^{3+} ($P \leq 0.05$; Fig. 2B). The greatest decrease of LP occurred at 10 µM MeJA regardless of application time and with the lowest MeJA dose (5 µM) when applied at the same time as Al^{3+}, these values being lower than the control ($P \leq 0.05$; Fig. 2B). Bluegold leaves and roots increased LP (13.7- and 1.8-fold, respectively) under toxic Al compared to the control ($P \leq 0.05$; Fig. 2C and D). However, these organs exhibited lower LP at the lowest MeJA treatment when MeJA was applied simultaneously with Al^{3+} compared to its prior application ($P \leq 0.05$; Fig. 2C and D). Similarly, at 10 µM and 50 µM MeJA applied prior to Al^{3+}, a reduction in LP was observed in leaves and roots (7.8- and 1.6-fold, respectively) compared to simultaneous MeJA application ($P \leq 0.05$; Fig. 2C and D).

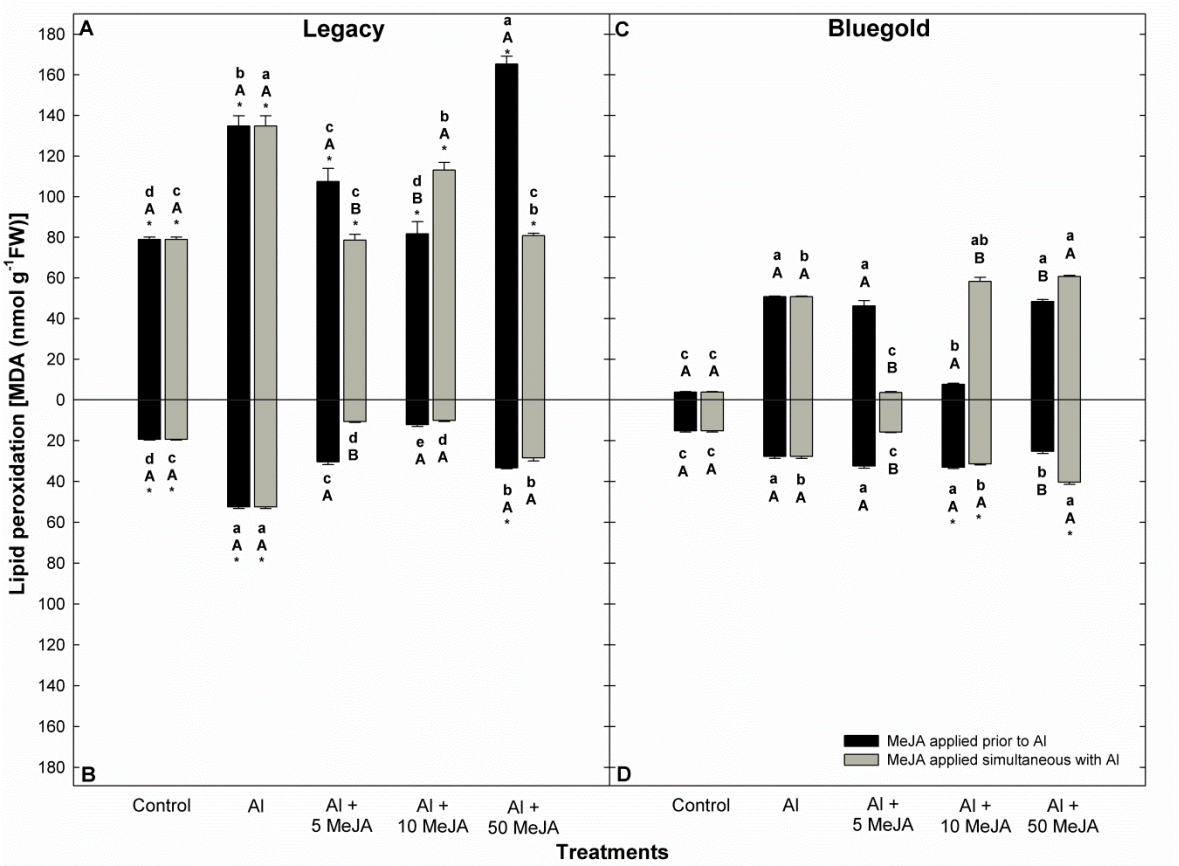

Fig. 2 Lipid peroxidation [MDA (nmolg^{-1} FW)] in leaves (A, C) and roots (B, D) of Legacy and Bluegold cultivars. Values represent the average or means of 3 replicates ± S.E. and doses in μM of MeJA and Al. Different lowercase letters show statistically significant differences among the treatments at each time of MeJA application (prior or simultaneously) in the same cultivar. Different capital letters show significant differences between MeJA application times for the same treatment and cultivar. Asterisk (*) shows significant differences between cultivars at the same treatment and time of MeJA application ($P \leq 0.05$).

No statistically significant differences between Al^{3+} and control treatments were detected in the H_2O_2 concentration of Legacy leaves and roots ($P \leq 0.05$; Fig.3A and B). The most noticeable reduction in H_2O_2 concentration of Legacy leaves (5.7%) and roots (28.8%) was with Al^{3+} and the lowest MeJA dose applied simultaneously as compared to its prior application ($P \leq 0.05$; Fig. 3A and B). Instead, in Bluegold the H_2O_2 concentration

increased in leaves (17.8%) and roots (37.2%) under Al treatment compared to the control ($P \leq 0.05$; Fig. 3C and D). The MeJA application generally decreased the H_2O_2 concentration compared to the Al treatment ($P \leq 0.05$; Fig. 3C and D). Bluegold leaves and roots decreased their H_2O_2 concentration at lower MeJA treatments applied prior to Al compared to Al treatment alone, reaching similar values to the control with the exception of the highest MeJA treatment in leaves ($P \leq 0.05$; Fig. 3C and D)

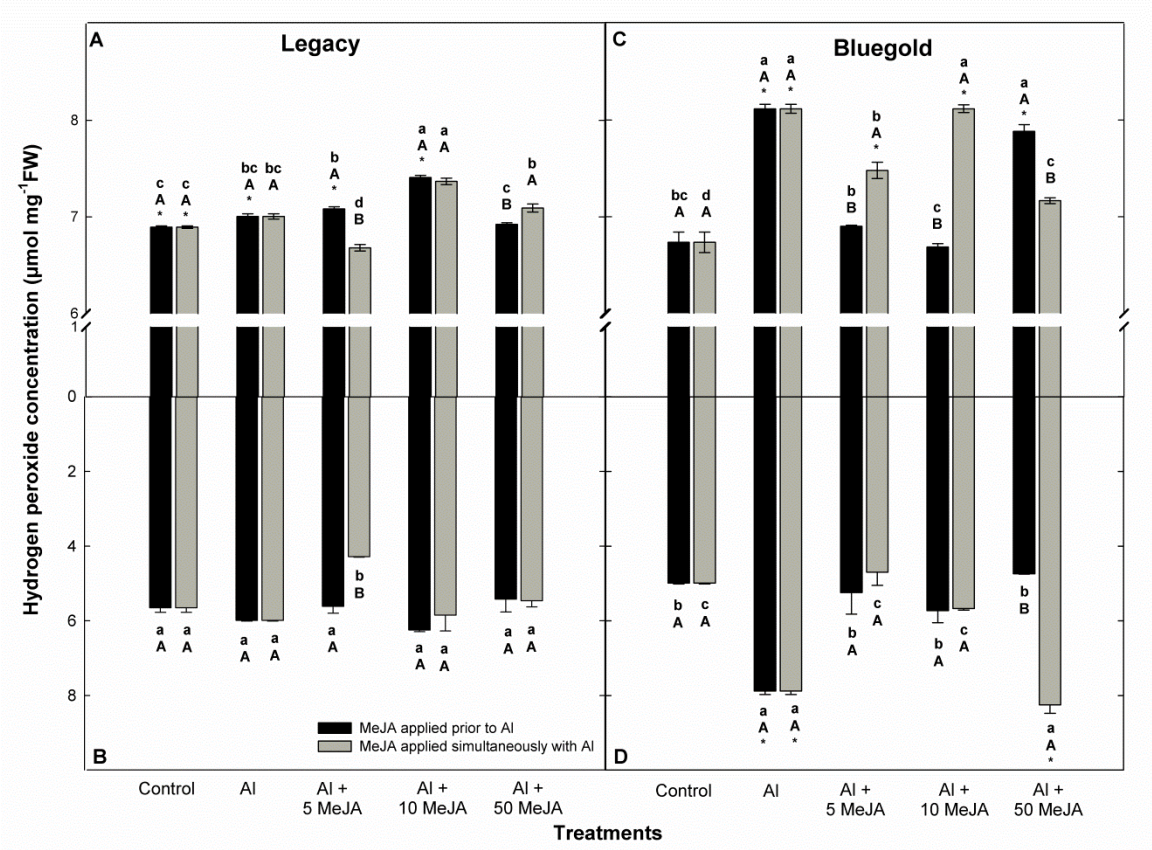

Fig. 3 H_2O_2 concentration (µmol mg^{-1} FW) in leaves (A, C) and roots (B, D) of Legacy and Bluegold cultivars. Values represent the average or means of 3 replicates ± S.E. and doses in µM of MeJA and Al. Different lowercase letters show statistically significant differences among the treatments at each time of MeJA application (prior or simultaneously) in the same cultivar. Different capital letters show significant differences between MeJA application times for the same treatment and cultivar. Asterisk (*) shows significant differences between cultivars at the same treatment and time of MeJA application ($P \leq 0.05$).

Generally, AA in Legacy leaves was higher than in the Bluegold cultivar in all treatments compared to the control ($P \leq 0.05$; Fig. 4A and C). By contrast, Bluegold roots showed higher AA values than Legacy roots ($P \leq 0.05$; Fig. 4B and D). The AA of Legacy leaves was enhanced in all treatments compared to the control, and the values were very similar between them ($P \leq 0.005$; Fig. 4A). In Legacy roots, the AA increased by 21.6% with Al^{3+} application ($P \leq 0.05$; Fig. 4B). The MeJA application in Legacy increased the AA of roots compared to the control, with the exception of the 10 µM MeJA treatment regardless of application time, and 50 µM MeJA applied prior to Al^{3+} ($P \leq 0.05$; Fig. 4B). A slight increase in the AA of Bluegold leaves was frequently observed in all treatments compared to the control ($P \leq 0.05$; Fig. 4C). In Bluegold roots, the AA was significantly increased by Al^{3+} alone and Al + 5 µM MeJA treatments regardless of the time of MeJA application compared to the control ($P \leq 0.05$; Fig. 4D).

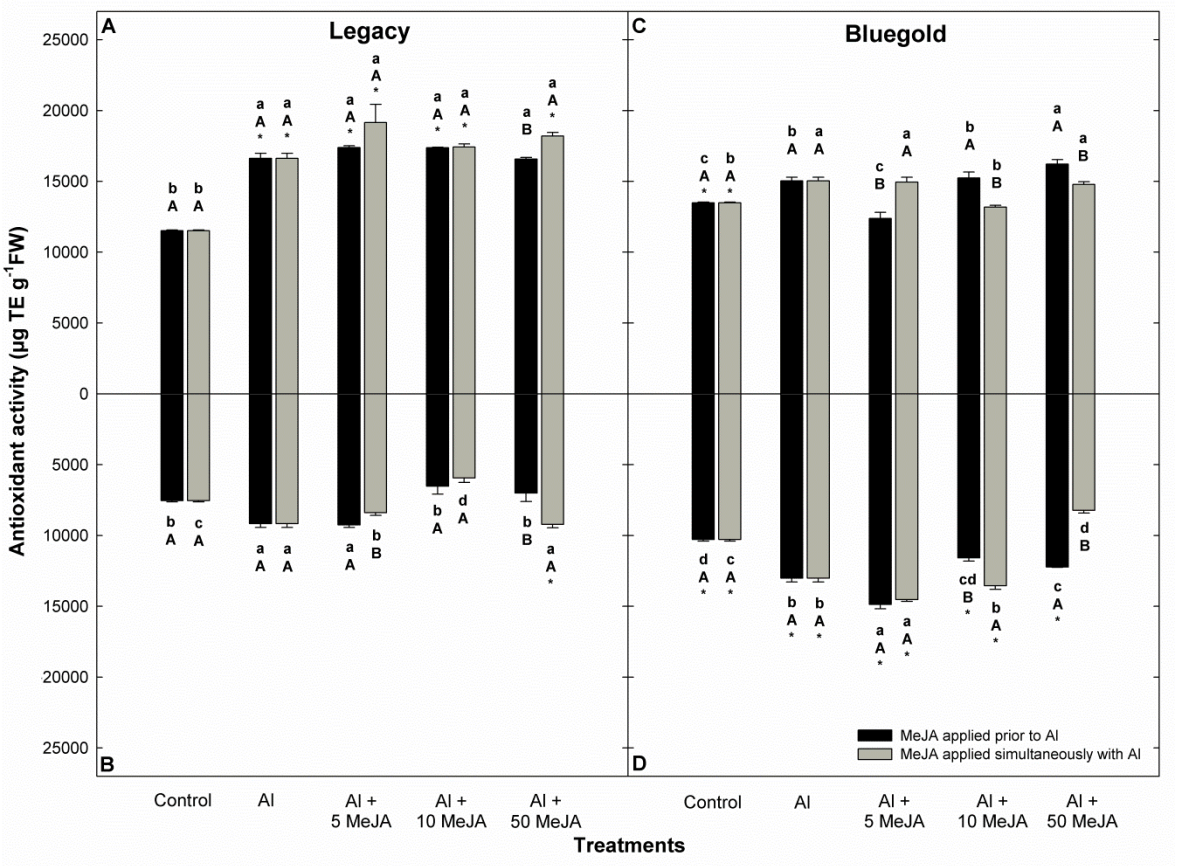

Fig. 4 Antioxidant activity (µg TEg^{-1} FW) in leaves (A, C) and roots (B, D) of Legacy and Bluegold cultivars. Values represent the average or means of 3 replicates ± S.E. and doses in µM of MeJA and Al. Different lowercase letters show statistically significant differences among the treatments at each time of MeJA application (prior or simultaneously) in the same cultivar. Different capital letters show significant differences between MeJA application times for the same treatment and cultivar. Asterisk (*) shows significant differences between cultivars at the same treatment and time of MeJA application ($P \leq 0.05$).

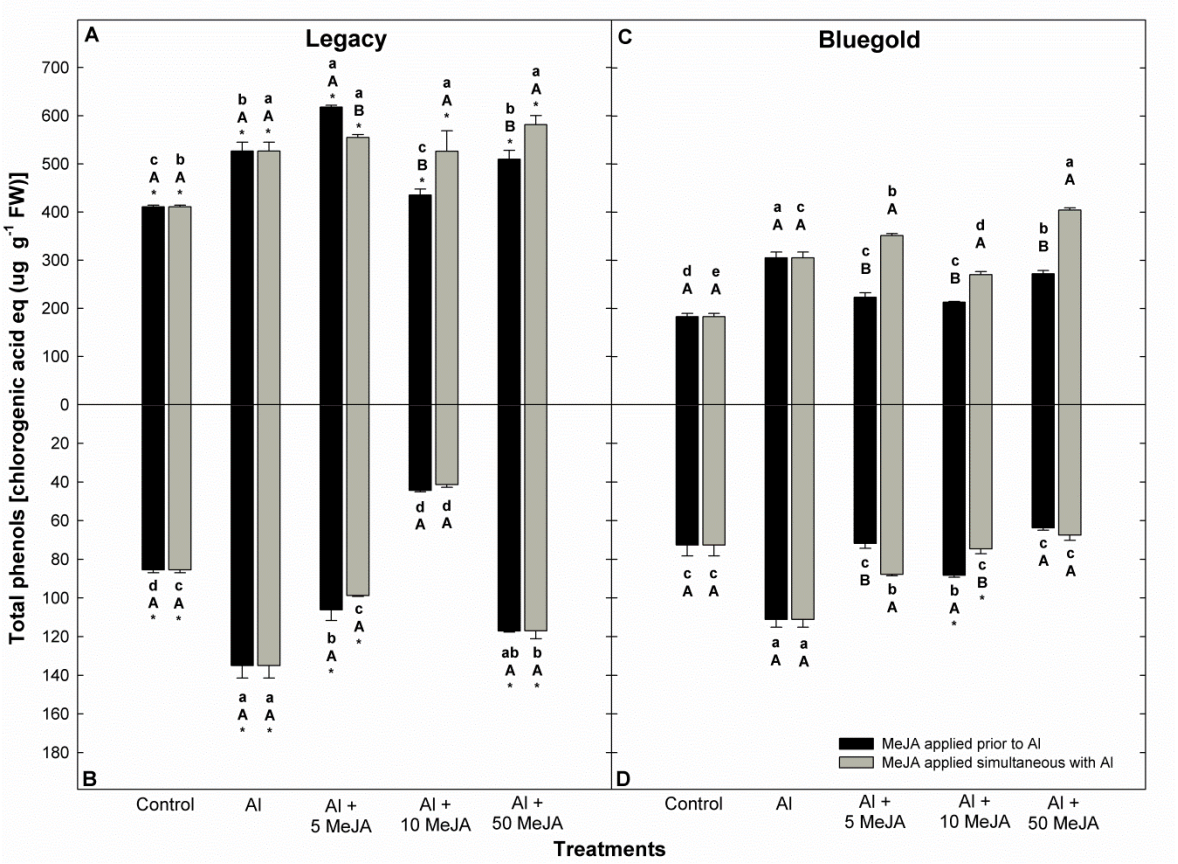

Fig. 5 The total phenols [chlorogenic acid eq (ug g^{-1} FW)] in leaves (A, C) and roots (B, D) of Legacy and Bluegold cultivars. Values represent the average or means of 3 replicates ± S.E. and doses in µM of MeJA and Al. Different lowercase letters show statistically significant differences among the treatments at each time of MeJA application (prior or

simultaneously) in the same cultivar. Different capital letters show significant differences between MeJA application times for the same treatment and cultivar. Asterisk (*) shows significant differences between cultivars at the same treatment and time of MeJA application ($P \leq 0.05$).

