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Effect of Al and Cd on oxidative stress and production of secondary metabolites of phenolic nature of highbush blueberry (*Vaccinium corymbosum* L.) cultivated *in vitro*.

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“Effect of Al and Cd on oxidative stress and production of secondary metabolites of phenolic nature of highbush blueberry (*Vaccinium corymbosum* L.) cultivated *in vitro*”

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Esta Tesis esta dedicada a Dios…

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RESUMEN

El estudio de los efectos de los metales pesados en plantas ha sido ampliamente descrito en la literatura, en donde se ha investigado su efecto en diferentes parámetros fisiológicos y bioquímicos. Sin embargo existe escasa información sobre la posibilidad de modular el perfil de compuestos fenólicos con respuesta antioxidante u otras propiedades de interés científico, a partir una modificación de la composición de un medio de cultivo o de la solución del suelo. En este contexto elementos presentes de manera natural en el suelo o incorporados por actividades antropogénicas (fertilización, aplicación de enmiendas, entre otras), como son los casos del Al y Cd, surgen como alternativa de estudio y análisis para desarrollar metodologías de manejos de cultivos de interés comercial en diferentes matrices (suelo, solución nutritiva y/o cultivos *in vitro*), potenciando la producción de principios bio-activos con propiedades farmacéutica, cosmetológicas y alimenticias.

La investigación desarrollada en esta tesis se centró en los efectos que puede causar la incorporación de elementos trazas como Al y Cd, en el estrés oxidativo, la capacidad antioxidante y el perfil de compuestos fenólicos, de plántulas de arándano cultivadas *in vitro*. El desarrollo de este estudio significó evaluar el comportamiento del arándano, cuando este es sometido a diferentes concentraciones de Al y Cd en tratamientos independientes y combinados, a fin de determinar cómo estos elementos producen cambios en la actividad antioxidante, el perfil de compuestos fenólicos y el estrés oxidativo en plántulas de arándano. Para evaluar la actividad antioxidante se utilizaron técnicas como DPPH y FRAP, mientras que para cuantificar e identificar el perfil de compuestos fenólicos se empleó el uso de HPLC y LC-MS.

En general la aplicación de Al y Cd de manera independiente o combinada modificó significativamente los parámetros fisiológicos anteriormente mencionados, siendo posible observar la capacidad que posee las plántulas de arándano, para contrarrestar los efectos negativos de ambos metales en su desarrollo, con diferentes mecanismos de respuesta.

La aplicación de diferentes concentraciones de Al al medio de cultivo (100 y 200 µM), permitió evaluar el contenido de malodialdehido (MDA), la capacidad antioxidante y el contenido de compuestos fenólicos. Luego de 3 semanas de estudio, se observó un mayor contenido de MDA en los tejidos de las plántulas alcanzando un peak a los 14 días de exposición. En el mismo periodo de tiempo aumentó la respuesta antioxidante y se incrementó el contenido de los ácidos clorogénico y elágico (determinados por HPLC), que corresponden a los principales compuestos fenólicos presentes en el arándano.

En un ensayo de tres semanas de duración, se evaluó la respuesta antioxidante y la producción de compuestos fenólicos del arándano, debido a una aplicación inicial de Cd (50-100 µM) al medio de cultivo. Se observaron cambios significativos en la respuesta antioxidante mediante el ensayo FRAP y en el contenido de MDA, parámetro que aumentó durante el estudio, indicando que el Cd^{2+} generó un daño oxidativo a las plántulas cultivadas *in vitro*. El principal compuesto fenólico identificado fue el ácido clorogénico, compuesto que vio aumentada su abundancia por efecto de la adición de Cd^{2+}, también fue posible identificar otros compuestos de origen fenólico como el ácido vallínico y Quercetina (determinados por LC-MS). Es indudable que las variaciones en el perfil de compuestos fenólicos, se debe a la presencia de Cd^{2+} en el medio de cultivo.

Se evalúo el efecto combinado (Al + Cd) en el estrés oxidativo, capacidad antioxidante y concentración de compuestos fenólicos en plántulas de arándano cultivadas *in vitro* durante 4 semanas. Los resultados indicaron que la aplicación conjunta de Al + Cd produjo estrés oxidativo, un incremento en la capacidad antioxidantes y en la concentración total de compuestos fenólicos. Los principales compuestos fenólicos que incrementaron su concentración después de la aplicación de los metales son los ácidos clorogénico y elágico.

De los resultados obtenidos en los diferentes estudios realizados en esta tesis podemos concluir que elementos trazas como Al y Cd inducen estrés oxidativo tanto de manera independiente como combinados, la cual correlaciona significativamente con la concentración total de compuestos fenolicos, lo que sugiere que estos compuestos tienen un rol importante en la respuesta frente al daño por estrés oxidativo. De acuerdo a lo anterior, la aplicación de elementos traza al medio de crecimiento produce cambios en el perfil y concentración de compuestos fenólicos, los cuales podrían ser modulados para aumentar la concentración de productos de interés cuando la planta se encuentra en un medio de crecimiento, controlando la adición de pequeñas concentraciones de elementos como Al y Cd.

ABSTRACT

The study of the effects of heavy metals in plants has been extensively described in the literature, where the effect has been investigated in various physiological and biochemical parameters. However, there is scant information on the possibility of modulating the profile of phenolic compounds with antioxidant response and other properties of scientific interest, from a change in the composition of culture medium or soil solution. In this context elements naturally present in soil or incorporated by anthropogenic activities (fertilization, application of amendments, etc.), as in the case of Al and Cd, emerge as an alternative study and analysis to develop methodologies for managements crop commercial interest in different matrices (soil, nutrient solution and/or *in vitro* culture), fostering the production of bioactive principles with pharmaceutical, nutritional and cosmetological properties.