The TP values of leaves and roots were commonly higher in Legacy than in Bluegold ($P \leq 0.05$; Fig. 5A, B, C, and D). In Legacy leaves significant differences were observed between treatments and control, with the exception of the treatment of Al+10 µM MeJA (with MeJA applied previous as Al), where no differences were found ($P \leq 0.05$; Fig. 5A). The highest TP of Legacy leaves was obtained at 5 µM MeJA, applied prior to Al, and this was higher than the control (33.7%) and Al (14.9%) treatments ($P \leq 0.05$; Fig. 5A). In Legacy roots the TP concentrations were enhanced 36% under toxic Al, and 16.7% under Al + 5 µM MeJA, and 27% with Al + 50 µM MeJA compared to the control, independently of the MeJA application time ($P \leq 0.05$; Fig. 5B). In Legacy roots, lowest TP concentration (3-fold) were obtained at the treatment of Al+10 µM MeJA compared with Al treatment at both application times ($P \leq 0.05$; Fig. 5B). In Bluegold leaves and roots, TP concentrations increased 1.7- and 1.5-fold respectively in plants subjected to Al^{3+} in relation to the control ($P \leq 0.05$; Fig. 5C). When MeJA was applied simultaneously with Al^{3+}, leaf TP concentrations significantly increased in all Al+MeJA treatments over the control, with the largest increase (2.2-fold) being at 50 µM Al+MeJA ($P \leq 0.05$; Fig. 5C). In Bluegold roots, TP concentration significantly increased with Al treatment alone (35%), but decreased when MeJA and Al were applied simultaneously reaching values similar to the control ($P \leq 0.05$; Fig. 5D).

SOD activity in leaves was higher in Bluegold than in Legacy ($P \leq 0.05$; Fig. 6A and C). There was practically no change in its activity among the treatments in Legacy leaves, with the exception of the Al^{3+}treatment, where a 1.6-fold increase was observed, whereas for the lowest and highest MeJA doses applied simultaneously with Al^{3+} 1.8- and 1.9-fold increases respectively were found ($P \leq 0.05$; Fig. 6A). SOD activity in Legacy roots decreased significantly (1.7-fold) with the Al^{3+} treatment compared to the control independently of time application, increasing at 5 µM MeJA until reaching control values ($P \leq 0.05$; Fig. 6B). A significant 5-fold decrease in SOD activity was also observed at 10

µM MeJA applied prior to Al compared to simultaneous MeJA application ($P \leq 0.05$; Fig. 6B). In Bluegold leaves, SOD activity significantly increased (2.9-fold) with Al treatment compared to the control ($P \leq 0.05$; Fig. 6C). The highest value of SOD activity was obtained at 10 µM MeJA applied simultaneously with Al^{3+}, representing a 2.2- and 6.4-fold increase compared to the Al^{3+} treatment alone and to control, respectively ($P \leq 0.05$; Fig. 6C). At 5 µM MeJA applied simultaneously with Al^{3+}, SOD activity was somewhat lower than at 10 µM MeJA, showing an increase of 1.4- and 4.2-fold with respect to Al^{3+} treatment alone and to control, respectively. In Bluegold roots, almost all the SOD values were similar to those of Al treatment and control, with the exception of 10 µM MeJA applied simultaneously with Al^{3+}, where a 2-fold increase of SOD activity was observed ($P \leq 0.05$; Fig.6D).

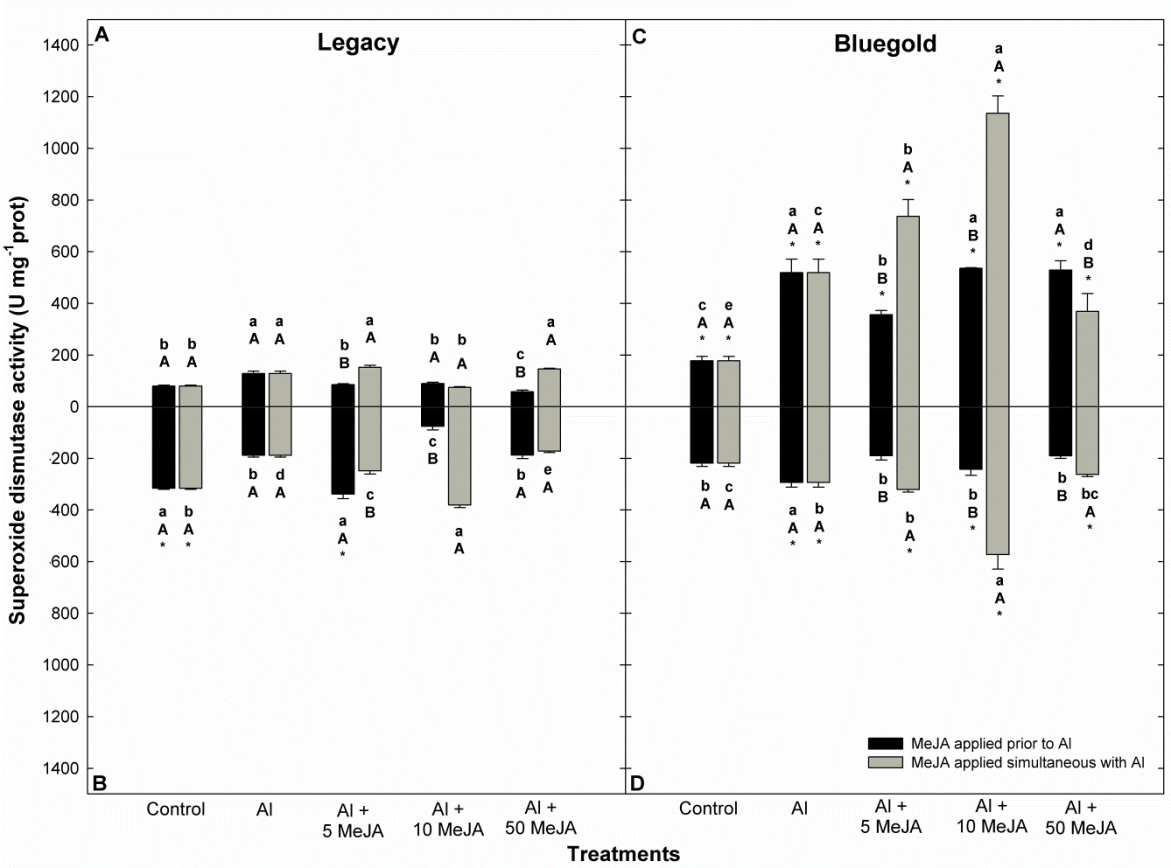

Fig. 6 Superoxide dismutase activity (U mg^{-1} prot) in leaves (A,C) and roots (B,D) of Legacy and Bluegold cultivars. Values represent the average or means of 3 replicates ± S.E. and doses in µM of MeJA and Al. Different lowercase letters show statistically significant differences among the treatments at each time of MeJA application (prior or

simultaneously) in the same cultivar. Different capital letters show significant differences between MeJA application times for the same treatment and cultivar. Asterisk (*) shows significant differences between cultivars at the same treatment and time of MeJA application ($P \leq 0.05$).

Total flavonoids (TF) of Legacy did not show any change among treatments or in their application time ($P \leq 0.05$; Table. 1). By contrast, Bluegold TF decreased (25%) in the presence of toxic Al, but the application of the lowest MeJA dose applied simultaneously with Al^{3+} counteracted this effect ($P \leq 0.05$; Table 1). Phenolic compounds of Legacy and Bluegold showed differences in concentrations of chlorogenic acid, rutin, coumaric acid, ferulic acid and myricetin. Caffeic acid was not detected in Bluegold leaves, while quercetin and kaempferol were not detected in either cultivar ($P \leq 0.05$; Table. 1). In roots of both cultivars, phenolic compounds were not detected as they were below the detection limit of the equipment used.

Chlorogenic acid in Legacy doubled with the application of Al alone compared to the control ($P \leq 0.05$; Table 1). The effect of MeJA application was more evident at the lowest MeJA dose when applied prior (3-fold) or simultaneously (1.7-fold) to Al^{3+} compared to the control ($P \leq 0.05$; Table. 1). By contrast, a reduction in chlorogenic acid was observed in Bluegold at both MeJA application times and in all treatments compared to the control ($P \leq 0.05$; Table. 1). An increase in caffeic acid in Legacy with Al and MeJA application was observed at both application times ($P \leq 0.05$; Table. 1). The high values of caffeic acid were found at Al^{3+} (2.8-fold) regardless of the MeJA application time, whereas at the lowest MeJA dose a 1.8-fold increase in the previous and 3-fold in the simultaneous MeJA application was detected ($P \leq 0.05$; Table. 1). Rutin, coumaric and ferulic acids in Legacy decreased with Al treatment (4.7-, 1.8-, and 2.5-fold, respectively), but when the lowest dose of MeJA was applied simultaneously with toxic Al, values similar to the control were achieved ($P \leq 0.05$; Table 1). Myricetin concentration in Legacy was augmented (1.8-fold) by adding the lowest MeJA dose at both application times compared to Al^{3+} treatment ($P \leq 0.05$; Table. 1). In Bluegold, rutin increased with MeJA application regardless of the

application time ($P \leq 0.05$; Table. 1). Coumaric acid and myricetin practically did not change in Bluegold, whereas ferulic acid decreased in all treatments independently of the MeJA application time ($P \leq 0.05$; Table. 1).

Table 1 Total flavonoid (µg rutin eq g^{-1} FW) and phenolic compounds (mg or µg g^{-1} FW) in Legacy and Bluegold leaves. Values represent the means of 3 replicates ± SD. ND = no detected. Different lowercase letters show statistically significant differences among the treatments at each time of MeJA application (priot or simultaneously) on the same cultivar. Different capital letters show significant differences between MeJA application times for the same treatment and cultivar. Asteriks (*) shows significant differences between cultivars at the same treatment and time of MeJA application ($P < 0.05$)

Cultivars	Al (µM)	MeJA (µM)	Application time of MeJA	Total flavonoids (µg rutin eq g^{-1} FW)	Clorogenic acid (mg g^{-1} FW)	Caffeic acid (mg g^{-1} FW)	Rutin (mg g^{-1} FW)	Coumaric acid (µg g^{-1} FW)	Ferulic acid (µg g^{-1} FW)	Myricetin (µg g^{-1} FW)
Legacy	0	0		211.2 ± 3.0 (aA*)	4.4 ± 0.2 (dA)	0.5 ± 0.0 (eA)	8.9 ± 0.3 (aA)	361.8 ± 29.7 (aA*)	291.8 ± 11.3 (aA*)	180.2 ± 9.2 (cA)
	100	0		210.1 ± 9.3 (aA*)	10.3 ± 0.4 (bA*)	1.4 ± 0.0 (bA)	1.9 ± 0.0 (cA)	201.5 ± 8.2 (bA)	117.4 ± 4.9 (bA)	241.1 ± 12.0 (bA)
	100	5	Prior to Al	217.4 ± 10.5 (aA*)	13.8 ± 0.6 (aA*)	1.9 ± 0.1 (aA)	2.9 ± 0.2 (cB)	383.9 ± 26.7 (aA*)	285.2 ± 10.7 (aB*)	439.5 ± 22.7 (aA*)
	100	10		207.2 ± 3.1 (aB*)	6.2 ± 0.2 (cB)	0.7 ± 0.0 (dB)	6.5 ± 0.2 (bA)	401.4 ± 14.9 (aA*)	260.8 ± 9.4 (aA*)	206.4 ± 7.17 (bAc)
	100	50		186.5 ± 5.4 (aB*)	6.4 ± 0.2 (cA)	1.1 ± 0.0 (cA)	6.6 ± 0.4 (bB)	391.8 ± 8.2 (aA*)	281.1 ± 2.9 (aB*)	246.1 ± 24.5 (bA)
	0	0		211.2 ± 3.0 (aA*)	4.4 ± 0.2 (cA)	0.5 ± 0.0 (cA)	8.9 ± 0.3 (aA)	365.2 ± 25.0 (aA*)	291.0 ± 20.7 (bA*)	180.2 ± 9.2 (cA)
	100	0		210.1 ± 9.3 (aA*)	10.3 ± 0.4 (aA*)	1.4 ± 0.0 (aA)	1.9 ± 0.0 (cA)	201.5 ± 8.2 (bA)	117.4 ± 4.9 (cA)	241.1 ± 12.0 (bA)
	100	5	Simultaneous	204.4 ± 8.2 (aA*)	7.3 ± 0.4 (bB)	0.9 ± 0.1 (bB)	8.8 ± 0.6 (aA)	421.8 ± 21.7 (aA*)	414.4 ± 35.8 (aA*)	429.6 ± 15.9 (aA*)
	100	10	to Al	235.3 ± 8.5 (aA*)	10.2 ± 0.4 (aA*)	1.4 ± 0.1 (aB)	4.0 ± 0.3 (bB)	423.2 ± 20.7 (aA*)	267.4 ± 2.8 (bA*)	245.6 ± 23.1 (bA)
	100	50		226.7 ± 12.5 (aB*)	7.2 ± 0.6 (bA)	0.9 ± 0.0 (bA)	8.4 ± 0.4 (aA)	420.7 ± 17.6 (aA*)	409.2 ± 40.99 (aA*)	279.1 ± 3.4 (bA*)
Bluegold	0	0		93.0 ± 2.3 (aA)	15.5 ± 1.0 (aA*)	ND	24.4 ± 1.7 (bA*)	240.7 ± 4.4 (cA)	161.4 ± 11.1 (aA)	239.4 ± 1.7 (aA*)
	100	0		69.3 ± 5.1 (bA)	6.8 ± 0.4 (bA)	ND	24.6 ±1.2 (bA*)	231.3 ± 6.5 (cA*)	120.0 ± 2.6 (cA)	240.8 ± 18.6 (aA)
	100	5	Prior to Al	79.6 ± 4.4 (abB)	5.1 ± 0.1 (bB)	ND	32.3 ± 1.9 (aB*)	237.5 ± 14.2 (cB)	131.9 ± 8.7 (bA)	230.7 ± 2.1 (aA)
	100	10		52.5 ± 3.7 (bA)	5.1 ± 0.1 (bB)	ND	33.4 ± 0.3 (aA*)	325.3 ± 2.5 (aA)	124.4 ± 2.3 (cA)	235.9 ± 0.9 (aA)
	100	50		86.6 ± 8.4 (aA)	5.4 ± 0.1 (bB)	ND	32.6 ± 1.6 (aA*)	287.6 ± 5.7 (bA)	123.7 ± 3.7 (cA)	238.8 ± 7.9 (aA)
	0	0		93.0 ± 2.3 (aA)	15.5 ± 1.0 (aA*)	ND	24.4 ± 1.7 (cA*)	240.7 ± 4.4 (bA)	161.4 ± 11.1 (aA)	239.4 ± 1.7 (aA*)
	100	0	Simultaneous to Al	69.3 ± 5.1 (bA)	6.8 ± 0.4 (bA)	ND	24.6 ±1.2 (cA*)	231.3 ± 6.5 (bA)	120.0 ± 2.6 (bA)	240.8 ± 18.6 (aA)
	100	5		96.3 ± 3.2 (aA)	7.5 ± 0.1 (bA)	ND	41.4 ± 2.8 (aA*)	265.7 ± 6.6 (aA)	120.9 ± 7.7 (bA)	233,5 ± 2.4 (aA)
	100	10		61.4 ± 2.1 (bA)	7.9 ± 0.2 (bA)	ND	33.4± 1.6 (bA*)	227.2 ± 11.3 (bB)	88.6 ± 0.7 (cB)	236.7 ± 9.2 (aA)
	100	50		70.2 ± 2.1 (bB)	7.7 ± 0.4 (bA)	ND	32.9 ± 1.3 (bA*)	252.2 ± 10.1 (abB)	107.9 ± 7.0 (bcA)	236.1 ± 4.9 (aA)

3.5 Discussion

This study focused on the effect of time and dose of a MeJA application on the antioxidant performance in blueberry cultivars under toxic Al. Substantial differences in the doses and application times were observed, demonstrating that simultaneous application and a low dose of MeJA (5 µM) was able to reduce the Al toxicity of *V. corymbosum* by reducing the Al concentration and oxidative damage (LP and H_2O_2 concentration), whereas the antioxidant performance (phenols and SOD activity) was differentially activated in leaves and roots according the Al resistance of the cultivars (Fig. 1, 2, 3, 5, 6 and Table 1).

Typical symptoms of Al toxicity in leaves and roots of blueberry plants were observed when plants were subjected to toxic Al (Fig. 1, 2 and 3). This is consistent with the data reported by Reyes-Díaz et al. (2009; 2010), Inostroza-Blancheteau et al. (2012), and Manquián et al. (2013). Changes in AA systems due to Al stress were reported by Inostroza-Blancheteau et al. (2011), where high Al concentration in *V. corymbosum* plants increased LP, showing a high gene expression of glutathione S-transferase (GST) and aldehyde dehydrogenase (ALDH) associated with the enhancement of Al toxicity according to the blueberry cultivars Al resistance or Al sensitivity. Our findings demonstrated that the highest AA and phenolic concentration is organ-dependent, being higher in leaves (Fig. 4A and C) followed by roots (Fig. 4B and D) and fruits (Ehlenfeldt and Prior, 2001; Ribera et al. 2010). In this sense, in Legacy (Al-resistant) leaves, the Al treatment increased chlorogenic and caffeic acids and myricetin, whereas rutin and ferulic acid as well as coumaric acid were reduced in the same treatment (Table 1). By contrast, in Bluegold (Al-sensitive) leaves, caffeic acid was not detected (Table 1). These data are consistent with those of Manquián et al. (2013), who reported an increase in chlorogenic acid and rutin by Al stress in Legacy, but this result is not consistent with the reduced rutin concentration found in our study (Table 1). On the other hand, it has been reported that, as constituents of cell walls, phenolic acids protect against biotic and abiotic stresses (Eraso and Hartley, 1990). In blueberry cultivars the richness and abundance of phenolic compounds depend on the species (Wang et al. 2015). Lowbush blueberry (*V. angustifolium*) is richer in

chlorogenic acid and quercetin glycosides (Harris et al. 2007; Wang et al. 2015), whereas in rabbiteye blueberry (*V. ashei*) flavan-3-ols, proanthocyanidins, chlorogenic acid and flavonol glycosides were the major phenolic compounds in leaf extracts (Matsuo et al. 2010; Wang et al. 2015). In this sense, Wang et al. (2015) analyzed leaves from 104 blueberry cultivars, identifying 28 phenolic compounds. Based on the results of a hierarchical cluster dendrogram analysis, the 104 blueberry cultivars were clustered into three groups, and Legacy and Bluegold were in different groups. This may explain the differences observed in our study, where the chlorogenic acid in the leaves was higher in Legacy than in Bluegold. In addition, the absence of any phenol compounds in Legacy (quercetin and kaempferol) and in Bluegold (quercetin, kaempferol and caffeic acid) may also explain this difference (Table 1).

Our findings also demonstrated that the reduction in the oxidative damage by the application of the lowest dose of MeJA applied simultaneously with toxic Al, triggered an increase in the antioxidant mechanism responses as: total phenols (Fig. 5) and any phenolic compounds (Table 1) as well as SOD activity (Fig. 6) in both cultivars. These results are consistent with those reported by Rudell et al. (2002); Jung (2004); Chen et al. (2006); Keramat et al. (2009); Wang et al. (2009); Ruiz-García et al. (2012); Poonam et al. (2013); Chen et al. (2014).

By contrast, the higher MeJA+Al^{3+} doses induced oxidative damage similar to those demonstrated by the Al treatment alone (Fig. 2 and 3). Furthermore, with these MeJA doses, antioxidant parameters did not provide evidence of a better response (Fig. 4). Despite the few reports about MeJA application under stress in woody plants, most of them used higher doses of MeJA than those used in our study. In this context, studies performed with MeJA in *Gossypium hirsutum* (Cotton), *Pyrus bretschneideri* (pear) and *Betula pubescens* under various stresses used doses of MeJA from 2.5 to 50 mM (Gao et al. 2004; Mäntylä et al. 2014; Konan et al. 2014). Nonetheless, Konan et al (2014) found that cotton plants treated with 20 mM of MeJA and biotic stress showed toxicity symptoms and altered total phenolic concentrations. Similar results regarding MeJA toxicity under biotic stress have been reported in other species by Heijari et al. (2008) and Moreira et al. (2009). Lower concentrations of MeJA (0.1-10 µM) applied to the woody species *Kandelia*

obovata subjected to Cd toxicity showed that regardless of the MeJA dose, lipid peroxidation decreased without significant differences among them, but Cd concentration increased compared to the Cd treatment alone. Based on this evidence, we selected 5 µM MeJA as the lowest dose of this phytohormone.

The time application of MeJA was a crucial factor for reducing the Al concentration in leaves, regardless of the Al resistance of the study cultivars (Fig. 1A and C). In *Phaseolus coccineus* plants the application on leaves of 10 µM MeJA prior (1h and 24h) to the addition of toxic Cu (50 and 100 µM) indicated that 1h prior MeJA application was more efficient at decreasing the Cu concentration at 50 µM Cu, whereas at 100 µM Cu, a MeJA application 24h prior was more effective than 1h (Hanaka et al. 2016). Nevertheless, Konan et al. (2014) reported that 5 mM and 10 mM MeJA applied 72 or 48 h prior to pathogen stress increased total phenols compared with pre-treatment at 24 h. Therefore, the time of MeJA application and stress intensity are key factors for response to MeJA application in plants.

The interaction between toxic metals and JAS is limited, and the mechanisms are mostly unknown (Keramat et al. 2009; Piotrowska et al. 2009). It is known that the increase in metal toxicity and oxidative damage can be decreased by the participation of antioxidant mechanisms induced by JAS (Maksymiec and Krupa, 2002; 2007b; Keramat et al. 2009; Piotrowska et al. 2009; Chen et al. 2014). Based on our results, we suggest that MeJA stimulated the antioxidant mechanisms to counteract the damage induced by toxic Al (Fig. 6 and Table 1). A high affinity for Al ions joining carboxylic groups of phenolic compounds in the cell wall limits the entry of available Al^{3+} inside the cells, decreasing the effect of toxic Al (McDonald et al. 1996). Therefore, we think that the retention of Al in the cell wall by MeJA application may be one of the first responses to minimize Al damage in the Al-resistant cultivar due to an increase in phenolic compounds with the lowest MeJA doses applied simultaneously with toxic Al (Table 1). However, in the case of the Al-sensitive cultivar subjected to the same treatment as above, the reduction in the Al concentration in tissues showed a relationship with the activation of the enzymatic activity (SOD) (Fig. 6C and D). Comparing the doses of MeJA used in our study with those reported for other fruit species, it appears that our doses are lower, suggesting that doses are

highly dependent on the stress condition and plant species (Wang 1999; Larronde et al. 2003; Menga et al. 2009; Ismail et al. 2012). Our results suggest that there is an optimal range of MeJA for each species that can counteract a determined stress; outside this range phytotoxicity occurs (Keramat et al. 2009). Furthermore, at higher doses MeJA could saturate the MeJA receptors in the membrane as has been reported in *Arabidopsis* subjected to saline and pathogen stress (An et al. 2008; Yoon et al. 2010). Another alternative might be the activation of defense mechanisms stimulated by joint Al action with simultaneous MeJA application, inducing changes in the ROS concentration. It has been reported that Al and MeJA could use H_2O_2 as a second messenger under stressful conditions (Hu et al. 2009; Liu et al. 2014). In *Cassia tora* roots grown with 10 µM Al and 10 µM MeJA was observed increases in H_2O_2 accumulation, lignin production in the root cell wall, AA activation, phenylalanine ammonia-lyase (PAL) and lipoxygenases (LOX) (Xue et al. (2008). It is important to note that MeJA and Al are linked to the early activation of programmed cell death (PCD) (Pan et al. 2001; Zhang and Xiang, 2008), suggesting that both Al and MeJA use the apoplastic H_2O_2 to trigger PCD. In this context, Zhang and Xiang, (2008) and Huang et al. (2014) determined the start time of ROS production in cells of *A. thaliana* and peanuts under MeJA and Al stress able to trigger activation of the antioxidant mechanisms. These authors also reported changes related to early ROS production under MeJA application and Al stress, respectively. Further, Sivaguru et al. (2013) described that Al-induced ROS production could be involved in the signaling, regulation and expression of the *SbMATE* (*Sorghum bicolor* multidrug and toxic compound extrusion) located in the root plasma membrane and related to citrate efflux, which regulates the entry of Al. Our results suggest that H_2O_2 could regulate a higher Al uptake in blueberry (Fig.1 and Fig. 3) given that the H_2O_2 concentration had greater values under Al^{3+}in both cultivars (Fig. 3). This behavior was more evident in the Al-sensitive cultivar (Bluegold) than in the Al-resistant cultivar (Legacy) (Fig. 3). Interestingly, our findings also showed that Al concentration in tissues decreased concomitantly with a decrease in H_2O_2 concentration at the lowest MeJA doses applied simultaneously with the toxic Al (Fig. 1 and 3). Which, it could be attributed to increased in the SOD activity and possible a higher CAT activity, that catalyze reactions to get rid of the ROS ($O_2^{\bullet-}$ and H_2O_2) forming non-toxic compounds (H_2O) (Gill and Tuteja, 2010).

Differential responses in the leaves and roots of blueberry plants under toxic Al and MeJA application were also found in total phenols and phenolic compounds (Fig. 5 and Table 1). Under Al toxicity and MeJA application these compounds were more abundant in leaves than in roots (Fig.5 and Table 1). In this sense, we suggest that in leaves phenolic compounds are induced as an antioxidant mechanism to counteract Al stress, while in roots this could be related to a high organic acid production to counteract the harmful effect of Al (Fig. 5). These suggestions agree with those of Hanaka et al. (2016), who reported stimulation of organic acid in *Phaseolus coccineus* treated with Cu and MeJA during short- and long-term exposure.

3.6 Conclusion

Simultaneous Al and MeJA application induced AA in both cultivars compared to prior MeJA application to toxic Al. Additionally, the Al-resistant cultivar increased mainly non-enzymatic compounds to counteract Al toxicity, whereas the Al-sensitive cultivar increased the SOD activity. Under Al toxicity and MeJA application, however, phenols were more abundant in leaves than in roots, suggesting that in leaves these compounds are induced as an antioxidant mechanism to counteract Al stress, while in roots this could be related to a high organic acid production. Low doses of MeJA applied simultaneously with toxic Al increased Al resistance in Legacy and decreased the oxidative damage in Bluegold, suggesting a decrease of Al-sensitivity in the latter cultivar. Therefore, the application of a low dose of MeJA could be a good alternative for reducing the negative effects of Al toxicity in blueberry, decreasing Al concentration in tissues and strengthening the antioxidant mechanisms. Contrarily, higher doses of MeJA could be potentially toxic. Finally, more studies are needed to determine the molecular mechanisms involved in the protective effect of MeJA against Al toxicity.

Acknowledgments: We are very grateful for FONDECYT Project Nº 1120917 which supported this work and PhD fellowship Nº21110919, both from the Comisión Nacional de

Investigación Científica y Tecnológica (CONICYT) of the Government of Chile, as well as the 2013 DI 13-2017 and 2015 DI 15-2015 Projects from the Dirección de Investigación at the Universidad de La Frontera, Temuco, Chile. We would like to thank Graciela Muñoz Pozo for her valuable assistance in the laboratory, and Dr. Helen Lowry for revising the language of the manuscript. Finally, we wish to thank the Doctorado en Ciencias de Recursos Naturales Program and the Laboratorio de Fisiología y Bioquímica Vegetal at the Universidad de La Frontera, Temuco, Chile.

References

An SH, Choi HW, Hwang IS, Hong JK, Hwang BK (2008) A novel pepper membrane-located receptor-like protein gene CaMRP1 is required for disease susceptibility, methyl jasmonate insensitivity and salt tolerance. Plant Mol Biol 67:519-533

Barceló J, Poschenrieder C (2002) Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. Environ Exp Bot 48:75-92

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Castrejón A, Eichholz I, Rohn S, Kroh LW, Huyskens-Keil S (2008) Phenolic profile and antioxidant activity of highbush blueberry (*Vaccinium corymbosum* L.) during fruit maturation and ripening. J Food Chem 109:564-572

Chen H, Jones AD, Howe GA (2006) Constitutive activation of the jasmonate signaling pathway enhances the production of secondary metabolites in tomato. FEBS Lett 580: 2540-2546

Chen J, Yan Z, Li X (2014) Effect of methyl jasmonate on cadmium uptake and antioxidative capacity in *Kandelia obovata* seedlings under cadmium stress. Ecotox

Environ Saf 104:349-356

Cheng GW, Breen PJ (1991) Activity of phenylalanine ammonia-lyase (PAL) and concentrations of anthocyanins and phenolics in developing strawberry fruit. J Amer Soc Hort Scl 116:865-869

Chinnici F, Bendini A, Gaiani A, Riponi C (2004) Radical scavenging activities of peels and pulps from cv. Golden delicious apples as related to their phenolic composition. J Agr Food Chem 52:4684-4689

Creelman RA, Mullet JE (1995) Jasmonic acid distribution and action in plants: Regulation during development and response to biotic and abiotic stress. Proc Natl Acad Sci USA 92:4114-4119

Creelman RA, Mullet JE (1997) Oligosaccharins, brassinolides, and jasmonates: nontraditional regulators of plant growth, development, and gene expression. Plant Cell 9:1211-1223

Delhaize E, Ma JF, Ryan PR (2012) Transcriptional regulation of aluminium tolerance genes. Trends Plant Sci 17:341-348

Du Z, Bramlage WJ (1992) Modified thiobarbituric acid assay for measuring lipid oxidation in sugar-rich plant tissue extracts. J Agric Food Chem 40:1556-1570

Ehlenfeldt MK, Prior RL (2001) Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry. J Agric Food Chem 49:2222-2227

Eraso F, Hartley RD (1990) Monomeric and dimeric constituents of plant cell walls possible factors influencing wall biodegradability. J Sci Food Agric 51:163-170

Gao XP, Wang XF, Lu YF, Zhang LY, Shen YY, Liang Z, Zhang DP (2004) Jasmonic acid is involved in the water-stress-induced betaine accumulation in pear leaves. Plant Cell Environ 27:497-507

Giannopolitis CN, Ries SK (1977) Superoxide dismutases. I. Occurrence in higher plants. Plant Physiol 59:309-314

Guo TR, Zhang GP, Zhang YH (2007) Physiological changes in barley plants under combined toxicity of aluminum, copper and cadmium. Colloids Surf B 57:182-188

Hanaka A, Wójcik M, Dresler S, Mroczek-Zdyrska M, Maksymiec W (2016) Does methyl jasmonate modify the oxidative stress response in *Phaseolus coccineus* treated with Cu?. Ecotox Environ Saf 124:480-488

Harris CS, Burt AJ, Saleem A, Le PM, Martineau LC, Haddad PS, Bennett SAL, Arnason JT (2007) A single HPLC-PAD-APCI/MS method for the quantitative comparison of phenolic compounds found in leaf, stem, root and fruit extracts of *Vaccinium angustifolium*. Phytochem Anal 18:161-169

Heijari J, Nerg AM, Kainulainen P, Vuorinen M, and Holopainen JK (2008) Long-term effects of exogenous methyl jasmonate application on Scots pine (*Pinus sylvestris*) needle chemical defense and diprionid sawfly performance. Entomol Exp Appl 128:162-171

Hu X, Li W, Chen Q, Yang Y (2009) Early signal transduction linking the synthesis of jasmonic acid in plant. Plant Signal Behav 4:696-697

Huang W, Yang X, Yao S, LwinOo T, He H, Wang A, Li C, He L (2014) Reactive oxygen species burst induced by aluminum stress triggers mitochondria-dependent programmed cell death in peanut root tip cells. Plant Physiol Biochem 82:76-84

Inostroza-Blancheteau C, Reyes-Díaz M, Aquea F, Nunes-Nesi A, Alberdi M, Arce-Johnson P (2011) Biochemical and molecular changes in response to aluminium-stress in highbush blueberry (*Vaccinium corymbosum* L). Plant Physiol Biochem 49:1005-1012

Inostroza-Blancheteau C, Rengel Z, Alberd M, Mora M, Aquea F, Arce-Johnson P, Reyes-Díaz M (2012) Molecular and physiological strategies to increase aluminum resistance in plants. Mol Biol Rep 39:2069-2079

Ismail A, Riemann M, Nick P (2012) The jasmonate pathway mediates salt tolerance in grapevines. J Exp Bot 63:2127-2139

Jung SJ (2004) Effect of chlorophyll reduction in *Arabidopsis thaliana* by methyl jasmonate or norflurazon on antioxidant systems. Plant Physiol Biochem 42:225-231

Keramat B, Kalantari KM, Arvin MJ (2009) Effects of methyl jasmonate in regulating cadmium induced oxidative stress in soybean plant (*Glycine max* L.). Afr J Microbiol Res 3:240-244

Kochian LV, Pineros MA, Liu J, Magalhaes JV (2015) Plant Adaptation to acid soils: The molecular basis for crop aluminum resistance. Annu Rev Plant Biol 66:571-598

Konan YKF, Kouassi KM, Kouakou KL, Koffi E, Kouassi KN, Sekou D, Kone M, and Kouakou TH (2014) Effect of Methyl Jasmonate on Phytoalexins Biosynthesis and Induced Disease Resistance to *Fusarium oxysporum f. sp. Vasinfectum* in Cotton (*Gossypium hirsutum* L.). Intl J Agron 2014:1-11

Larronde F, Gaudillière JP, Krisa S, Decendit A, Deffieux G, Mérillon JM (2003) Airborne methyl jasmonate induces stilbene accumulation in leaves and berries of grapevine plants. Am J Enol Vitic 54:60-63

Li Y, Nie Y, Zhang Z, Ye Z, Zou X, Zhang LH, Wang Z (2014) Comparative proteomic analysis of methyl jasmonate-induced defense responses in different rice cultivars. Proteomics 14:1088-1101

Liu J, Li Z, Wang Y, Xing D (2014) Overexpression of ALTERNATIVE OXIDASE1a alleviates mitochondria-dependent programmed cell death induced by aluminium phytotoicity in Arabidopsis. J Exp Bot 65:4465-4478

Loreto F, Velikova V (2001) Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. Plant Physiol 127:1781-1787

Ma B, Wan J, Shen ZG (2007) H_2O_2 production and antioxidant responses in seeds and early seedlings of two different rice varieties exposed to aluminum. Plant Growth Regul 52:91-100

Maksymiec W, Krupa Z (2002) Jasmonic acid and heavy metals in *Arabidopsis* plants- a similar physiological responses to both stressors? J Plant Physiol 159:509-515

Maksymiec W, Krupa Z (2007a) Effects of methyl jasmonate and excess copper on root and leaf growth. Biol Plant 51:322-326

Maksymiec W, Wójcik M, Krupa Z (2007b) Variation in oxidative stress and photochemical activity in *Arabidopsis thaliana* leaves subjected to cadmium and excess copper in the presence or absence of jasmonate and ascorbate. Chemosphere 66:421-427