The research developed in this thesis focused on the effects that can cause the incorporation of trace elements such as Al and Cd, on oxidative stress, antioxidant capacity and profile of phenolic compounds of blueberry seedlings cultured *in vitro*. The development of this study meant to evaluate the behavior of the blueberry, when this is subjected to different concentrations of Al and Cd in combination separate treatments and to determine how these factors cause changes in the antioxidant activity, the profile of phenolic compounds and oxidative stress in seedlings of blueberry. To evaluate the antioxidant activity was measured by using DPPH and FRAP, whereas to quantify and identify the profile of phenolic compounds using HPLC and LC-MS were used.

In general the application of Al and Cd independently or in combination significantly change the above physiological parameters, being possible to observe the capacity that blueberry seedling to counter act the negative effects of both metals in its development, with different mechanisms of response.

The application of different Al concentrations in the culture medium (100-200 µM), and its relation with the content of Malondialdehyde (MDA), the antioxidant capacity and phenolics content was studied. After 3 weeks of study, a higher content of MDA was observed in tissues of seedlings reaching a peak at 14 days of exposure. In the same period of time increased the antioxidant responseand the content of chlorogenic and ellagic acids (determined by HPLC), corresponding to the main phenolic compounds in blueberry increased.

In a three-week test period, the antioxidant response and the production of phenolic compounds was evaluated blueberry, due to initial application of Cd (50-100 mM) in the culture medium. Significant changes were observed in antioxidant response by FRAP assay and the content of MDA, parameter increased during the study, indicating that Cd^{2+} generated oxidative damage to plantlets grown *in vitro*. The phenolic compound was identified principal chlorogenic acid, compound abundance was increased due to the addition of Cd^{2+}, it was also possible to identify other phenolic compounds as vallinic acid source and quercetin (determined by LC-MS). It is clear that the profile of phenolic compounds change with the presence of Cd^{2+} in the culture medium.

The combined effect of (Al +Cd) was evaluated in the oxidative stress, antioxidant capacity and concentration of phenolic compounds in blueberries plantlets cultured *in vitro*

for 4 weeks. The results indicated that the combined effect of Al+Cd produced oxidative stress, an increase in antioxidant capacity and the total concentration of phenolic compounds. The major phenolic compounds that increased the concentration after the application of the metals are ellagic and chlorogenic acids.

From the results obtained indifferent studies in this thesis we can conclude that trace elements such as Al and Cd induce oxidative stress both independently and in combination, which correlated significantly with the total concentration of phenolic compounds, suggesting that these compounds have an important role in the response to oxidative stress damage. According to the above application of trace elements to the growth medium causes changes in the profile and concentration of phenolic compounds, which could be modulated to increase the concentration of products of interest when the plant is in a growth medium, controlling adding small concentrations of elements such as Al, and Cd.

ABBREVIATIONS

Al	Aluminium element
Al^{3+}	Aluminium ion
≡Al-OH	Aluminol
APX	Ascorbate peroxidase
CAT	Catalase
Cd	Cadmiun element
Cd^{2+}	Cadmiun ion
CEC	Cationic exchange capacity
DNA	Desoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EDTA	Etilendiamintetracetic acid
≡Fe-OH	Ferrol
FRAP	Ferric reducing antioxidant power
GSH	Glutathione
HM	Heavy Metal
HPLC	High performance liquid chromatography
H_2O_2	Peroxide hidrogen
LC-MS	Liquid Chromatography Mass Spectrometry
MDA	Malondialdehyde
MRM	Multiple reactions monitoring
OM	Organic Matter
PAL	Phenylalanine ammonia lyase
POD	Peroxidase
ROS	Reactive oxigen species
SOD	Superoxide Dismutasa
≡Si-OH	Silanol
TPC	Total Phenolic Content
TPTZ	Tripyridyltriazine
TEs	Trace Elements
TSP	Triple superphosphate

OUTLINE DE LA TESIS

La investigación reportada en esta tesis fue realizada en los laboratorios de Fisiología y Biotecnología Vegetal y Fisicoquímica de Suelos en Facultad de Química y Biología, Universidad de Santiago de Chile.

El capítulo I de esta tesis proporciona una visión general de los efectos de elementos trazas, principalmente Al^{3+} y Cd^{2+}, sobre diferentes parámetros fisiológicos determinados en distintos tipos de plantas y como propiedades fisicoquímicas del suelo, tales como, pH, contenido de arcillas, materia orgánica y óxidos de Fe, pueden modificar la disponibilidad de estos elementos, llegando incluso a una condición de fitotoxicidad. Este trabajo se preparó para ser próximamente enviado a *South African Journal of Botany* **(The Responses of Plants to Trace Elements. A Review Focusing on Aluminium and Cadmium)**.

Las modificaciones en el perfil metabólico y cambios en parámetros fisiológicos, enfocado a estrés oxidativo y respuesta antioxidante, por cambios en el contenido de Al^{3+}, se resumen en el capítulo II, de estos resultados se publicó un artículo en el *Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas* **(Effect of aluminum on antioxidant activity and phenolic compounds content in *in vitro* cultured blueberries)**.

El capítulo III se estudió el comportamiento del arándano sometido a diferentes concentraciones de Cd^{2+} en un lapsus de tiempo de 3 semanas. Se estableció que este elemento genera especies reactivas de oxígeno (ROS por sus siglas en inglés), aunque el mecanismo es diferente a lo observado para el Al^{3+} (capítulo II). Este trabajo sera enviado a la revista *Molecules*, bajo el título de "**Effect of the availability of Cd^{2+} on the non-enzymatic antioxidant response and the phenolic compounds profile of blueberry (*Vaccinium corymbosum* L.) plantlets cultivated *in vitro*".**

El capítulo IV describe el efecto en la peroxidación lipídica (estrés oxidativo) y respuesta antioxidante del arándano cuando es sometido a medios de crecimientos enriquecidos con Al y Cd (sistemas binarios). Los resultados obtenidos serán enviado al journal *Environmental and Experimental Botany.*

GENERAL INTRODUCTION

The soil is the most important factor in a plant's growth and development. Depending on its origin and its physical, chemical and biological characteristics, it conditions the disposition and concentration not only of nutrients but also contaminants, which makes most of the plant's nutritive flows.