Manquián K, Zúñiga GE, Barrientos H, Escudey M, Molina M (2013) Effect of aluminum on antioxidant activity and phenolic compounds content in *in vitro* cultured blueberries. Bol Latinoam Caribe Plant Med Aromat 12:603-611

Matsuo Y, Fujita Y, Ohnishi S, Tanaka T, Hirabaru H, Kai T, Sakaida H, Nishizono S, Kouno I (2010) Chemical constituents of the leaves of rabbiteye blueberry (*Vaccinium ashei*) and characterisation of polymeric proanthocyanidins containing phenylpropanoid units and A-type linkages. Food Chem 121:1073-1079

Mäntylä E, Blande JD, Klemola T (2014) Does application of methyl jasmonate to birch mimic herbivory and attract insectivorous birds in nature? Arthropod Plant Interact 8:143-153

McDonald M, Mila I, Scalbert A (1996) Precipitation of metal ions by plant polyphenols: optimal conditions and origin of precipitation. J Agric Food Chem 44:599-606

Menga X, Hana J, Wang Q, Tian S (2009) Changes in physiology and quality of peach fruits treated by methyl jasmonate under low temperature stress. Food Chem 114:1028-1035

Moreira X, Sampedro L, Zas R (2009) Defensive responses of *Pinus pinaster* seedlings to exogenous application of methyl jasmonate: Concentration effect and systemic response. Environ Exp Bot 67:94-100

Pan JW, Zhu MY, Chen H (2001) Aluminum-induced cell death in root-tip cells of barley. Environ Exp Bot 46:71-79

Pauwels L, Morreel K, De Witte E, Lammertyn F, Van Montagu M, Boerjan W, Inzé D, Goossens A (2008) Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured Arabidopsis cells. Proc Natl Acad Sci USA 105:1380-1385

Piotrowska A, Bajguz A, Godlewska-żyłkiewicz B, Czerpak R, Kamińska M (2009) Jasmonic acid as modulator of lead toxicity in aquatic plant *Wolffia arrhiza* (Lemnaceae). J Exp Bot 66:507-513

Poonam S, Kaur H, Geetika S (2013) Effect of jasmonic acid on photosynthetic pigments and stress markers in *Cajanus cajan* (L.) Mill sp. seedlings under copper stress. Am J Plant Sci 4:817-823

Reyes-Díaz M, Alberdi M, Mora ML (2009) Short-term aluminum stress differentially affects the photochemical efficiency of photosystem II in Highbush Blueberry genotypes. J Amer Soc Hort Sci 134:14-21

Reyes-Díaz M, Inostroza-Blancheteau C, Millaleo R, Cruces E, Wulff-Zottele C, Alberdi M, Mora ML (2010) Long-term aluminum exposure effects on physiological and biochemical features of Highbush Blueberry cultivars. J Amer Soc Hort Sci 135: 212-222

Ribera AE, Reyes-Díaz M, Alberdi M, Zuniga GE, Mora ML (2010) Antioxidant compounds in skin and pulp of fruits change among genotypes and maturity stages in Highbush blueberry (*Vaccinium corymbosum* L.) grown in southern Chile. J Soil Sci Plant Nutr 10:509-536

Rudell DR, Mattheis JP, Fan X, Fellman JK (2002) Methyl jasmonate enhances

anthocyanin accumulation and modified production of phenolics and pigments in ‘‘Fuji’’ apples. J Amer Soc Hort Sci 127:435-441

Ruhland CT, Day TA (2000) Effects of ultraviolet-B radiation on leaf elongation, production and phenylpropanoid concentrations of *Deschampsia antarctica* and *Colobanthus quitensis* in Antarctica. Physiol Plant 109:244-251

Ruiz-García Y, Romero-Cascales I, Gil-Muñoz R, Fernández-Fernández JI, López-Roca JM, Gómez-Plaza E (2012) Improving grape phenolic content and wine chromatic characteristics through the use of two different elicitors: Methyl jasmonate versus benzothiadiazole. J Agric Food Chem 60:1283-1290

Sadzawka AM, Grez R, Carrasco MA, Mora ML (2004) Métodos de análisis de tejidos vegetales. Comisión de normalización y acreditación, sociedad chilena de la ciencia del suelo, In: Editorial salesianos impresores, Santiago, Chile, pp 105

Schaller A, Stintzi A (2009) Enzymes in jasmonate biosynthesis - Structure, function, regulation. Phytochemistry 70:1532-1538

Shaff JE, Schultz BA, Craft EJ, Clark RT, Kochian LV (2010) GEOCHEM-EZ: a chemical speciation program with greater power and flexibility. Plant Soil 330:207-214

Shao HB, Chu LY, Shao MA, Jaleel CA, Mi HM (2008) Higher plant antioxidants and redox signaling under environmental stresses. C R Biologies 331:433-441

Sivaguru M, Liu J, Kochian LV (2013) Targeted expression of SbMATE in the root distal transition zone is responsible for sorghum aluminum resistance. Plant J 76:297-307

Slinkard K, Singleton VL (1977) Total phenol analysis: automation and comparison with manual methods. Am J Enol Vitic 28:29-55

Spollansky TC, Pitta-Alvarez SI, Giulietti AM (2000) Effect of jasmonic acid and aluminum on production of tropane alkaloids in hairy root cultures of *Brugmansia candida*. Electron J Biotechn 3:72-75

Staswick PE (2008) JAZing up jasmonate signaling. Trends Plant Sci 13:66-71

Wang SY (1999) Methyl jasmonate reduces water stress in strawberry. J Plant Growth Regul 18:127-134

Wang FZ, Wang QB, Kwon SY, Kwak SS, Su WA (2005) Enhanced drought tolerance of transgenic rice plants expressing a pea manganese superoxide dismutase. J Plant Physiol 162:465-472

Wang SY, Chen C, Wang CY (2009) The influence of light and maturity on fruit quality and flavonoid content red raspberries. Food Chem 112:676-684

Wang LJ, Wu J, Wang HX, Li SS, Zheng XC, Du H, Xu YJ, Wang LS (2015) Composition of phenolic compounds and antioxidant activity in the leaves of blueberry cultivars. J Funct Foods 16:295-304

Xue YJ, Tao L, Yang ZM (2008) Aluminum-induced cell wall peroxidase activity and lignin synthesis are differentially regulated by jasmonate and nitric oxide. J Agric Food Chem 56:9676-9684

Yamamoto Y, Kobayashi Y, Devi SR, Rikiishi S, Matsumoto H (2002) Aluminum toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. Plant Physiol 128:63-72

Yoon JY, Hamayun M, Lee SK, and Lee IJ (2010) Methyl jasmonate alleviated salinity stress in soybean. J Crop Sci Biotechnol 12:63-68

Zhang L. Xing D (2008) Methyl jasmonate induces production of reactive oxygen species and alterations in mitochondrial dynamics that precede photosynthetic dysfunction and subsequent cell death. Plant Cell Physiol 49:1092-1111

CHAPTER IV

Protective effect of methyl jasmonate on photosynthetic performance and its association with antioxidants in two blueberry cultivars with contrasting Al-resistance exposed to Al-toxic

(Submitted to Photosynthesis Research)

Protective effect of methyl jasmonate on photosynthetic performance and its association with antioxidants in two blueberry cultivars with contrasting Al-resistance exposed to Al-toxic.

4.1 Abstract

Vaccinium corymbosum (highbush blueberry) is an species rich in antioxidant activity (AA) that grown in soil with low pH (<5.5). In this soil, the presence of toxic aluminum (Al^{3+}) could induce AA to counteract the metabolic disturbance. On the other hand, Al-toxicity could be decreased by the methyl jasmonate (MeJA) application in leaves increases AA in blueberry cultivars. The aim of the study was to determine the protective effect of MeJA on photosynthetic performance and its association with antioxidants in two blueberry cultivars with contrasting Al-resistance exposed to Al-toxic. The treatments were: a) Without Al and MeJA (Control), b) 100 µM Al (Al^{3+}), c) 5 µM MeJA (MeJA) and d) 100 µM Al + 5 µM MeJA (Al+MeJA). The samples were harvested at 0, 24, and 48 h for analyzing Al-concentration, lipid peroxidation (LP), H_2O_2 concentration, AA, superoxide dismutase SOD and catalase (CAT) activity, total phenols (TP), chlorogenic acid, and pigments and *in vivo* measured CO_2 assimilation and stomatal conductance. The Al^{3+} alone increased the Al-concentration and oxidative damage in the Al-sensitive cultivar. Although the antioxidant responses were increased, photosynthetic performance was strongly decreased, whereas in Al-resistant cultivar did not occur. Under Al+MeJA application, the Al-concentration and oxidative damage strongly decreased in both cultivars during the first hours de Al-exposition. Thus, concomitantly, AA, SOD and CAT activity, TP and chlorogenic acid were stimulated, protecting photosynthetic performance in both cultivars. The MeJA application decreases Al-uptake and stimulates the AA counteract toxic Al effects, decreasing the alterations on photosynthetic performance Al-induced.

Keywords Jasmonate; pH acid; photosynthesis; pigments

4.2 Introduction

Acid soils with pH<5.5 represents the 30-40% of the world's arable land and up to 70% of the world's potentially arable land (Haug 1983). In these soils, acidity allows the solubilisation of the phytotoxic aluminum (Al^{3+}) (Kochian 1995), which limits physiological and metabolic plant functions (Liet al. 2012). The toxic effect of Al^{3+} appears early in roots, being manifested by an inhibition of their cell division and elongation. This prevents water absorption and nutrient transport, essential for the cellular and plant metabolism, ultimately reflected into a reduction in plant productivity and quality (Kochian 1995; Rout 2001). At the cellular level, Al-toxic alter the function of the plasma membrane and organelles, because the Al^{3+} binds to the membrane, inducing disorders, particularly an increase of reactive oxygen species (ROS) and thereby oxidative stress and lipid peroxidation (LP) (Darkó et al. 2004; Inostroza-Blancheteau et al. 2008). To counteract the Al-toxic damage induced by oxidative stress, plants can activate antioxidant systems (Yamamoto et al. 2002; Ma et al. 2007). Enzymatic activities as catalase (CAT), peroxidase (APX), and superoxide dismutase (SOD) and non-enzymatic phenols have been reported as ROS scavengers in the Al-detoxification in many plants (Mukhopadyay et al. 2012). However, when the antioxidant systems are not sufficiently effective to counteract the toxic Al-effects, main processes such as photosynthetic performance, including the photochemical efficiency of photosystem II (PSII) and CO_2 assimilation are altered (Ridolfi and Garrec 2000). The Al-toxicity may also reduce total chlorophyll, disrupting the photochemical capacity of photosystem II. In this context, studies in herbaceous and woody plants have been shown that Al-toxicity decreases photochemical efficiency of PSII as have been demonstrated in wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), pummelo (*Citrus grandis*) plants (Moustakas et al. 1995; Peixoto et al. 2002; Jiang et al. 2008). It has been observed that the maximum quantum yield of the PSII (*Fv/Fm*) and the effective quantum yield of PSII (ΦPSII) were reduced in tangerine (*Citrus reshni*) (Chen et al. 2005) and in tobacco seedlings (*Nicotiana tabacum*) (Li et al. 2012), respectively. Likewise, electron transport rate (ETR) and non-photochemical quenching (NPQ) were also declined in wheat (Moustakas et al. 1995) and in rye (*Secale cereal*) (Silva et al. 2012), respectively. In addition, in highbush blueberry cultivars Reyes-Díaz et al. (2009, 2010) found decreases in the photochemical efficiency of PSII, when plants were subjected to Al-toxic in a

nutrient solution. In other hand, decreases in the CO_2 assimilation are reported by Al-toxicity in barley (*Hordeum vulgare*) (Ali et al. 2011), blueberry (*Vaccinium corymbosum*) (Reyes-Díaz et al. 2011) and eucalyptus (*E. grandis* × *E. urophylla, E. urophylla* × *E. camaldulensis, and E. urophylla*) (Yang et al. 2015). Besides, stomatal conductance, water use efficiency (WUE) and photosynthetic pigments (chlorophyll and carotenoids concentration) are diminished by effect of Al-toxic in soybean (*Glycine max*), cacao (*Theobroma cacao*), and in rye plants according to Zhang at al. (2007); Ribeiro et al. (2013) and Silva et al. (2012), respectively.

Currently, the protective effect of the phytohormones as jasmonate (JAS), or methyl jasmonate (MeJA) on plants under various stresses, including toxic metals (TM) has been reported (Ismail et al. 2012; Larronde et al. 2003; Keramat et al. 2009; Reyes-Díaz et al. 2016). However, the studies about the protective effects of JAS on plants under Al-toxicity are scarce, with the exception of a few works of Spollansky (2000) in *Brugmansia x candida*, Xue et al. (2008) in tora (*Cassia tora*), Roselló et al. (2015) in rice, and Ulloa-Inostroza et al. (2016) in highbush blueberry. The reports on the interaction between TM and MeJA on plants have been controversial. Thus, favorable plant responses under TM have been observed with the MeJA application on herbaceous and woody plant species, showing an increase of antioxidant responses (enzymatic and non-enzymatic) and a decrease in the oxidative stress (LP and ROS) (Piotrowska-Niczyporuka et al. 2009; Keramat et al. 2009; Chen et al. 2014; Ulloa-Inostroza et al. 2016). Contrarily, negative effects by higher MeJA doses application with arsenic in peppers (*Capsicum frutescens*) and cadmium (Cd) in *Kandelia obovata* stress has been reported, where increased LP and content of hydrogen peroxide (H_2O_2) and decreased antioxidant responses were found (Yan et al. 2013; Chen et al. 2014). Important responses to JAS application are the changes in the photosynthetic apparatus functionality in *K. obovata* under cadmium-stress (Chen et al. 2014), runner bean (*Phaseolus coccineus*) with copper(Cu)-exposition (Hanaka et al. 2016), canola (*Brassica napus*) and soybean with arsenic- and nickel-stress, respectively (Farooq et al. 2016; Sirhindi et al. 2016). These reports showed protective effect of MeJA, controlling the decrease of photosynthetic performance among them CO_2 assimilation, stomatal conductance, and fluorescence parameters of the PSII (*Fv/Fm*, ΦPSII, and ETR) provoked by TM stress. Popova et al. (2003) demonstrated that a pre-treatment with MeJA

application provided protection of photosynthesis against paraquat stress, reducing oxidative damage. However, the MeJA treatment alone resulted in decreased levels of chlorophyll *a*+*b*, photosynthesis, ΦPSII, and improved some antioxidant enzymes. Thus, they suggested that MeJA is able to play a protective role against paraquat-toxicity. Yan et al. (2013) observed that in peppers seedlings under Cd-toxicity that 0.1 μmol^{-1} MeJA dose was able to counteract toxic Cd at 7 days. Likewise, Ma et al. (2014) reported that the application of 0.25 μM MeJA in wheat mitigated the decline of net photosynthetic rate, stomatal conductance, and water-use efficiency induced by drought stress. In the same report, MeJA also enhanced the activities of SOD, APX, CAT, and reduced LP in wheat.

A previous study of our group has showed a decrease of oxidative damage in blueberry cultivars under Al-toxicity with MeJA application (Ulloa-Inostroza et al. 2016). This study concluded that a low MeJA dose (5 μM) applied simultaneously with toxic Al at short-term decrease its toxic effect, increasing the antioxidant compounds in two contrasting Al-resistance cultivars. Based on the changes induced by the Al-toxicity, which disturb negatively photosynthetic performance, it is important to find forms to mitigate the Al-damage in blueberry plants, being MeJA application a good alternative to protect the photosynthetic apparatus against to negative effects of Al. Therefore, our aim was to study the protective effect of MeJA on photosynthetic performance and its association with antioxidants in two blueberry cultivars with contrasting Al-resistance exposed to Al-toxic.

4.3 Materials and methods

4.3.1 Plant material

Two-year-old blueberry (*V. corymbosum*) cultivars with contrasting Al-resistance (Legacy: Al-resistant and Bluegold: Al-sensitive), according previously classification of Reyes-Díaz et al. (2009, 2010), were used in this study. They were provided by Berries San Luis (Quillém, Lautaro, Chile; 38° 29` S, 72° 23` W). The cultivars were produced *in vitro* and grown in a substrate of oat shell:sawdust:pine needles at a 1:1:1 proportion. Uniform size and healthy plants were selected for the experiment.

4.3.2 Growth conditions in nutrient solution

The experiment was carried out in a greenhouse of the Instituto de Agroindustria, Universidad de La Frontera, Temuco, Chile (38°45'S, 72°40'W). Blueberry cultivars shrubs were grown in 18-L pots with Hoagland's nutrient solution under controlled conditions: temperature 25 ± 0.2 °C, 400 μmol m^{-2} s^{-1} photosynthetic photon flux, and 70 % relative humidity. The plants were pre-conditioned during seven days in nutrient solution aerated continuously with an aquarium pump.

4.3.3 Treatments and experimental design

The experimental design was completely randomized with three replicates per treatment giving a total of 54 plants of both cultivars. At the start of the experiment, Al was applied as $AlCl_3$ (100 μM) with Al^{3+} 26.8 % as free metal (Geochem speciation, Shaff et al. 2010) to the Hoagland nutrient solution (Hoagland and Arnon 1959) maintained at pH 4.5 (20 °C) under continuous aeration. MeJA was applied by spraying on leaves according to Ulloa-Inostroza et al. (2016). Plants were treated with and without toxic Al and MeJA application as follow: a) without Al and MeJA (Control), b) 5 μM MeJA (MeJA),c) 100 μM Al (Al^{3+}), and d) 100 μM Al + 5 μM MeJA (Al+MeJA) for 0, 24 and 48h. After each time, plants were harvested and leaves were frozen at -80°C until analyses.

4.3.4 Chemical parameters

The Al concentration in leaves was determined after drying the plant material in a forced air oven (70 ºC) until obtaining dry weight, which were weighed and incinerated at 500 ºC for 8 h and digested with 2M hydrochloric acid. The Al concentrations were determined in a spectrophotometer simultaneous multi-element atomic absorption (model 969; UNICAM, Cambridge, UK) according to Sadzawka et al. (2004).

4.3.5 Biochemical parameters

The LP was determined in fresh leaves as indicator of oxidative stress and thereby Al-resistance or Al-sensitivity of blueberry plants. Thiobarbituric acid reacting substance (TBARS) assay was used according to the modified method by Du and Bramlage (1992).

The quantification of the malondialdehyde (MDA) was measured at 532, 600 and 440 nm in a UV-VIS spectrophotometer.

The H_2O_2 concentration was measured at 390 nm in leaf samples in a UV–VIS spectrophotometer according to Loreto and Velikova (2001) and Ulloa-Inostroza et al. (2016). It was expressed as H_2O_2 µmol g^{-1} FW.

The total AA in leaves was determined by using the 2.1-diphenyl1-1-picrylhydrazyl (DPPH) method according to Chinnici et al. (2004) and Reyes-Díaz et al. (2010). The leaf extracts were measured at 515 nm in a UV-VIS spectrophotometer and were expressed by Trolox equivalents (TE).

The SOD was assayed according to Giannopolitis and Ries (1977) by monitoring the superoxide radical-induced nitro blue tetrazolium (NBT) reduction at 560 nm in a UV-VIS spectrophotometer. Enzymatic activity was expressed as protein content determined by Bradford's method (Bradford 1976).

The CAT activity was measured by monitoring the conversion of H_2O_2 to H_2O and O_2 (Pinhero et al. 1997). The quantification enzyme activity was estimated by H_2O_2 consumption for 60 s at 240 nm in a UV-VIS spectrophotometer. Enzymatic activity values were standardized by the total protein content by Bradford method (Bradford 1976).

Total phenols (TP) were determined with the Folin-Ciocalteu reagent using the method described by Slinkard and Singleton (1977). The absorbance of all samples was measured at 765 nm using a UV-VIS spectrophotometer and the results were expressed as milligrams of chlorogenic acid equivalent per gram of fresh weight.

Chlorogenic acid was measured by HPLC-analysis as described by Ribera et al. (2010). The signals were detected at 320 nm. The mobile phase was: (A) acidified water (phosphoric acid 10%) and (B) 100% Acetonitrile, and the gradient were as follows: 0-9 min of 100% A, 9.1-19.9 min of 81% A and 19% B, 20-25 min of 100% B.

4.3.6 Physiological parameters

The *in vivo* net photosynthesis and stomatal conductance were measured with a portable infrared gas analyzer (Licor-6400, LI-COR Bioscience, Inc., Lincoln, Nebraska, USA) according to Reyes-Díaz et al. (2010). Intrinsic water-use efficiency (WUE) was calculated through the photosynthesis and stomatal conductance, where WUE= photosynthesis (µmol CO_2 m^{-2} s^{-1}) / stomatal conductance (mol H_2O m^{-2} s^{-1}) (Locke and Ort 2014).

Photosynthetic pigments concentrations were extracted from leaves with 100 (v/v) HPLC grade acetone at 4ºC under a green safelight and they centrifuged at 4ºC according to Lichtenthaler and Wellburn (1983). The pigments were quantified by HPLC according to Garcia-Plazaola and Becerril (1999).

4.2.7 Statistical analysis

The results are based on three replicates. All data passed the normality and equal variance tests according to Kolmogorov-Smirnov normality test. Statistical data analyses were carried out by three-way ANOVA (where factor was treatment, analysis time, and cultivar) using Sigma Stat 2.0 software. Significantly different means were determined using Tukey's multiple comparison test (statistical significance $P \leq 0.05$).

4.4 Results

In both cultivars, the Al concentrations in leaves were increased under Al^{3+} treatment alone during the first hours of the Al-exposition compared to the control, being significantly higher in the Al-sensitive cultivar (Bluegold) ($P \leq 0.05$; Fig. 1). In the Al-resistant cultivar (Legacy), the Al^{3+} treatment alone showed a statistically significant increase from 0.6- to 1.7-fold at 24 and 48 h, respectively in comparison to control ($P \leq 0.05$; Fig. 1a), whereas in the Al-sensitive cultivar, these increases were higher reaching until 14-fold at 48 h in the relation to control ($P \leq 0.05$; Fig. 1b). Al-concentration was significantly decreased with Al+MeJA application to 0.8-fold at 48 h compared with the Al treatment in Al-resistant ($P \leq 0.05$; Fig. 1a), while Al-sensitive showed similar values to the control ($P \leq 0.05$; Fig. 1b).

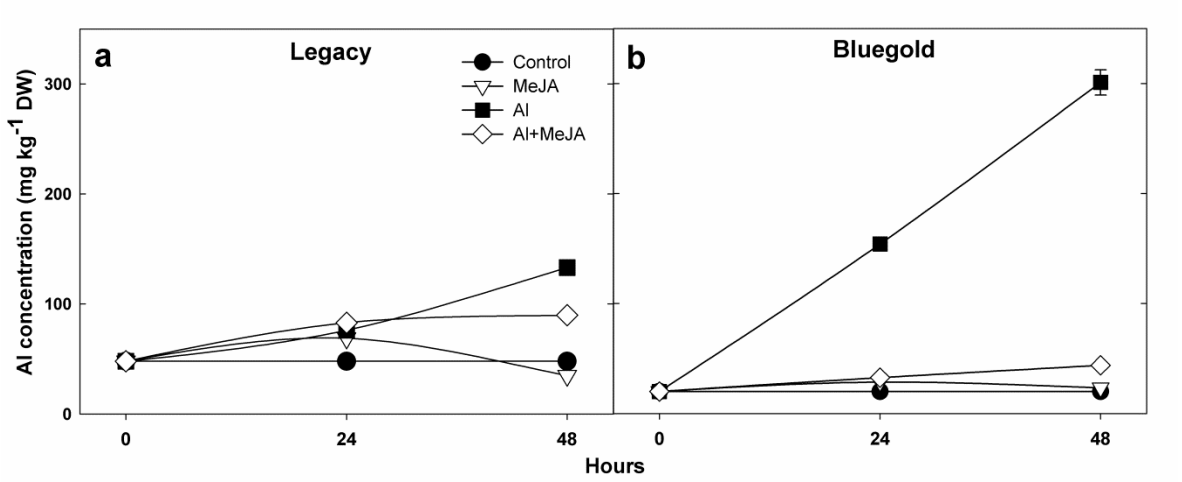

Fig. 1 Kinetic of mitigation by MeJA on the aluminum concentration (mg kg^{-1} DW) in leaves of Al-resistant (A) and Al-sensitive (B) cultivars. Values represent the average or means of 3 replicates ± S.E. and doses in 5 μM of MeJA and 100 μM Al ($P \leq 0.05$)

The LP was increased in direct relation with Al^{3+} exposition time in both cultivars, being notoriously higher in the Al-sensitive cultivar ($P \leq 0.05$; Fig. 2a and b). In both cultivars the Al+MeJA application did not show a statistically significant different in comparison to the control at 24 h the Al-exposition ($P \leq 0.05$; Fig. 2a and b). However, the LP decreased notoriously at 48 h for about 21% and 37% in comparison to the Al alone in the Al-resistant and Al-sensitive cultivars, respectively ($P \leq 0.05$; Fig. 2 a and b). The H_2O_2 concentration in the Al-resistant cultivar was significantly higher than Al-sensitive cultivar ($P \leq 0.05$; Fig. 2c and d). Increase around ~ 8% in H_2O_2 concentration were observed in the Al-resistant cultivar at 48 h with the MeJA treatment alone and Al^{3+} treatment alone in comparison to control ($P \leq 0.05$; Fig. 2c). Higher values of H_2O_2 concentration in Al-sensitive cultivar were obtained during the assay with Al^{3+} alone (4.6%) as compared to the control ($P \leq 0.05$; Fig. 2d), being similar at 24 h with the Al+MeJA treatment, whereas at 48 h decreased the H_2O_2 concentration in a 37% in comparison to the Al^{3+} alone ($P \leq 0.05$; Fig. 2d).

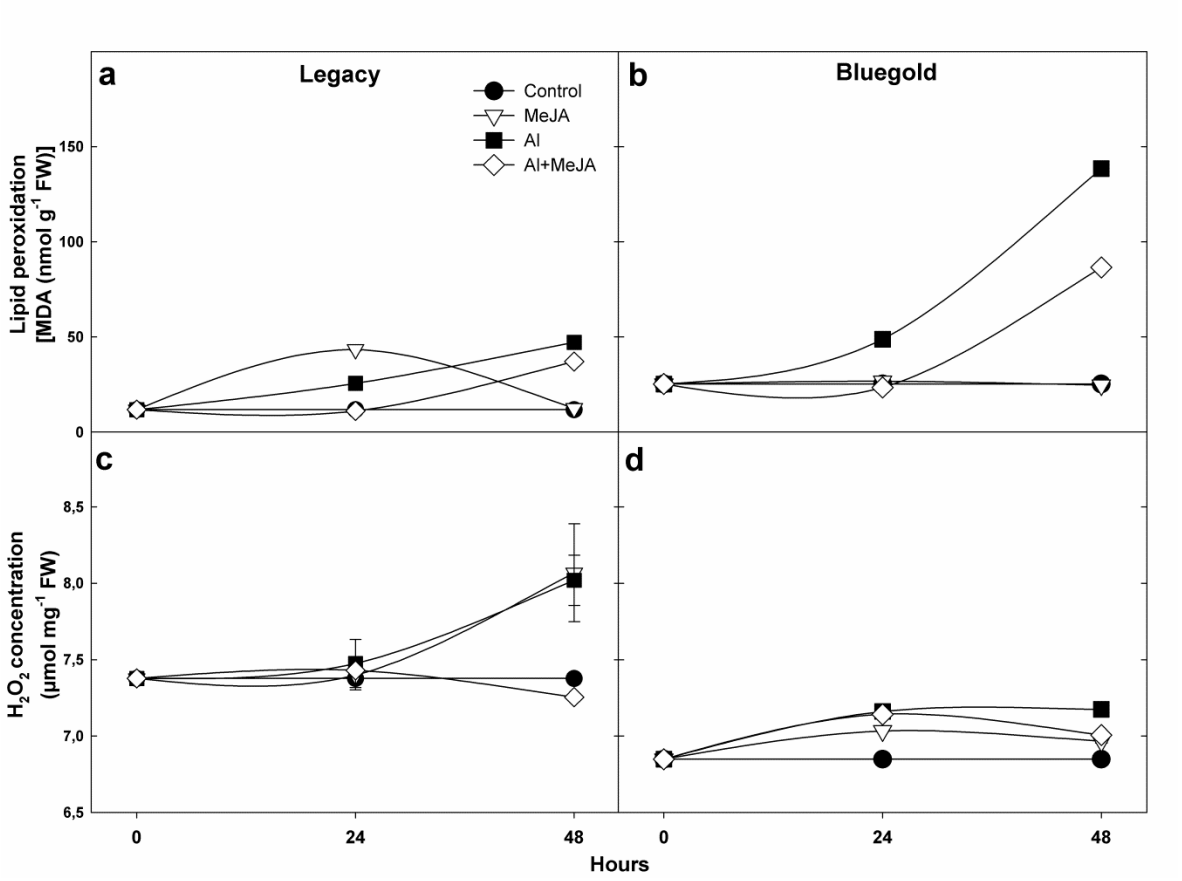

Fig. 2 Kinetic of mitigation by MeJA on the lipid peroxidation [MDA (nmol g^{-1} FW)] (A and B) and H_2O_2 concentration (µmol mg^{-1} FW) (C and D) in leaves of Al-resistant (A and C) and Al-sensitive (B and D) cultivars. Values represent the average or means of 3 replicates ± S.E. and doses in 5 µM of MeJA and 100 µM Al ($P \leq 0.05$)

In both cultivars, the AA in leaves was enhanced under Al^{3+} treatment alone during the first hours of Al-exposition compared to the control, being higher in the Al-resistant cultivar in each treatment during the assay ($P \leq 0.05$; Fig. 3a and b). In Al-resistant cultivar, a strong increase in the AA (31%) was observed with Al^{3+} treatment alone (at 24 and 48 h) compared the control ($P \leq 0.05$; Fig. 3a). In the Al-sensitive cultivar, the increases were from 33 to 40% at 24 and 48 h, respectively in relation to control ($P \leq 0.05$; Fig. 3b). These increases were lowered with Al+MeJA application in around 14% in both cultivars at 24 h. Nevertheless, in both cultivars at 48 h were statistically similar in AA to their respective

Al^{3+} alone treatment ($P \leq 0.05$; Fig. 3a and b).

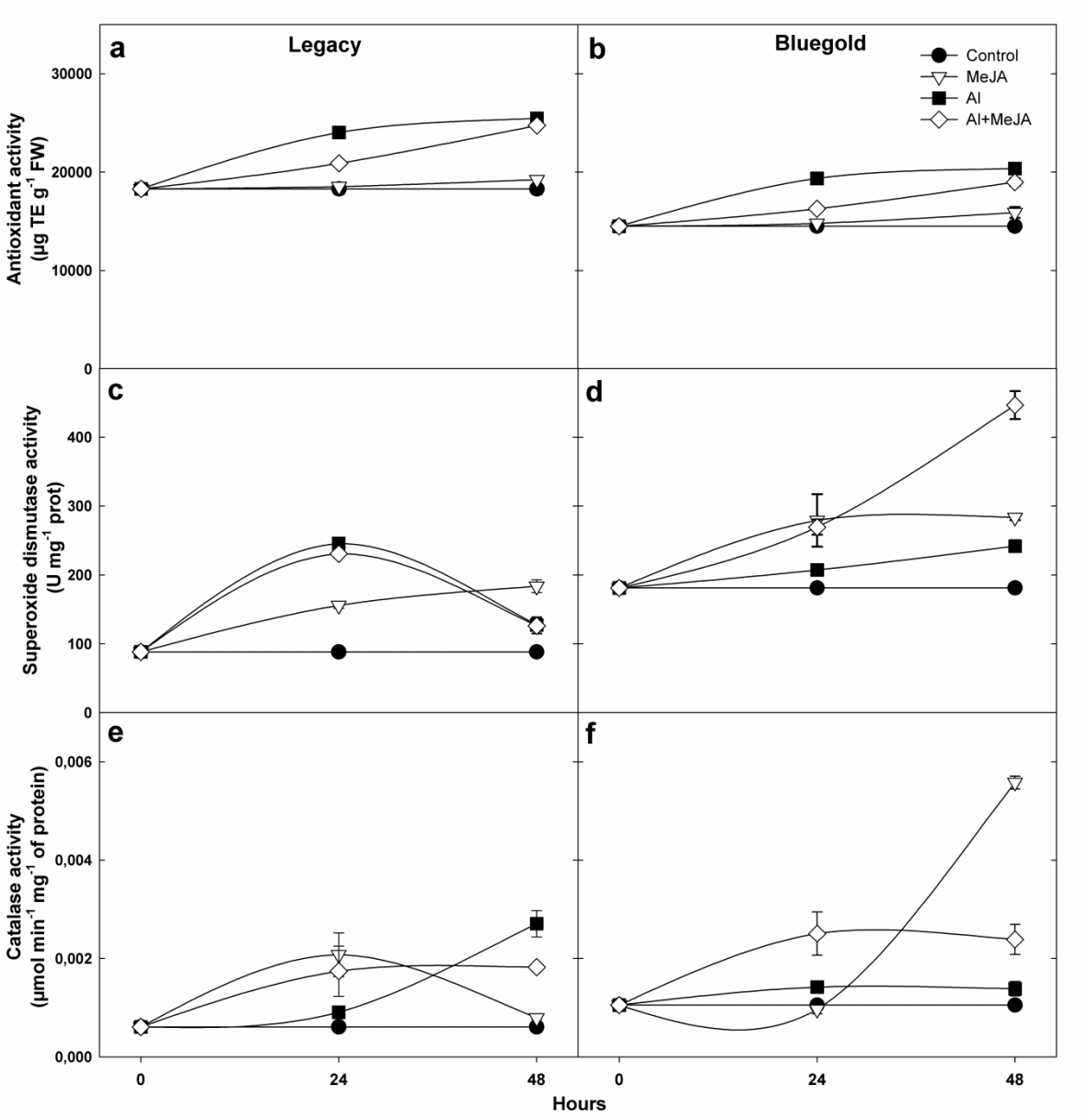

Fig. 3 Kinetic of mitigation by MeJA of the antioxidant activity (µg TE g^{-1} FW) (A and B), superoxide dismutase activity (U mg^{-1}prot) (C and D), and catalase activity (µmol min^{-1} mg^{-1} of prot) (E and F) in leaves of Al-resistant (A, C, and E) and Al-sensitive (B, D, and F) cultivars. Values represent the average or means of 3 replicates ± S.E. and doses in 5 µM of MeJA and 100 µM Al ($P \leq 0.05$)

A higher SOD activity was observed in the Al-sensitive in relation toAl-resistant cultivar ($P \leq 0.05$; Fig. 3c and d) in the control treatments. Similar tendency were observed in the SOD activity with the MeJA application in both cultivars ($P \leq 0.05$; Fig. 3c and d). In the Al-resistant increased the at 24 h and 48 h in 0.7- and 1-fold, while in the Al-sensitive was kept during to the 48 h an increased from 55% in comparison to their respectively control ($P \leq 0.05$; Fig. 3c and d). In the Al-resistant cultivar, high value of SOD activity was found in the Al+MeJA treatment at 24 h with an increase of 1.6-fold, whereas at 48 h a lower increase the SOD activity (12%) in relation to the control was observed ($P \leq 0.05$; Fig. 3c). In the Al-sensitive cultivar, the SOD activity was gradually increased during assay, showing a strong increase at 48 h in the Al^{3+} (33%) and Al+MeJA (1.4-fold) in comparison to control ($P \leq 0.05$; Fig. 3d).