Acid soils are found throughout the world. It is estimated that about 40% of the world's soils are being used for agriculture and of that about 12% have pH below 5.5 (Guo *et al.*, 2007). The soils in the central and south zones of Chile are of volcanic origin, which are characterized by having a high capacity of chemical elements retention of agricultural and environmental importance (nutrients, HM, pesticides). This type of soil has pH values under 6.0, as well as, a low concentration of interchangeable basis (Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}), and as consequence has a high level of phytotoxic aluminum (Mora *et al.*, 2002, 2009). In addition, this high acidity is accelerated by the high pluviometry in the zones/areas and the use of fertilizers with acid reaction (urea, ammonia phosphate). The use of fertilizers as source of nitrogen, phosphorus and potassium, among those that stand out as the most used which are urea, triple superphosphate (TSP), diamonic monoammonium phosphate, are important sources of trace elements. Various research has shown that the use of fertilizers, are the most important sources for incorporation of As, Zn, Cd, Pb, Cr Ni, Se, V and Cu to the soil (Chang and Page, 2000; Nicholson *et al.*, 2003; Williams *et al.*, 1973; Otero *et al.*, 2005). By this, it can be shown that there is evidence of high contamination of trace elements to the soils via different ways, which are also affecting the organisms and plants that grow in these soils.

Amongst the elements that are often named because of how they affect the soils and the waters, include cadmium (Cd) for its high toxicity and mobility, which is shown in the

soil/plant system (Sanita and Gabbrielli., 1999). The industrial, the agricultural activity with phosphatic fertilizers and biosolids have enriched the soils with this element (Zheljazkov *et al.,* 2005). In Chile, Bonomelli *et al.,* 2002 analyzed the presence of Cd in phosphatic fertilizers imported to Chile during the years 1999-2000, finding a high metal content in TSP type fertilizers. Similar results were obtained by Molina *et al*., (2009) who found high concentrations of Cd in this type of fertilizers, concluding that the prolonged use of phosphatic fertilizers can increase the levels of HM, as well as, Cd in soils used for agriculture (Molina *et al.,* 2009).

In plants, the exposure to cadmium induced various photo-toxicity symptoms, such as chlorosis, biomass reduction, inhibition of root elongation, which kills them (Milone *et al.,* 2003; Lux *et al.,* 2011). It has been discovered that the presence of Cd generates free radicals which damage the plants tissue and depending on its concentration and the type of plant that is exposed to cadmium, this can inhibit or stimulate activity in various anti-oxidant enzymes, before the symptoms of toxicity are even visible (Fu and Huang., 2001; Liu *et al.,* 2007). Another side effect of the presence of cadmium is the fact that it is related to disruption in the capture and distribution of nutrients in the plants (Sandalio *et al.,* 2001; Rascio *et al.,* 2010).

The concentration of heavy metals affects the plants in different ways when they develop in an environment that is contaminated, because some plants possess a high tolerance to heavy metals and others that are more sensitive to them. The heavy metals induce the generation of species reactive to oxygen (ROS) which produce damage at the cell membrane level, lipid oxidation, proteins and DNA (Boscolo *et al.,* 2003; Arora *et al.,* 2002)

It has been described that plants show strategies that allows them to control the

species reactive to oxygen. One of these strategies is based in the production of secondary metabolites with anti-oxidant properties. The secondary metabolites are synthesized by plant as a defense mechanism against biotic stress. However, it is possible that when a plant is affected by the presence of a high concentration of heavy metals, when these metabolites play a role in the physiological function of said plant (Close and McArthur, 2002; Keilig *et al.,* 2009).

Of the different bioactive secondary metabolites, we can find the phenolic compounds, which make up a vast group of metabolites, with different activity and chemical structure. Nowadays, these compounds require more attention due to their contribution to health, how many of the beneficial properties found in plants and vegetables, especially linked to anti-oxidant activities, are tightly linked to the presence and content of secondary metabolites of phenolic nature (Vanisree *et al.,* 2004; Jung *et al.,* 2007; Seeram *et al.,* 2005).

The antioxidant activity shown by phenolic compounds, is of interest from the technological and nutritional point of view, for example the phenolic compounds intervene as natural antioxidants in food, which reduces the use of synthetic additives and from the nutritional point of view the antioxidant compounds are associated with the role of protecting against cancer, cardiovascular problems and the aging process (Sakihama *et al.,* 2002; Niggeweg *et al.,* 2004; Prasad *et al.,* 2009).

The higher demand of plants with antioxidant properties and bioactive compounds has generated an increase in production, beginning to be an important part in today's agricultural production, which means that plants are being cultivated in places where soil handling, which in part means that fertilizers and pesticides are being used and applied in order to improve the fertility and property of the soils.

The blueberry is a minor fruit tree native to North America, considered among the berries group, which was introduced in Chile in the nineteen eighties. In Chile, the farming of blueberry is mainly concentrated between the VI and the X regions. The tall blueberry (*Vaccinium corymbosum* L) grows very well in soils with pH between 4.8 and 5.5, as well as, it is very well valued in different countries by its antioxidant and antibiotic properties. Due to the fact that in the last few years this species has positioned itself in the country's agriculture, it is believed that it is important to cultivate and handle the growth conditions for this plant, so it will allow us to evaluate the factors directly related to the production of secondary metabolites and its antioxidant properties.