In general, the CAT activity was higher in the Al-sensitive cultivar ($P \leq 0.05$; Fig. 3e and f). The MeJA application alone stimulated earlier the CAT activity in the Al-resistant cultivar (at 24 h in 2.3-fold) than in the Al-sensitive, which was induced at 48 h (4.5-fold) in relation to their respective controls ($P \leq 0.05$; Fig. 3e and f). In both cultivars, the Al^{3+} alone showed a slightly increase of CAT activity at 24 h in comparison to control ($P \leq 0.05$; Fig. 3e and f). However, at 48 the CAT activity was only increased in Al-resistant cultivar (3.5-fold) in comparison to its respective control ($P \leq 0.05$; Fig. 3e). The CAT activity of both cultivars in Al+MeJA treatment was increased in around 1.6-fold in both cultivars at 24 h, maintaining the activity at 48 h in relation to their controls ($P \leq 0.05$; Fig. 3e and f).

The TP concentrations were higher in the Al-resistant cultivar than the sensitive ones ($P \leq 0.05$; Fig. 3a and b). In the MeJA treatment, TP were strongly enhanced in Al-resistant cultivar at 24 h and 48 h compared to control, being lower the increases obtained in Al-sensitive cultivar at the same times ($P \leq 0.05$; Fig. 3a and b). Low increase was exhibited in TP under Al^{3+} alone in both cultivars in comparison with the controls ($P \leq 0.05$; Fig. 3a and b). The Al+MeJA treatment only was increased (20%) at 48 h compared to control in Legacy cultivar ($P \leq 0.05$; Fig. 3a), while TP of Bluegold cultivar slightly increase at 24 (27%) and 48 h (39%) in relation to control ($P \leq 0.05$; Fig. 3a and b). Chlorogenic acid concentrations in the Al-sensitive cultivar were statistically significant reduced in the Al^{3+} alone and MeJA treatments at 24 h, with exception to 48 h when MeJA treatment was

applied ($P \leq 0.05$; Fig. 3d). Contrarily, the Al-resistant cultivar showed an augment in chlorogenic acid concentration in Al+MeJA and Al^{3+} treatments at 24 h, decreasing afterwards ($P \leq 0.05$; Fig.3c).

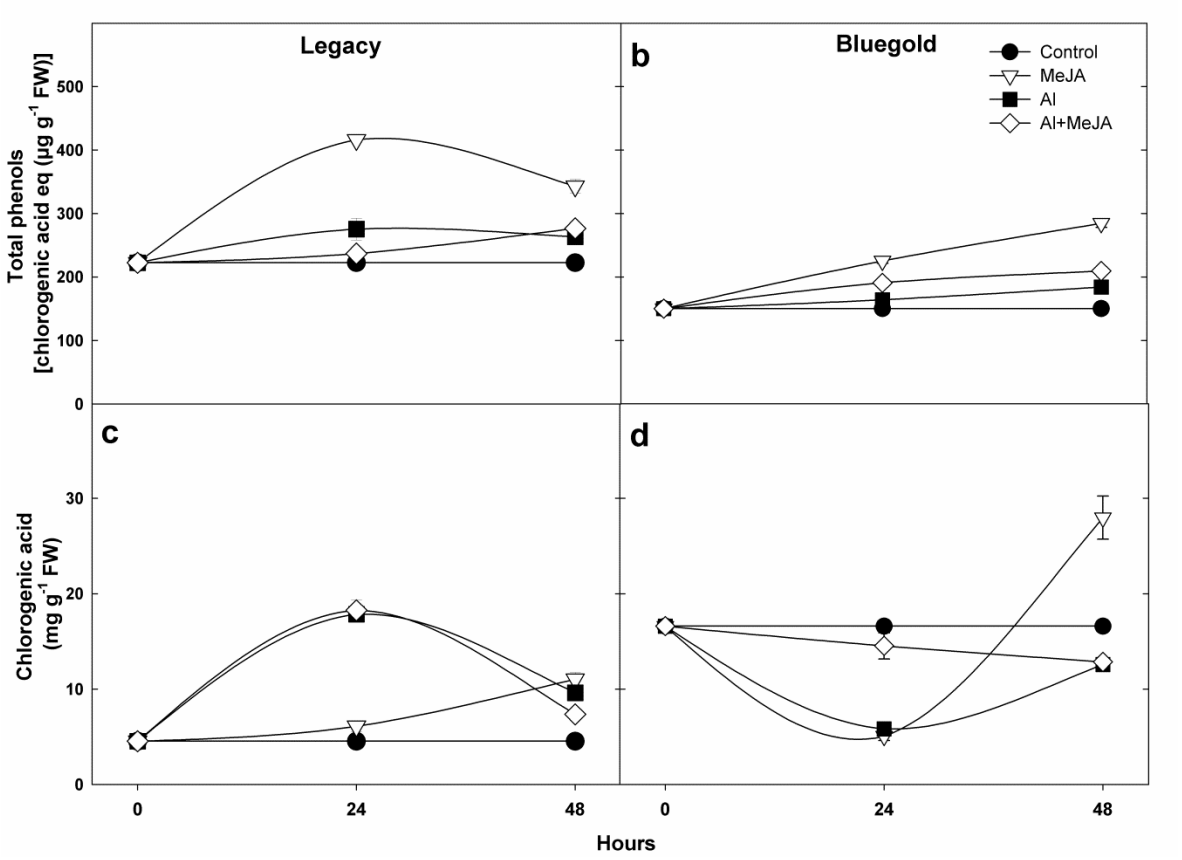

Fig. 4. Kinetic of total phenols [chlorogenic acid (µg g^{-1} FW)] (A, B) and chlorogenic acid (mg g^{-1} FW) (C, D) in leaves of Al-resistant (A and C) and Al-sensitive (B and D) cultivars. Values represent the average or means of 3 replicates ± S.E. and doses in 5 µM of MeJA and 100 µM Al. ($P \leq 0.05$)

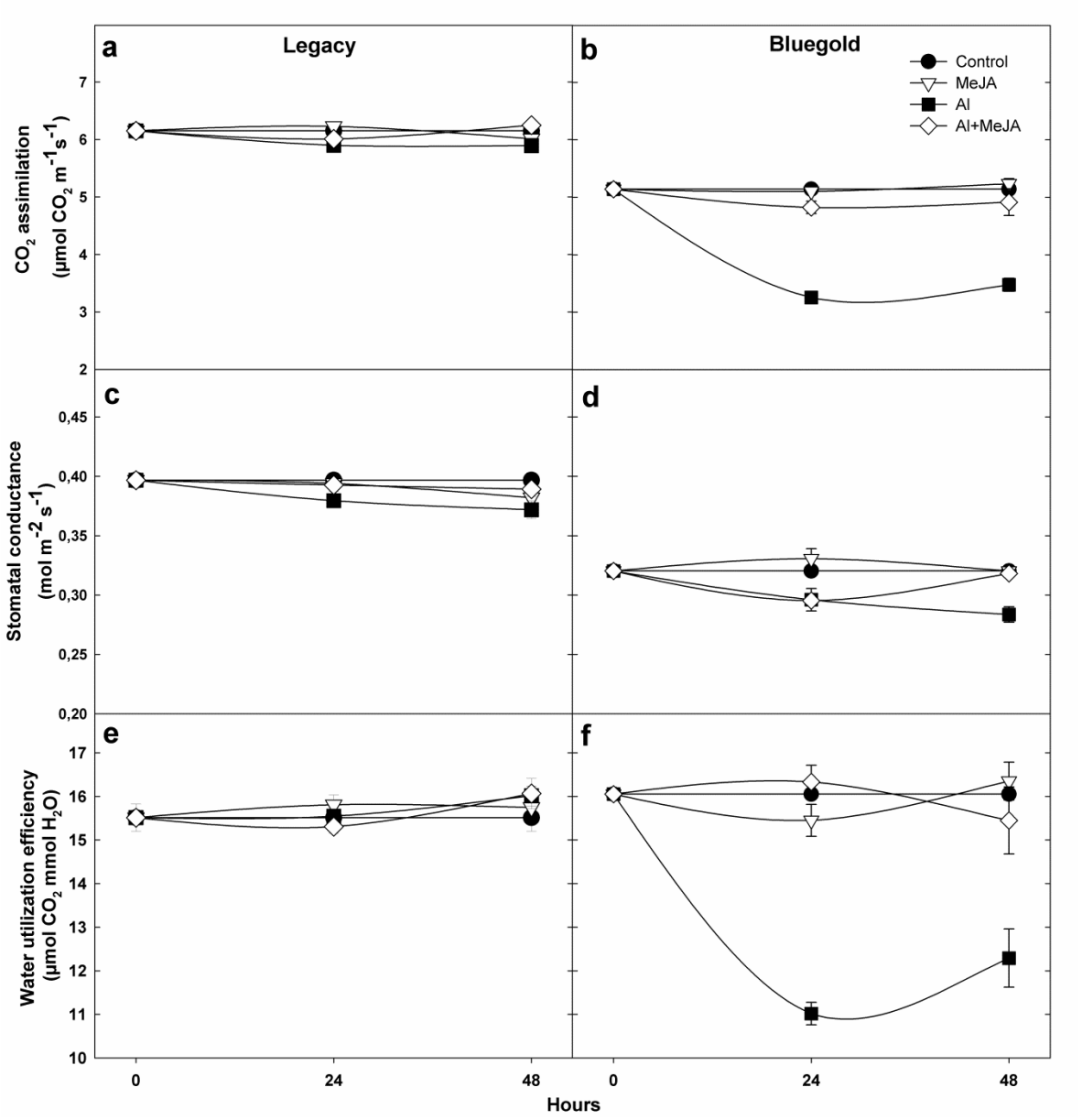

Fig. 5 Kinetic of the CO_2 assimilation (µmol CO_2 $m^{-1}s^{-1}$) (A, B), stomatal conductance (mol m^{-2} s^{-1}) (C, D), and water utilization efficiency (µmol CO_2 mmol H_2O) (E, F) in leaves of Al-resistant (A, C, and E) and Al-sensitive (B, D, and F) cultivars. Values represent the average or means of 3 replicates ± S.E. and doses in 5 µM of MeJA and 100 µM Al ($P \leq 0.05$)

The CO_2 assimilation and stomatal conductance were higher in the Al-resistance cultivar

than in Al-sensitive under all treatments ($P \leq 0.05$; Fig. 5a and b). The Al-resistant cultivar did not show any changes in the CO_2 assimilation, stomatal conductance and WUE between treatments ($P \leq 0.05$; Fig. 5a, c, and e). In Al-sensitive cultivar, the Al^{3+} treatment strongly decrease the CO_2 assimilation and WUE at 24 and 48 h in relation to control ($P \leq 0.05$; Fig. 5b and f). Nonetheless, under Al+MeJA treatment both parameters were maintained similar to the control ($P \leq 0.05$; Fig. 5b and f). In Al-sensitive cultivar, the Al^{3+} treatment decreased gradually the stomatal conductance at 24 (10%) and 48 h (13%) in comparison to the control ($P \leq 0.05$; Fig. 5d). Nevertheless, Al+MeJA application favored the obtention of stomatal conductance rates similar to the control at 48 h ($P \leq 0.05$; Fig. 5d).

The pigments were higher in the Al-sensitive than in the Al-resistant cultivar ($P \leq 0.05$; Table 1). In Al^{3+} alone treatment, xanthophyll cycle of Al-resistant cultivar was decreased as follow: neoxanthin (16%), violaxanthin (20%), and antheraxanthin (18%) at 24 h, whereas at 48 h lutein, neoxanthin, and violaxanthin decreased in aroud of 28% in relation to the control ($P \leq 0.05$; Table 1). In Al+MeJA treatment, all the pigments were increased with exception to lutein at 24 h and chlorophyll *a/b* at 48 h in comparison to Al^{3+} treatment ($P \leq 0.05$; Table 1). In the Al-sensitive cultivar, beta carotene was decreased at 24 and 48 h by around of 23%, while antheraxanthin was only estimulated at 24 h around 50% in Al^{3+} alone and Al-MeJA treatments and violaxanthin (18%) in the Al^{3+} treatment at the same time in comparison to the control ($P \leq 0.05$; Table 1). Chlorophyll *a*+b (22%), beta caroteno (24%), lutein (18%) and neoxanthin (32%) decreased in Al^{3+} treatment at 48 h in relation to control, while with Al+MeJA treatment these pigments were similar to the control ($P \leq 0.05$; Table 1).

Table 1. Chlorophyll *a+b*, chlorophyll *a/b*, and carotenoids concentration (µg g^{-1} FW) in two blueberry cultivars with contrasting Al-resistance and the MeJA application leaves and under Al-toxicity for different timesanalyze. Values represent the average or means of 3 replicates ± S.E. and doses in 5 µM of MeJA and 100 µM Al. Different lowercase letters show statistically significant differences among the treatments at each time of MeJA application (0 or 24 or 48) in the same cultivar. Different capital letters show significant differences between time analyses for the same treatment and cultivar. Asterisk (*) shows significant differences between cultivars at the same treatment and time of analyses ($P < 0.05$). The start of experiment (0 time) for each parameter did not show statistically significant differences with the control at each time (24 and 48 h) and therefore they were not indicated in the table.

Cultivars	Hour	Treatment	Chlorophyll *a+b*	Chlorophyll *a/b*	Beta carotene	Lutein	Neoxanthin	Violaxanthin	Antheraxanthin
Legacy	24	Control	598.1 ± 31.3 bA	1.5 ± 0.0 bA	110.8 ± 6.1 bA	240.9 ± 17.7 aA	64.6 ± 2.4 bA	78.3 ± 3.5 aA	3.8 ± 0.2 bcA
	24	MeJA	494.4 ± 25.1 bA	1.6 ± 0.0 bA	98.4 ± 5.1 bA	229.1 ± 5.4 aA	52.6 ± 3.2 cB	60.0 ± 3.5 bB	5.1 ± 0.3 aB
	24	Al	578.1 ± 27.9 bA	1.4 ± 0.1 bB	101.7 ± 6.4 bA	225.9 ± 7.0 aAB	53.8 ± 1.5 cB	62.7 ± 3.4 bB	3.1 ± 0.1cA
	24	Al+MeJA	730.4 ± 32.1 aA	2.0 ± 0.1 aA *	148.9 ± 5.2 aA	279.7 ± 14.7 aA	80.1 ± 4.8 aA	87.9 ± 3.5 aA	4.5 ± 0.2 abB
	48	Control	598.1 ± 31.3 bA	1.5 ± 0.0 bA	110.8 ± 6.1 abA	240.9 ± 17.7 aA	64.6 ± 2.4 aA	78.3 ± 3.5 aA	3.8 ± 0.2 cA
	48	MeJA	509.0 ± 30.1 bA	1.5 ± 0.0 bA	106.0 ± 0.5 bA	225.2 ± 12.2 abA	50.8 ± 3.1 bB	57.2 ± 3.5 bB	6.7 ± 0.1 aA
	48	Al	520.4 ± 19.7 bA	2.0 ± 0.0 aA	102.5 ± 2.0 bA	176.0 ± 6.4 bB	46.0 ± 2.7 bB	55.3 ± 1.7 bB	3.4 ± 0.1cA
	48	Al+MeJA	700.6 ± 7.7 aA	1.7 ± 0.0 bB *	128.0 ± 3.3 aB	269.3 ± 4.5 aA	71.4 ± 0.2 aAB	82.9 ± 1.5 aA	5.5 ± 0.0 bA
Bluegold	24	Control	888.7 ± 24.2 aA*	1.6 ± 0.0 aA	187.3 ± 2.9 aA*	332.3 ± 11.5 aA*	87.2 ± 2.5 aA*	89.9 ± 1.7 bA*	13.2 ± 0.7 bA*
	24	MeJA	853.6 ± 13.5 aA*	1.6 ± 0.0 aB	177.4 ± 9.5aA *	330.0 ± 12.4 aA*	80.3 ± 0.8 aA*	82.4 ± 4.0 bA*	20.1 ± 0.1 aB*
	24	Al	807.2 ± 9.4 aA*	1.5 ± 0.0 aB	146.8 ± 3.7 bB*	348.7 ± 7.3 aA*	81.6 ± 3.6 aA*	106.2 ± 4.8 aA*	19.1 ± 1.1 aA*
	24	Al+MeJA	798.6 ± 25.2 aA*	1.5 ± 0.0 aA	145.0 ± 6.2 bB	351.6 ± 21.3 aA*	78.6 ± 2.5 aA	92.4 ± 4.0 bA	20.0 ± 0.9 aA*
	48	Control	888.7 ± 24.2 aA*	1.6 ± 0.0 aA	187.3 ± 2.9 aA*	332.3 ± 11.5 aA*	88.0 ± 4.1 aA*	91.2 ± 3.4 aA	12.2 ± 1.1 bA*
	48	MeJA	573.1 ± 23.4 cB	1.3 ± 0.0 cbA	108.7 ± 6.4 dB	270.7 ± 10.3 bB	60.3 ± 4.9 bB	59.1 ± 3.2 bB	24.6 ± 1.5 aA*
	48	Al	696.6 ± 23.7 bB*	1.8 ± 0.0 aA	143.1 ± 3.2 cB*	271.2 ± 10.3 bB*	60.1 ± 3.8 bB*	84.4 ± 3.9 aB*	12.5 ± 0.5 bB*
	48	Al+MeJA	792.2 ± 37.0 aA	1.5 ± 0.0 abA	155.3 ± 12.2 aB	324.5 ± 18.9 aA*	82.5 ± 2.1 aA*	89.3 ± 3.0 aA	13.1 ± 0.8 bB*