Therefore, considering the development that the blueberry has had in our country and the importance that it has generated in the last few years due to the contributions this fruit provides to our health because of its antioxidant properties, it is important to research the effects that handling the soil has (by incorporating Cd and Al in mono and multicomponent system) on the production of secondary metabolites of phenolic nature, by cultivating plants thru *in vitro*, conditioning the previously mentioned factors, in order to determine its real effect and to be able to modulate the plant in a line of interest.

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HYPOTHESIS

Taking into account the high phytotoxic Al contents found in the acid soils and the toxicity that shows Cd on the plants, both metals will induce oxidative stress in the blueberry plants, stress characterized by incresing levels of secondary metabolites of phenolic nature.

GENERAL OBJECTIVE

To evaluate, through *in vitro* cultivation, the impact of Cd and A1 in the production of secondary metabolites of phenolic origin in blueberry.

SPECIFIC OBJETIVES

1) To evaluate the effects of Cd and A1, in the oxidative status in *Vaccinium corymbosum* cultivated *in vitro*

2) To determine the effect of Cd and Al in the antioxidant capacity of ethanolic extracts in the cultivation of *V. corymbosum* cultivated *in vitro*.

3) To determine the effect of Cd and Al in the phenolic metabolic in ethanolic extract of *V. corymbosum* cultivated *in vitro*.

4) To establish the combined effect of Al-Cd toxicity over the oxidative stress and production of secondary metabolites in the different crops.

Chapter 1

The Response of Plants to Trace Elements. A Review

Focusing on Aluminium and Cadmium

Abstract

Contamination with trace elements has increased in recent times as a direct consequence of industrialization and technification at the world level. The different sources of trace elements incorporation in the environment include production sectors such as mining, agriculture, and industry which alter the normal conditions of these elements in the soil and irrigation water, consequently increasing the total content and potentially the bioavailability of heavy metals to plants. Many factors affect trace elements bioavailability in soil, being the most relevant; the total metal concentration, pH, redox conditions, organic matter, clays and hydrated oxides.

Trace elements concentration affects in different ways the plants that grow in a contaminated environment, because there are plants that have high tolerance for heavy metals and others that are sensitive to them. Heavy metals induce the generation of reactive oxygen species (ROS) that cause damage at the cell membrane, lipid oxidation, protein, and DNA levels. It has been described that plants have strategies that allow them to control ROS. One of these strategies is based on the production of secondary metabolites having anti-oxidant properties. The phenolic compound are synthesized by the plants as a method of defense against biotic stress, but it is also possible that they play a role in the physiologic functioning of the plants when they are in the presence of a high concentration of heavy metals. This review shows the complexity of the toxicity Mechanisms of trace elements, such as changes in the availability of free species of heavy metals can induce oxidative stress by multiple mechanisms, from the generation of ROS until inhibition of some enzymes

Keywords: Heavy metals, Lipid peroxidation, Phenolic Compound, ROS, Soil, Plant

Introduction

Soil contamination by heavy metals (HM) is considered one of the main problems affecting both developing and developed countries (Chang and Page 2000). In recent years, the soil composition has changed due to man-caused activities. Industrial, agricultural residues and mining activities have led to an increase in the concentration of heavy metals (HM) in soils (Li *et al.*, 2008, Boularbah *et al.*, 2006).

The HM as Cr, Cd, Pb and others are called trace elements (TE), because the quantities that are in traces in environmental matrices. These elements are not biodegradable and they easily accumulate in the soil, becoming harmful to plants, animals and humans when their concentrations are above a critical level. Among the different trace elements we can find a group of them which are essential micronutrients for plants (Fe, Mn, Cu, Co, Zn, Ni, Mo), due to their involvement in various metabolic processes (Salt *et al.*, 1998; Schutzendubel and Polle 2002). Then, there are those that are considered as non-essential (Pb, Cr, Cd, Hg, Al, etc.) and they are potentially toxic to plants (Schutzendubel and Polle 2002). The behavior and speciation of the HM in soil depends on various factors such as organic matter, moisture, aeration, biological activity, redox potential, pH and others, which determine the shape and therefore the bioavailability and mobility of HM (Bonomelli *et al.*, 2002).

The heavy metals can physiologically inhibit processes such as respiration, photosynthesis, cell elongation, capture water and nutrients. When a plant has physiological or visible symptoms of toxicity by HM, it is the last link in a chain of events that caused an increase of these contaminants to phytotoxic levels in the environment (Scora and Chang, 1997). These events are conditioned in two steps: 1) the incorporation of heavy metals in groundwater and soil through various natural and anthropogenic sources, standing out as

the most important sources of pollution to highly productive sectors such as agriculture, mining and industry (Li *et al.*, 2008, Boularbah *et al.*, 2006) and 2) the deployment by the plant's mechanisms of absorption of nutrients (radical exudates), which indirectly incorporates HM and trace elements being these translocated to various parts of the plants, altering its different states and physiological processes (Larcher, 2003; Meyer *et al.*, 2000; Poschenrieder *et al.*, 2006) (Figure 1).

Plants absorb essential and non-essential elements for its life cycle from soils, in response to the concentration gradients induced by selective ion absorption by the roots or by diffusion of ions in the soil. The levels of accumulation of elements in plants differ within the same species or between species (McGrath *et al.*, 2002).

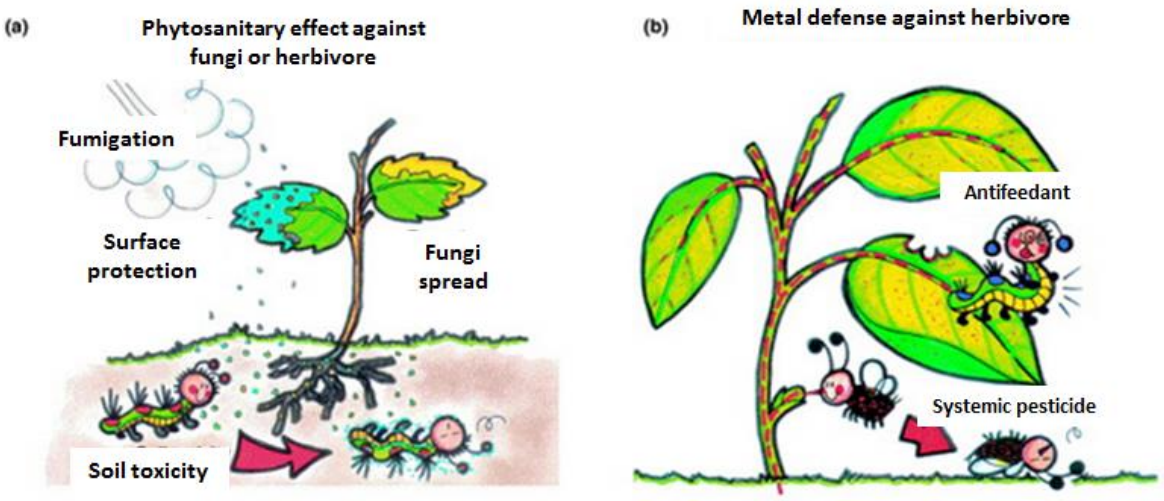

Figure 1. Different ways for high metal concentrations to protect plants against biotic stress. (a) Metal-containing compounds are used by farmers in phytosanitary treatments as leaf sprays or soil amendments. (b) According to the metal defense hypothesis, the high metal concentrations that accumulate in leaf tissues of metal hyper accumulators either deter (antifeedant) or intoxicate (plant systemic pesticide) herbivores or pathogens. (Taken from Poschenrieder *et al.*, 2006).

Plants may adopt different strategies against the presence of metals in the environment (Baker, 1981; Gunsé *et al.*, 2003). Some based their resistance to metals with the strategy of an efficient exclusion of metal, restricting transport to the aerial part. Others accumulate the metal in the aerial part in a non-toxic way to the plant. The exclusion is more characteristic of sensitive and tolerant species to metals, and accumulation is more common in species that always appear in contaminated soils (Baker, 1981).

Among the mechanisms to deal with the toxicity by HM, are those that limit uptake of metals by the roots, helping the plant to tolerate some amount of toxic elements using organic acids exudation which chelate metal in the rhizosphere, preventing this to be absorbed by the root (Schutzendubel and Polle, 2002). Tolerance to heavy metals is also reinforced by the action of mycorrhizal fungi present in some types of plants (Schutzendubel and Polle, 2002).

The heavy metals can be transported via apoplast and being immobilized on the cell walls. The HM becomes a threat to the plants, mainly when they reach the cell cytosol. Therefore, the ability of root cells to control the transport of heavy metals through the membranes determines tolerance by plants.

Three molecular mechanisms associated with the toxicity of heavy metals have been described: the production of reactive species to the oxygen (ROS), the blocking of essential functional groups and the displacement of metal ions in biomolecules (Schützendübel and Polle, 2002).

The objectives of this work are showing the impact and the effect of heavy metals in plants, analyzing the incorporation of these contaminants routes to the different substrates and its impact on the production of secondary metabolites related with the degree of tolerance from a general perspective.

SOIL AND METALS

Factors affecting metal availability

The trace elements pools present in soils are not fully available for the growth of plants. The total traces elements pool present in the soils can be classified into four categories; 1) the solubilized elements in the soil solution, 2) those which form precipitates, 3) the slurp in clays, oxides of Fe and organic matter and 4) those who are part of the soil parent material, these fractions are in a constant dynamic equilibrium with each other (Norvell, 1991; Davis and Leckie, 1978). These equilibria may be strongly influenced by the physicochemical characteristics of the soil, where the plant availability of nutrients, micronutrients and trace elements in general depend on the total content of these present in soil, pH and clay Fe oxide and organic matter, in addition to the redox conditions (Fageria *et al.*, 1991; Marschner, 1995; Whitehead, 2000).

Research on volcanic soils treated with mud and sludge ashes and oriented to determine the retention capacity of heavy metals (Cu, Zn, Cd, Pb, Mo and Mn), showed that the percentage of heavy metals that are retained in the soil varies between 96.0 to 99.9% (Escudey *et al.*, 2007; Pinochet *et al.*, 2002).

However in the rhizosphere (region of soil that is subject to the influence of the roots and is characterized by an area of high microbial activity), chemical and biological conditions are quite different from the "bulk" of the soil, therefore the bioavailability of heavy metals depend on the physicochemical nature of the soil, which governs both the sorption and mobility of these elements in depth and the strategies of plants to capture nutrients (Table 1), mainly through root exudates, which include short-chain organic acids (Legrand *et al.*,

2005; Dakora and Phillips, 2002, Lexmond, 1980; Sauve *et al.*, 1996; McBride *et al.*, 1997; Sauve *et al.*, 1997; Peijnenburg *et al.*, 2000).

Table 1.Essential nutrient elements used by plants: element, symbol and primary chemical forms.