4.5 Discussion

This study is focused on protective effect of MeJA on photosynthetic performance and its association with antioxidants in two blueberry cultivars with contrasting Al-resistance exposed to toxic Al. In our study, the MeJA application reduced gradually Al-concentration and oxidative damage in both cultivars, favoring the photosynthetic performance during the first hours of Al-exposition, which was more evident in the Al-sensitive cultivar (Fig. 1, 2, 3, 4 and 5 and Table 1). In this cultivar, Al-toxicity alone disturbed the photosynthetic performance, showing the highest reductions of CO_2 assimilation, stomatal conductance, and WUE compared with Al untreated plants (Fig. 5b, d and f). We believe that the toxic Al that can replace the iron (Fe) between quinones (QA and QB) according to Li et al. (2012). Thus, this Al could induce an unbalance redox, altering the electron transport chain, the H^+ transference from stroma to lumen, the NADPH production, and even the lower H^+ level in the lumen, reducing the ATP by lower H^+ transport through H^+ pumps. All previous conditions could decrease the NADPH and ATP needed for the Calvin cycle, reducing the fixation and reduction of CO_2. Possibly, the increment of ROS ($O_2^{\bullet -}$ and H_2O_2) would depolarize the plasma membrane of stomatal guard cells and activate the calcium increase to the guard cells cytoplasm. This calcium enhancement would induce the output of chloride and potassium from stomatal guard cells and would modulate its closure as indicate by Taiz and Zeiger (2002). Moreover, our findings showed that MeJA application improved these responses (Fig. 5b, d and f). The MeJA effect could be primarily attributed to the reduction in the Al-concentration in leaves (Fig. 1). In this context, it is reported that blueberry plants exhibited a lower root Al-uptake and limited Al-mobilization to leaves by MeJA application under Al-toxicity (Ulloa-Inostroza et al. 2016). This Al reduction may decrease the direct damage in the redox balance in PSII due to the substitution of Fe by Al between quinones (QA and QB) as postulated by Li et al. (2012). In addition, the reduction of the ROS production by MeJA application decreased the direct oxidative damage in the photosynthetic apparatus by Al-toxicity (Fig. 2 and 5). Our experiment allows to suggest that MeJA and Al-toxic stimulated differential antioxidant responses in the studied cultivars, depending on their degree of Al-resistance. In general, in the Al-resistant cultivar

the phenolic compounds were mainly strengthened, keeping the Al^{3+} linked to them (by hydroxyl and carboxyl groups) and decreasing its availability (Fig. 4a and c), while in the Al-sensitive cultivar a higher enzymatic activity were mostly triggered to reduce the oxidative damage induced by Al-stress (Fig. 3c, d, e, and f). It is important to highlight that both antioxidant responses were stimulated in blueberry plants by MeJA application under Al-stress, limiting the Al mobilization and its toxic effects (Fig. 3 and 4). Likely, a high linkage of Al and phenolic compounds were able to keep back and restrict the Al entry in the cell, limiting the Al-exposition to chloroplasts, similarly in *Arabidopsis* by Yang et al. (2002). Recently, Ulloa-Inostroza et al. (2016) analyzed phenolic compounds in Legacy and Bluegold, showing that chlorogenic acid, caffeic acid, ferulic acid and myricetin were associated with higher Al-resistance in Legacy under Al+MeJA, while in Bluegold (Al-sensitive) cultivar, these compounds were decreased or not present. Despite of the reduced Al-mobilization by MeJA application mentioned above, it is also possible that some Al could attain at the chloroplasts. To minimize the photosynthetic damage caused by these Al in the chloroplasts, others alternatives of Al detoxification may be activated. An increase of compouds xanthophyll cycle (Table 1), enzymes of the water-water cycle as SOD (Fig. 3c and d), and CAT (Fig. 3e and f), could be increased by the MeJA application to neutralize the ROS induced by toxic Al. Probably, in our study, the anteraxanthin and lutein from xanthophyll cycle participated in the dissipation of the excess energy absorbed by chlorophylls, protecting the antenna. Surprisingly, zeaxanthin highly photoprotective was not detected. It is importan to mention, that the major difference found in our study with respect to the xanthophyll pigments were the time necessary to increase the α- or β-carotenoids in both blueberry cultivars. Products derived from β-carotene were increased (neoxanthin, violaxanthin, anteraxanthin) earlier (24 h) than those derived from α-carotene (lutein), which increased later (48 h) (Table 1). Very few reports about the carotenoid concentration with toxic metals and JAS application exist. As example, in the aquatic plant *Wolffia arrhizal* exposed to Pb and JAS the total carotenoid concentrations were increased (Piotrowska at el. 2009). In addition, in the present work the Al toxic and MeJA application decreased the ROS ($O_2^{\bullet -}$ and H_2O_2) production by the action of enzyme is involved in the water-water cycle as SOD. We think that the increase in SOD activity could be associated to isoforms of the SOD as copper/zinc SOD (Cu/ZnSOD), iron (FeSOD), or manganese

SOD (MnSOD) present in the chloroplasts. Later, the CAT (Fig. 3e and f) and probably ascorbate peroxidase (thylakoid-APX) converts the H_2O_2 back into water, decreasing the damages induced by ROS. Thus, possibly the ROS not depolarized the plasma membrane of the stomatal guard cells and not changed the cytoplasmic calcium, chloride, and potassium concentration. Therefore, the stomatal guard cells were maintained open allowing the exchange of CO_2.

Based on these results we propose that the MeJA could trigger some signal, allowing a decrease in the time enzymatic (SOD and CAT) responses to counteract the toxic Al effects. Likewise, an earlier enzymatic activity that was induced by MeJA application might help to induce a greater Al-resistance associated to SOD and CAT activities. Inostroza-Blancheteau et al. (2011) showed that during the first six-hours of Al-stress in the Al-resistant blueberry cultivar (Brigitta), the SOD and CAT activities were higher 3- and 2 fold, respectively, whereas a slightly increase in the Al-sensitive cultivar after 24 h of Al-exposition (100µM Al) was found. Reyes-Díaz et al. (2011) found that at 15 days of Al-exposition to similar Al dose of above the Al-sensitive blueberry (Bluegold) cultivar, presented higher SOD activity. Therefore, the time shortened the enzimatic activity by MeJA application under Al toxic in the Al-sensitive cultivar.

4.6 Conclussion

The MeJA application under Al^{3+} has a high protective effect on photosynthetic performance in the Al-sensitive and Al-tolerant cultivars of blueberry; (1) decreasing Al-damage by lower Al-accumulation in the leaves, (2) stimulating the antioxidant response through the phenolic compounds (Al-phenols bind) and SOD and CAT activities, detoxifing and decreasing the damages induced by ROS, and (3) dispelling the excess energy absorbed by chlorophylls, protecting the antenna through from xanthophyll cycle (anteraxanthin and lutein). All this, could be able to protect the normal photosynthetic performance, particularly in the Al-sensitive cultivar.

Acknowledgments: We are very grateful for FONDECYT Project Nº 1120917 which supported this work and PhD fellowship Nº21110919, both from the Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) of the Government of Chile, as well as the DI 13-2017, DI 15-2015, and DI 16-2011 Projects from the Dirección de Investigación at the Universidad de La Frontera, Temuco, Chile. Finally, we wish to thank to Mariela Mora for her valuable assistance in the laboratory.

References

Aftab T, Khan MM, Idrees M, Naeem M, Moinuddin, Hashmi N (2011) Methyl jasmonate counteracts boron toxicity by preventing oxidative stress and regulating antioxidant enzyme activities and artemisinin biosynthesis in *Artemisia annua* L. Protoplasma 248:601-612

Ali S, Zeng F, Qiu L, Zhang G (2011) The effect of chromium and aluminum on growth, root morphology, photosynthetic parameters and transpiration of the two barley cultivars. Biol Plantarum 55:291-296

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Chen L-S, Qi Y-P, Liu X-H (2005) Effects of aluminum on light energy utilization and photoprotective systems in citrus leaves. Annals of Botany 96:35-41

Chen J, Yan Z, Li X (2014) Effect of methyl jasmonate on cadmium uptake and antioxidative capacity in *Kandelia obovata* seedlings under cadmium stress. Ecotox Environ Saf 104:349-356

Chinnici F, Bendini A, Gaiani A, Riponi C (2004) Radical scavenging activities of peels and pulps from cv. Golden delicious apples as related to their phenolic composition. J Agr Food Chem 52:4684-4689

Darkó É, Ambrus H, Stefanovits-Bányai E, Fodor J, Bakos F, Barnabás B (2004) Aluminium toxicity, Al tolerance and oxidative stress in an Al-sensitive wheat genotype and in Al-tolerant lines developed by in vitro microspore selection. Plant Sci 166:583–591

Du Z, Bramlage WJ (1992) Modified thiobarbituric acid assay for measuring lipid oxidation in sugar-rich plant tissue extracts. J Agric Food Chem 40:1556-1570

Farooq MA, Gill RA, Islam F, Ali B, Liu H, Xu J, He S, Zhou1 W (2016) Methyl jasmonate regulates antioxidant defense and suppresses arsenic uptake in *Brassica napus* L. Front Plant Sci. doi: 10.3389/fpls.2016.00468

Garcia-Plazaola JI, Becerril JM (1999) A rapid HPLC method to measure liphophilic antioxidant in stressed plants: simultaneous determination of carotenoids and tocopherols. Phytochemical Analyses 10:307-313

Giannopolitis CN, Ries SK (1977) Superoxide dismutases. I. Occurrence in higher plants. Plant Physiol 59:309-314

Hanaka A, Wójcik M, Dresler S, Mroczek-Zdyrska M, Maksymiec W (2016) Does methyl jasmonate modify the oxidative stress response in *Phaseolus coccineus* treated with Cu?. Ecotox Environ Saf 124:480-488

Haug A 1983. Molecular aspects of aluminum toxicity. CRC Crit Rev Plant Sci 1:345-373

Hoagland, D.R. and Arnon, D.I. 1959. The water culture method for growing plants without soil. California Agr. Expt. Sta. 347:1–32.

Ismail A, Riemann M, Nick P (2012) The jasmonate pathway mediates salt tolerance in grapevines. J Exp Bot 63:2127-2139

Inostroza-Blancheteau C, Soto B, Ulloa P, Aquea F, and Reyes-Díaz M (2008) Resistance mechanisms of aluminum (Al^{3+}) phytotoxicity in cereals: physiological, genetic and molecular bases. Revista de la Ciencia del Suelo y Nutrición Vegetal 8:57-71

Inostroza-Blancheteau C, Reyes-Díaz M, Aquea F, Nunes-Nesi A, Alberdi M, Arce-Johnson P (2011) Biochemical and molecular changes in response to aluminium-stress in highbush blueberry (*Vaccinium corymbosum* L). Plant Physiol Biochem 49:1005-1012

Jiang HX, Chen LS, Zheng J-G, HAN S, Tang Ning, Smith BR (2008) Aluminum-induced effects on photosystem II photochemistry in Citrus leaves assessed by the chlorophyll *a* fluorescence transient. Tree Physiology 28:1863-1871

Keramat B, Kalantari KM, Arvin MJ (2009) Effects of methyl jasmonate in regulating cadmium induced oxidative stress in soybean plant (*Glycine max* L.). Afr J Microbiol Res 3:240-244

Kochian, 1995; Kochian L (1995) Cellular mechanisms of aluminum toxicity and resistance in plants. Annu Rev Plant Physiol Plant Mol Biol 46:237-260

Larronde F, Gaudillière JP, Krisa S, Decendit A, Deffieux G, Mérillon JM (2003) Airborne methyl jasmonate induces stilbene accumulation in leaves and berries of grapevine plants. Am J Enol Vitic 54:60-63

Li Z, Xing F, Xing D (2012) Characterization of target site of aluminum phytotoxicity in photosynthetic electron transport by fluorescence techniques in tobacco leaves. Plant Cell Physiol 53:1295-1309

Lichtenthaler H, Wellburn AR (1983) Determinations of total carotenoids and chlorophyll *a* and *b* of leaf extracts in different solvents. Biochem So Trans 603:591-592

Locke AM, Ort DR (2014) Leaf hydraulic conductance declines in coordination with photosynthesis, transpiration and leaf water status as soybean leaves age regardless of soil moisture. J Exp Bot. doi:10.1093/jxb/eru380

Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. Plant Physiol 127:1781-1787

Ma B, Wan J, Shen ZG (2007) H_2O_2 production and antioxidant responses in seeds and early seedlings of two different rice varieties exposed to aluminum. Plant Growth Regul 52:91-100

Ma C, Wang LT, Zhang LT, Sun MM, Lin TB (2014) Photosynthetic responses of wheat (*Triticum aestivum* L.) to combined effects of drought and exogenous methyl jasmonate. Photosynthetica 52: 377-385

Manquián K, Zúñiga GE, Barrientos H, Escudey M, Molina M (2013) Effect of aluminum on antioxidant activity and phenolic compounds content in in vitro cultured blueberries. Bol Latinoam Caribe Plant Med Aromat 12:603-611

Moustakas M, Ouzounidou G, Lannoye R (1995) Aluminum effects on photosynthesis and elemental uptake in an aluminum-tolerant and non-tolerant wheat cultivar. J Plant Nutr 18:669-683

Mukhopadyay M, Bantawa P, Das A, Sarkar B, Bera B, Ghosh P, Mondal TK (2012) Changes of growth, photosynthesis and alteration of leaf antioxidative defence system of tea [*Camellia sinensis* (L.) O. Kuntze] seedlings under aluminum stress. Biometals 25:1141–1154

Peixoto HP, Da Matta FM, Da Matta JC (2002) Responses of the photosynthetic apparatus to aluminum stress in two sorghum cultivars. J Plant Nutr 25:821-832 J Plant Nutr

Pinhero RG, Rao MV, Paliyath G, Murr DP, Fletcher RA (1997) Changes in activities of antioxidant enzymes and their relationship to genetic and paclobutrazol-induced chilling tolerance of maize seedlings. Plant Physiol 114:695-704

Piotrowska A, Bajguz A, Godlewska-żyłkiewicz B, Czerpak R, Kamińska M (2009) Jasmonic acid as modulator of lead toxicity in aquatic plant *Wolffia arrhiza* (Lemnaceae). J Exp Bot 66:507-513

Popova L, Ananieva E, Hristova V, Christov K, Georgieva K, Alexieva V, Stoinova Zh (2003) Salicylic acid - and methyl jasmonate – induced protection on photosynthesis to

paraquat oxidative stress. Bulg J Plant Physiol 2003:133-152

Reyes-Díaz M, Alberdi M, Mora ML (2009) Short-term aluminum stress differentially affects the photochemical efficiency of photosystem II in Highbush Blueberry genotypes. J Amer Soc Hort Sci 134:14-21

Reyes-Díaz M, Inostroza-Blancheteau C, Millaleo R, Cruces E, Wulff-Zottele C, Alberdi M, Mora ML (2010) Long-term aluminum exposure effects on physiological and biochemical features of Highbush Blueberry cultivars. J Amer Soc Hort Sci 135: 212-222

Reyes-Díaz M, Meriño-Gergichevich C, Alarcón E, Alberdi M, Horst WJ (2011) Calcium sulfate ameliorates the effect of aluminum toxicity differentially in genotypes of highbush blueberry (*Vaccinium corymbosum* L.). Journal of Soil Science and Plant Nutrition 11:59-78

Reyes-Díaz M, Ulloa-Inostroza EM, González-Villagra J, Ivanov AG Kurepin LV (2016) Phytohormonal Responses to Soil Acidity. In eds. G.J. Ahammed, J.-Q. Yu. Plant Hormones under Challenging. Environmental Factors. doi 10.1007/978-94-017-7758-2_6

Ribeiro MAQ, Almeida AAFD, Mielke MS, Gomes FP, Pires MV, Baligar VC (2013) Aluminum effects on growth, photosynthesis, and mineral nutrition of cacao genotypes. J Plant Nutr 36:1161-1179

Ribera AE, Reyes-Díaz M, Alberdi M, Zuniga GE, Mora ML (2010) Antioxidant compounds in skin and pulp of fruits change among genotypes and maturity stages in Highbush blueberry (*Vaccinium corymbosum* L.) grown in southern Chile. J Soil Sci Plant Nutr 10:509-536

Ridolfi M, and Garrec JP (2000) Consequences of an excess Al and a deficiency in Ca and Mg for stomatal functioning and net carbon assimilation of beech leaves. Ann For Sci 57:209-218

Roselló M, Poschenrieder C, Gunsé B, Barceló J, Llugany M (2015) Differential activation of genes related to aluminium tolerance in two contrasting rice cultivars. J Inorg Biochem

152:160–166

Rout GR, Samantaray S, Das P (2001) Aluminium toxicity in plants: a review. Agronomie 21:3-21

Sadzawka AM, Grez R, Carrasco MA, Mora ML (2004) Métodos de análisis de tejidos vegetales. Comisión de normalización y acreditación, sociedad chilena de la ciencia del suelo, In: Editorial salesianos impresores, Santiago, Chile, pp 105

Shaff JE, Schultz BA, Craft EJ, Clark RT, Kochian LV (2010) GEOCHEM-EZ: a chemical speciation program with greater power and flexibility. Plant Soil 330:207-214

Silva S, Pinto G, Dias MC, Correia CM, Moutinho-Pereira J, Pinto-Carnide O, Santos C (2012) Aluminium long-term stress differently affects photosynthesis in rye genotypes. Plant Physiol Biochem 54:105-112

Sirhindi G, Mir MA, Abd-Allah EF, Ahmad P and Gucel S (2016) jasmonic acid modulates the physio-biochemical attributes, antioxidant enzyme activity, and gene expression in glycine max under nickel toxicity. Front Plant Sci 7:591

Slinkard K, Singleton VL (1977) Total phenol analysis: automation and comparison with manual methods. Am J Enol Vitic 28:29-55

Spollansky T (2000) Effect of jasmonic acid and aluminum on production of tropane alkaloids in hairy root cultures of Brugmansia candida. Elec J Biot 3:0717-3458

Taiz L, Zeiger E (2002) Plant Physiology, Third Edition. Sinauer Associates, Sunderland, MA. 690 pps.

Ulloa-Inostroza EM, Alberdi M, Meriño-Gergichevich C, Reyes-Díaz M (2016) Low doses of exogenous methyl jasmonate applied simultaneously with toxic aluminum improves the antioxidant performance of *Vaccinium corymbosum.* Plant Soil. doi: 10.1007/s11104-016-2985-z

Xue YJ, Ling T, Yang ZM (2008) Aluminum-induced cell wall peroxidase activity and lignin synthesis are differentially regulated by jasmonate and nitric oxide. J Agric Food Chem 56:9676-9684

Yan Z, Chen J, Li X (2013) Methyl jasmonate as modulator of Cd toxicity in *Capsicum frutescens* var. fasciculatum seedlings. Ecotox Environ Saf 98:203-209

Yang M, Tan L, Xu Y, Zhao Y, Cheng F, Ye S, Jiang W (2015) Effect of low pH and aluminum toxicity on the photosynthetic characteristics of different fast-growing eucalyptus vegetatively propagated clones. PLoS ONE 10(6):e0130963

Yang JL, Zhu XF, Peng YX, Zheng C, Li G. X, Liu, Zhi Shi Y, Zheng SJ (2011) Cell Wall Hemicellulose Contributes Significantly to Aluminum Adsorption and Root Growth in Arabidopsis. Plant Physiol 155;1885-1892

Yamamoto Y, Kobayashi Y, Devi SR, Rikiishi S, Matsumoto H (2002) Aluminum toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. Plant Physiol 128:63-72

Zhang X-B, Liu P, Yang YS, Xu GD (2007) Effect of Al in soil on photosynthesis and related morphological and physiological characteristics of two soybean genotypes. Botanical Studies 48:435-444

CHAPTER V

General discussion and conclusions

5.1. General discussion

Based in the results obtained in this theses we propose a model of MeJA action in blueberry plants under Al-toxicity, showing the influence of MeJA to counteract Al^{3+} effect on roots and leaves in blueberry, wich will permit a better discution of this work (in this Chapter, Fig. 1). In this model, it is shown that the lowest MeJA (5 μM) dose applied simultaneously with toxic Al (100 μM) was able to reduce the Al-absorption and translocation from root to leaves and the toxic Al effects, compared to Al-toxicity alone. Similar results have been reported with low MeJA doses, able to decrease the metal-concentration and oxidative damage in *Wolffia arrhizal* subjected to lead-toxicity, *Capsium frutescens* and *Kandelia obovata* cadmium-toxicity, compared with a higher MeJA doses (Piotrowska et al. 2009; Yan et al. 2013; Chen et al. 2014), which is in accordance with our findings (Chapter II and III Fig 1 and 2, Chapter II Fig III). A possible explication to the decrease in the Al uptake induced by MeJA could be the regulation of the Al transporters and/or of the exudation of organic acids in the blueberry roots (in this Chapter, Fig. 1). Unfortunately, reports about Al transporter and regulation under Al and MeJA application are currently not available. Concerning to organic acid transporters Yang et al. (2016), showed in Arabidopsis plants subjected to Al and MeJA application, that the ALMT-mediated (**Al**uminum-activated **M**alate **T**ransporter) malate exudation is regulated by COI1-mediated (coronatine-insensitive 1) JA signal in the studied root. The COI1 is an F-box protein that determines the target specificity of the E3 ubiquitin ligase SCFCOI1, which is required for JA-signaling processes (Katsir et al. 2008). Therefore, in the Arabidopsis plants the reduction of the relative primary root growth observed by using three doses of MeJA and Al application could be associated to MeJA incorporated in the nutrient solution. Likely, the site of MeJA application is an important factor to trigger Al resistance mechanisms to counteract the Al-damage (lipid peroxidation or root growth decrease). According our results the MeJA application by spraying on leaves reduced the Al injury (lipid peroxidation) in roots in the first our of the treatment, differently as the findings in Arabidopsis with a reduction in relative root growth at seven days (Yang et al. 2016). Thus, likely, when the stomata are open the MeJA application in the leaves, enabling the entry of MeJA and activating ROS and/or Ca^{2+} sensor regulating the Jas synthesis in the

chloroplasts. In addition, the MeJA incorporation in the nutrient solution could be oxidated to jasmonic acid, by losing its methyl group, joining the jasmonic acid to the Al. However, the stability of this union is not yet studied.

Other important factor that limited the Al-toxicity in blueberry plants is the antioxidant response, previously reported by our research group (Inostroza-Blancheteau et al. 2011; Manquián et al. 2013; Reyes-Díaz et al. 2009: 2010; 2016). In our work the lower MeJA dose applied simultaneously with Al^{3+}, increased antioxidant mechanisms according to Al-resistance of the cultivars (Chapter III, Fig. 6 and Table 1; Chapter IV, Fig. 3, 4, and Table 1 and in this Chapter, Fig. 1). In the Al-resistant cultivar phenolic compounds were stimulated, while in the Al-sensitive cultivar enzymatic compounds were mainly present. This suggest that the low MeJA dose application under Al-toxicity can trigger some signal that decrease the response times to counteract the Al-toxic effects and could award a greater Al-resistance in both blueberry cultivars. Thus, the increase in phenolic compouds could limit the Al-mobilization inside the cell, binding toxic Al to them, through of hydroxyl and carboxyl groups (in this Chapter, Fig. 1), carrying it to the vacuole as part of the internal exclusion mechanism. On the other hand, a higher enzymatic SOD and CAT activity may decrease the concentration of radical species as ($O_2^{\bullet -}$) and H_2O_2 respectively, converting in H_2O finally (in this Chapter, Fig. 1). A similar result was observed in *Brugmansia x candida* and *Cassia tora* plants exposed to Al toxicity and MeJA application, where the first increased the scopolamine and hyoscyamin (secondary metabolites) accumulation and second showed an upregulation of gen expressions of coenzyme A ligase (4CL, EC 6.2.1.12), lipoxygenases (LOX), and L-phenylalanine ammonialyase (PAL, EC 4.3.1.5) (Spollansky, 2000; Xue et al. 2008).

With respect to the Al+MeJA effect on photosynthetic performance, we suggest that the MeJA could help to protect the chloroplasts of Al-toxicity in two forms: through preventing the chloroplasts to Al-exposition or by a lesser Al-exposition on the chloroplasts. In the first case, it is possible, that the antioxidant response limited a total chloroplasts Al-exposition, without alter the photosynthetic performance. In the second case, the increase in photoprotective compounds of the xanthophyll cycle and enzymes involved in the water-water cycle as SOD, and CAT (Chapter IV Fig. 3 c, d, e and f), could minimize the

photosynthetic damage by the Al in the chloroplasts, due to the Al substitution by iron (Fe) between quinones (QA and QB), inducing a redox unbalance in PSI, limiting the photosynthetic efficiency by not the reduction of Fe^{+2} to Fe^{+3} (Li et al. 2012). Photoprotective compounds of the xanthophyll cycle such as anteraxanthin (during the all the experiment) and the lutein (during the last 24 h) of Al-exposition and MeJA application could have participated in the dissipation of the excess energy induced by toxic Al, protecting the antenna, specially in the Al-sensitive cultivar. It is reported, that in Citrus leaves, treated with Al the excitation energy may be in excess with respect to untreated leaves (Chen et al. 2005). In addition, the SOD and CAT activity could decrease the ROS ($O_2^{\bullet -}$ and H_2O_2) production, converting the H_2O_2 back into H_2O in blueberry plants with the MeJA application under Al-toxicity. The ROS has been associated with the modulation of stomatal closure, inducing the increase of cytoplasmic calcium concentration in stomatal guard cells, triggering the output of chloride and potassium of them (Taiz and Zeiger, 2002). We believe, that with Al-toxicity and MeJA application the lower ROS production limited the stomatal closure, due to not depolarization of the plasma membrane of the stomatal guard cells and not changed the cytoplasmic calcium, chloride, and potassium concentration, maintaining the exchange of CO_2 (Chapter III Table 1). Similar resuts was reported by Popova et al (2009) in *Hordeum vulgare* seedlings where the MeJA induced protection on photosynthesis againts paraquat oxidative stress, normalizing the photosynthesis parameters.

Therefore, the hypothesis proposed in the present research was fulfilled according to the main results in this thesis. Due to in this study, the action of the MeJA application reduced the Al-toxicity through enhancement of enzymatic and non-enzymatic antioxidant mechanisms, protecting photosynthetic performance in both cultivars (Al-resistant and Al-sensitive).

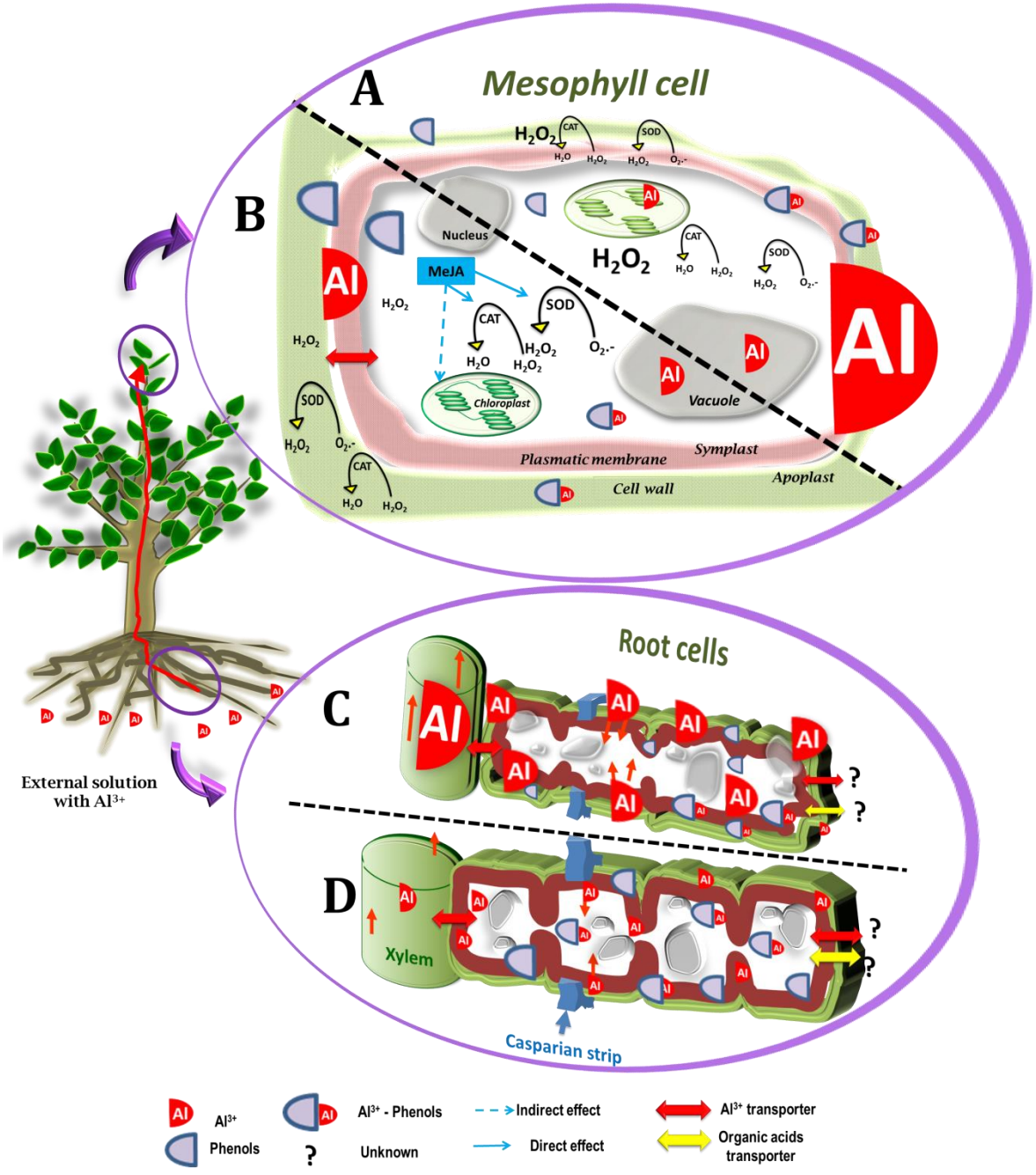

Fig 1 General model illustrating the changes induced by the MeJA application in blueberry plants under toxic Al. On the upper, a mesophyll cell divided by a segmenting line into two parts: without (A) and with MeJA (B) application. On the bottom a group of root cells separated in to two parts: without (C) and with (D) MeJA application on leaves of blueberry plants under a solution with Al^{3+}. In this model, the MeJA application in

blueberry reduces the Al taken up by the root from the nutrient solution by an unknown MeJA effect on Al-transporters regulation. This determines a lower Al-translocation to leaves where it is stored. In root and leaves, the Al^{3+} is chelated by phenolic compounds and its toxic effects are decreased by enzymatic activities stimulated by the MeJA application, decreasing the possibility of the Al-exposure to chloroplasts, favoring an adequate photosynthetic performance.

5.2 General Conclusions

Thus, according to the main results in this thesis, it is concluded that:

The lower MeJA dose (5 μM) applied simultaneously with Al^{3+} in blueberry cultivars with different Al-resistance decreased the Al-toxic effect in root and leaves. Thus, the MeJA application under Al-toxicity was able to stimulate the antioxidant mechanism to counteract the Al and its toxic effect, decreasing the Al-translocation from root to leaves.

The approach to the MeJA potential effects on photosynthetic performance in the blueberry cultivars under Al-toxicity revealed a better performance in both cultivars, being more evident in the Al-sensitive cultivar. This could be attributed to increased antioxidant mechanism and xanthophylls cycle, which likely limited the Al-exposition and its damage. Thus, it is possibly, that the lower Al-availability caused a lower damage in the chloroplasts by toxic Al, keeping a normal photosynthetic performance in the blueberry cultivars due to MeJA application under Al-toxicity.

5.3 Futures perspectives

The knowledge of physiological and biochemical processes presented here will contribute to understand of mechanisms of Al resistance and its link with phytohormonal response front to Al-toxicity in plants of agronomic interest.

References.

Chen L-S, Qi Y-P, Liu X-H (2005) Effects of aluminum on light energy utilization and photoprotective systems in citrus leaves. Annals of Botany 96:35-41

Chen J, Yan Z, Li X (2014) Effect of methyl jasmonate on cadmium uptake and antioxidative capacity in *Kandelia obovata* seedlings under cadmium stress. Ecotox Environ Saf 104:349-356

Inostroza-Blancheteau C, Reyes-Díaz M, Aquea F, Nunes-Nesi A, Alberdi M, Arce-Johnson P (2011) Biochemical and molecular changes in response to aluminium-stress in highbush blueberry (*Vaccinium corymbosum* L). Plant Physiol Biochem 49:1005-1012

Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proc Natl Acad Sci USA 105:7100-7105

Li Z, Xing F, Xing D (2012) Characterization of target site of aluminum phytotoxicity in photosynthetic electron transport by fluorescence techniques in tobacco leaves. Plant Cell Physiol 53:1295-1309

Manquián K, Zúñiga GE, Barrientos H, Escudey M, Molina M (2013) Effect of aluminum on antioxidant activity and phenolic compounds content in in vitro cultured blueberries. Bol Latinoam Caribe Plant Med Aromat 12:603-611

Piotrowska A, Bajguz A, Godlewska-żyłkiewicz B, Czerpak R, Kamińska M (2009) Jasmonic acid as modulator of lead toxicity in aquatic plant *Wolffia arrhiza* (Lemnaceae). J Exp Bot 66:507-513

Popova L, Ananieva E, Hristova V, Christov K, Georgieva K, Alexieva V, Stoinova Zh (2003) Salicylic acid - and methyl jasmonate – induced protection on photosynthesis to paraquat oxidative stress. Bulg J Plant Physiol 2003:133-152

Reyes-Díaz M, Alberdi M, Mora ML (2009) Short-term aluminum stress differentially

affects the photochemical efficiency of photosystem II in Highbush Blueberry genotypes. J Amer Soc Hort Sci 134:14-21

Reyes-Díaz M, Inostroza-Blancheteau C, Millaleo R, Cruces E, Wulff-Zottele C, Alberdi M, Mora ML (2010) Long-term aluminum exposure effects on physiological and biochemical features of Highbush Blueberry cultivars. J Amer Soc Hort Sci 135: 212-222

Reyes-Díaz M, Ulloa-Inostroza EM, González-Villagra J, Ivanov AG Kurepin LV (2016) Phytohormonal Responses to Soil Acidity. In eds. G.J. Ahammed, J.-Q. Yu. Plant Hormones under Challenging. Environmental Factors. doi 10.1007/978-94-017-7758-2_6

Spollansky TC, Pitta-Alvarez SI, Giulietti AM (2000) Effect of jasmonic acid and aluminum on production of tropane alkaloids in hairy root cultures of *Brugmansia candida*. Electron J Biotechn 3:72-75

Taiz L, Zeiger E (2002) Plant Physiology, Third Edition. Sinauer Associates, Sunderland, MA. 690 pps.

Xue YJ, Ling T, Yang ZM (2008) Aluminum-induced cell wall peroxidase activity and lignin synthesis are differentially regulated by jasmonate and nitric oxide. J Agric Food Chem 56:9676-9684

Yan Z, Chen J, Li X (2013) Methyl jasmonate as modulator of Cd toxicity in *Capsicum frutescens* var. fasciculatum seedlings. Ecotox Environ Saf 98:203-209

Yang Z-B, He C, Ma Y, Herde M, Ding Z (2016) Jasmonic acid enhances Al-induced root-growth inhibition. Plant Physiol DOI: 10.1104/pp.16.01756