Element	Symbol	Primary Forms Used by Plants
NON-MINERAL ELEMENTS		
Carbon	C	$CO_{2(g)}$
Hydrogen	H	$H_2O_{(l)}$, H^+
Oxygen	O	$H_2O_{(l)}$, $O_{2(g)}$
MINERAL ELEMENTS		
Nitrogen	N	NH_4^+, NO_3^-
Phosphorous	P	HPO_4^{2-}, $H_2PO_4^-$
Potassium	K	K^+
SECONDARY NUTRIENTS		
Calcium	Ca	Ca^{2+}
Magnesium	Mg	Mg^{2+}
Sulfur	S	SO_4^{2-}
MICRONUTRIENTS		
Iron	Fe	Fe^{3+}, Fe^{2+}
Manganese	Mn	Mn^{2+}
Zinc	Zn	Zn^{2+}
Copper	Cu	Cu^{2+}
Boron	B	$B(OH)_3^0$ (Boric Acid)
Molybdenum	Mo	MoO_4^{2-}
Chlorine	Cl	Cl^-

pH

The balance between the different trace elements speciation, its solubility, adsorption or exchange occurring with the sites present in the soil solid phase, it is intimately related to the soil pH value (Olomu *et al.*, 1973; Kalbasi *et al.*, 1978; Cavallaro and McBride, 1984; Sauve *et al.*, 1997). Several studies have shown that minor variations in this parameter

significantly affect the bioavailability of trace elements (Turner, 1994; McBride *et al.*, 1997), such as Mn, Cu, Zn, Cd and the in which the percentage of their free species are strongly affected by the pH of the soil (Fergus, 1954; McGrath *et al.*, 1988; Turner, 1994; Kalbasi *et al.*, 1978; McBride, 1982; Bar-Tal *et al.*, 1989 ;Msaky and Calvet, 1990; Sauve *et al.*, 1997; Jeffery and Uren, 1983). Studies in Arachishypogaea (peanut), Phaseolusvulgaris (beans) and Vignaun-guiculata (Cowpea) grown in contaminated soils with Zn^{2+}, and Mn^{2+} a phytotoxic response was observed, with a significant increase in these elements shoots, as pH decreased (Parker *et al.*, 2001; Davis-Carter and Shuman, 1993; Fergus, 1954; Vega *et al.*, 1992).

Other sensitive to pH changes in soil metals are Al^{3+} and Cd^{2+}, which are also affected in their speciation, solubility, complexation and adsorption (Payne and Pickering, 1975; Msaky and Calvet, 1990; Reddy *et al.*, 1995, Tolrá *et al.*, 2005; Guo *et al.*, 2007). In the case of Al, it is noteworthy that this metal is a constituent of the inorganic fraction of the soil, forming part of aluminosilicates (Escudey *et al.*, 2007).

The availability of Al^{3+} in soil solution is favored under conditions of pH less than 5.5, where the predominant species are Al^{3+}, $Al(OH)^{2+}$, $Al(OH)_2^{+}$, but above pH 7.5 the dominant species is $Al(OH)_4^{-}$ (McBride, 1994). Under conditions of pH less than 5.5 the Al rapidly alter the plant growth affecting mainly the root elongation, which hinder the capture of water and nutrients, whereby a concentration of free Al is considered one of the major factors limiting crop growth in acid soils (Tolrá *et al.*, 2005; Guo *et al.*, 2007).

The application of fertilizers such as triple superphosphate (TSP), has been one of the main inputs of Cd in soils (Molina *et al.*, 2010; Cordero *et al.*, 2004). Cadmium availability

strongly decreases at pH values above 6, where it starts to precipitate as hydroxide (Barančiková *et al.*, 2004). Cadmium also forms precipitates with HPO_3^{2-} and CO_3^{2-}, while variations in the concentrations of Ca^{2+} and Cl^- are impacting the availability in soil (Dudley *et al.*, 1988; 1991). In general in acid conditions the solubility increases Cd, where the ionic species (Cd^{2+}) of this element predominates, however the potential reduction of the Cd^{2+} is unable to generate ROS through Fenton reaction (Cuypers *et al.*, 2010). Studies on soybean cultivated in nutrient media enriched with Cd, determined that this metal is phytotoxic inducing oxidative stress by multiple mechanisms (Balestrasse *et al.*, 2004; Sandalio *et al.*, 2001; Balestrasse *et al.*, 2001).

Organic matter

The type and content of organic matter (OM) play a key role in complexation of metal ions present in soil solution, affecting availability for plants. This complexation can occur by different functional groups presenting the organic matter (Baker and Senft, 1995), such as:

- Nitrogenous aromatic groups, for example pyrrole and indole.
- Aromatic groups: as naphthalene and benzene.
- Reactive groups: primarily responsible for the properties of organic matter: hydroxyl, carboxyl, amino.

The interaction of the organic matter with the Al^{3+}, has been extensively studied (Stevenson, 1991; Garrido and Matus, 2012). Bioavailability and potential Al toxicity in soils and waters are highly dependent on chemical interactions with natural organic matter. There are generally two types of binding mechanisms in which interact different forms of Al with OM; those of electrostatic type occurring mainly between Al^{3+} and dissociated

functional groups of organic matter, mainly -COOH, -OH and-NH_2, besides the Al can form specific bonds with one or more specific functional groups through coordination with aromatic groups (Tam, 1987).

There are few studies that account for the interaction of Cd with OM compared to other heavy metals such as Cu. However, OM also affects the bioavailability of Cd in soils (Barančiková *et al.*, 2004). Soils with high content in OM are able to absorb up to 30 times more than clay soils, where the main interactions occur with Cd ionizable groups of fulvic acids (Barančiková *et al.*, 2004). It has been reported that this element has a high affinity for sulfhydryl groups, hydroxyl and those ligands that contain nitrogen (Benavides *et al.*, 2005).

Clays and hydrous oxides

Clay and hydrated oxides of Al and Fe have ionizable sites silanol (≡Si-OH), aluminol (≡Al-OH) and ferrol (≡Fe-OH) which play an important role in the availability of metals present in the soil solution, through different mechanisms where the processes of sorption (adsorption, absorption and precipitation) (Miller *et al.*, 1987; Pampura *et al.*, 1993; Kalbasi *et al.*, 1978; Basta and Tabatabai, 1992; Martínez and McBride, 1998). Despite the scarce studies that consider the interactions that occur between the inorganic fraction of soil and Al^{3+} and Cd^{2+}, it is possible to establish that increases in clay levels and hydrated oxides, by anthropogenic or natural factors, provides more places for the adsorption of these, thus reducing their availability (Ghanem and Mikkelsen, 1988; Barrow, 1993; Qiao and Ho, 1996).

METALS AND PLANTS

Plants have evolved a number of mechanisms for obtaining nutrients present in the soil and translocating each of its components, but these processes are not specific, and therefore the plant also includes non-essential elements (Redjala *et al.,* 2009).

Uptake mechanisms

The roots are responsible for the uptake of essential and non-essential elements in plants. This process involves a sequence of steps, not fully understood, allowing the transfer of these from the soil solution to the root surface and then into the root cells (Laurie and Manthey, 1994; Fan *et al.,* 1997). The complexity of the absorption processes of elements present in the soil, due to the constant changes that occur between the rhizosphere soil solution and microorganisms (Laurie and Manthey, 1994).

Bio-available metals supply for plant uptake.

Plants are able to influence the solubility and speciation of metals that are found in the rhizospheric area, either by the exudation of chelating agents or pH variations agents (Welch, 1995; Fan *et al.,* 1997). The uptake of the elements inside the plants can occur through the xylem and phloem, where the composition and plants age have an important role in ions movement speed and its toxicity (Fan *et al.,* 1997).

Mechanisms of incorporation of Al and Cd in plants

Incorporation mechanisms of essential and nonessential elements within the plant occur through water transportation (Redjala *et al.,* 2009) (Figure 2).

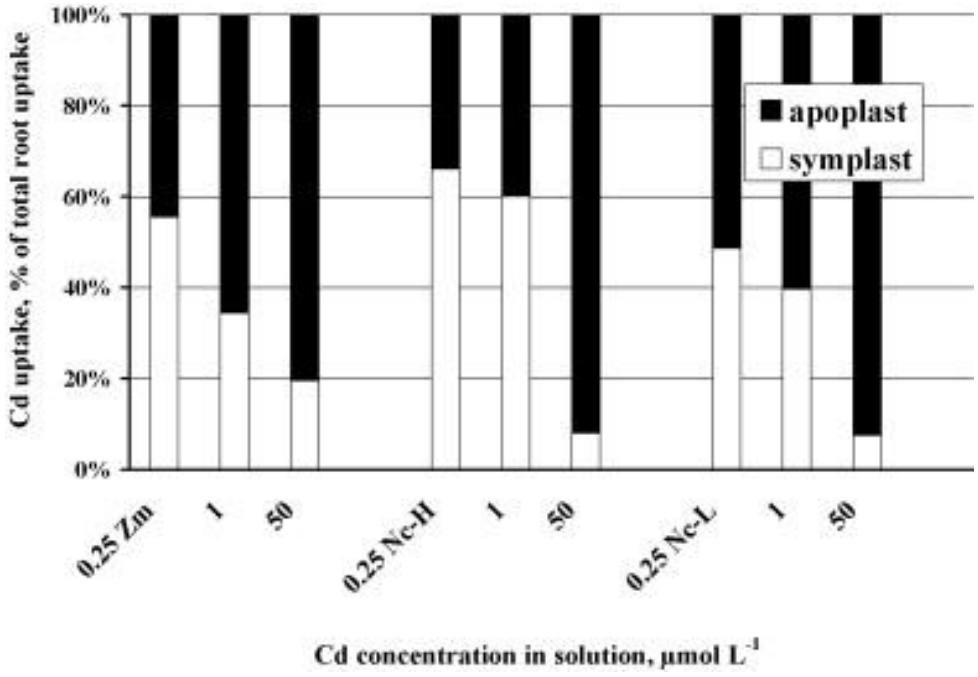

Figure 2. Contribution of apoplast and symplast to Cd uptake by roots of maize (Zm) and high- (Nc-H) and low- (Nc-L) Cd accumulating ecotypes of *Noccaea caerulescens* (Taken from Redjala *et al.,* 2009).

This step may occur through apoplast or symplast pathway (Figure 3).

- The apoplast pathway refers to the passage of water transversely through the cell walls and intercellular spaces.
- The symplast pathway refers to the passage of water into tissues transversely through cell walls and cytoplasm of cells.

In the case of aluminum, plants compartmentalize in vacuoles, once it was absorbed, and thereby reduce its phytotoxic effect. Silva *et al.,* 2002, suggests that the main aluminum transport mechanism occurs via apoplast (extracellular) where the net negative charge of the roots caused by the deprotonation of the carboxylic groups of polygalacturonic acid, the

main component of the cell wall and the outer surface of the membrane, plasma, leads to increased cationic exchange capacity (CEC) of the roots, facilitating the incorporation of Al (Valencia *et al.*, 2012).

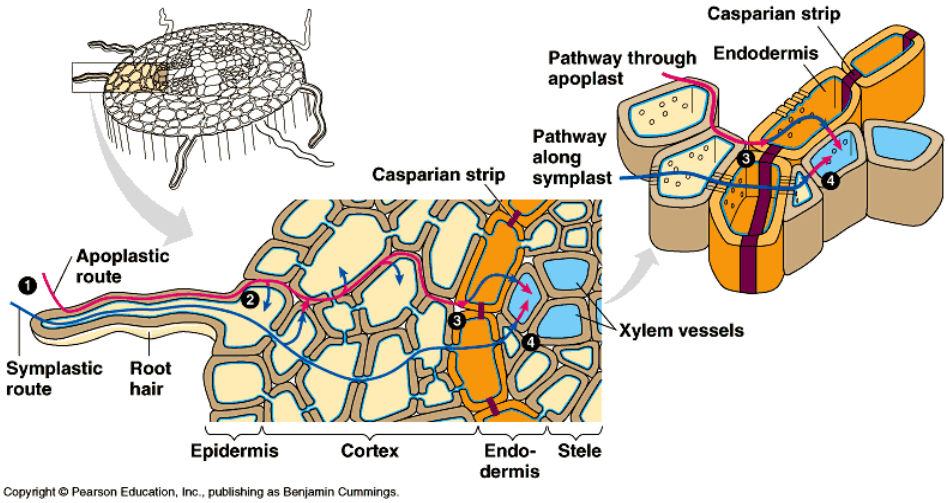

Figure 3. Lateral transport of minerals (micronutrients) and water in roots (Taken from White, 2001)

However, inside the plant, cells have a lower content of pectin and high degree of methylation, which reduces the CEC and the binding of Al^{3+} to the cell wall (Silva *et al.*, 2002), having as a consequence a lower accumulation of Al^{3+} to the interior of the cell (via symplast). In soils with high contents of free Al, the roots of the plants are characterized by a low CEC, being this adaptation a defense mechanism (Blamey *et al.*, 1990).

Studies in *Pisium sativum* L., suggest that Cd is accumulated in descending order in roots, stems, leaves, fruits and seeds (Blumm, 1977). In general, once that Cd enters to the root can move through the xylem via the apoplast and/or symplast forming complexes with nitrogen and sulfur donors compounds present in macromolecules and low molecular weight ligands, which were possibly biosynthesized in response to the presence of Cd^{2+}

(Clemens *et al.,* 2002). Since Cd can enter the plant via apoplast and / or symplast, this element can accumulate in other organelles compared to what happens with Al^{3+} (Godbold and Huttermann, 1985; Frausto da Silva and Williams, 2001), the symplastic pathways of Cd incorporation in plants are closely linked with those carrying Zn^{2+} and Ca^{2+}, so that variations in the concentrations of these nutrients (Huang *et al.,* 1996; Guerinot *et al.,* 2000).

Effect of heavy metals in plants: phytotoxicity symptoms

Elements such as Cu, Zn, Fe, Mn, Mo, Ni and Co are essential micronutrients to metabolic development of plants, but an excess of them in soils can be toxic for their development (Monni *et al.,* 2000). These elements along with Cr, Cd metals, Pb and others, are called trace elements, because the quantities that are in traces (10 mg kg^{-1} or mg ml^{-1}) or ultra traces (1 µg kg^{-1} or µg L^{-1}) in environmental matrices (Monni *et al.,* 2000).

The most widespread visual evidence of the toxicity of metals is a reduction in the growth of plants as the metal toxicity increases. However, as different metals have different sites of action within the plant, the response of the toxicity in visual form differs among metals.

Effects of cadmium on plants

The Cd is highly toxic to humans, animals and plants. The Cd limits in agricultural soil is 100 mg kg^{-1} of soil. In the plants exposure to Cd produces various symptoms of phytotoxicity, such as chlorosis, reduction of the biomass, inhibition of roots elongation and even death of the plant (Sanita di Toppi and Gabbrielli, 1999; Milone *et al.,* 2003; Guo *et al.,* 2008).

The presence of Cd in the growth medium of the plant interferes with incorporation, transport and uptake of essential elements such as Ca, Mg, K, and P, causing imbalances in the plant metabolism (Sandalio *et al.,* 2001; Li *et al.,* 2008).The Cd also reduces the absorption of nitrates and their transport from the root to the stem, and also to inhibit nitrate reductase activity in stems (Gouia *et al.,* 2000). Plants exposed to Cd show changes in stomatal aperture, photosynthesis and transpiration (Sandalio *et al.,* 2001). Studies have shown that phytotoxicity by Cd produces chlorosis due to a deficiency of Fe, PO_4^{3-}or reduced transport of Mn (Liu *et al.,* 2007; Benavides *et al.,* 2005; Gaballah *et al.,* 2012).

The photosynthetic apparatus is one of the target sites of Cd action in plants. The Cd can directly or indirectly interact with different components of the photosynthetic apparatus and can reduce the efficiency of electron transport inhibiting chlorophyll biosynthesis, leading to a reduction of CO_2 assimilation (Maksymiec *et al.,* 2007; Wang *et al.,* 2006). Other effects on the metabolism of the plant due to Cd are reduced ATPase activity of the plasma membrane, changes in the functionality of the plasma membrane (Fodor *et al.,* 1995), which has been observed in roots of sunflower and imbalances in chloroplast metabolism, inhibiting chlorophyll synthesis and reducing the activity of enzymes involved in CO_2 fixation (Sandalio *et al.,* 2001; Astolfi *et al.,* 2005; Maksymiec *et al.,* 2007). It has been found that the presence of Cd in plants increases ROS production, causing oxidative stress and increased lipid peroxidation in leaves and roots (Smeets *et al.,* 2005; Guo *et al.,* 2007; Cherif *et al.,* 2011).

Effect of Aluminum on the plant

Phytotoxicity by Al^{3+}, is shown especially in acid soils, where it is one of the main factors affecting culture production. The first symptoms of phytotoxicity by Al^{3+} are reduced elongation and cell division of roots in plants, which is reflected in a decrease in the absorption of nutrients and water (Miyasaka *et al.,* 1991). The concentration of Al^{3+} in plants grown under the presence of metal is greater in roots than in the aerial part. Symptoms of phytotoxicity of Al^{3+} have been observed in *Triticum aestivum*, where a decrease in root length after one hour exposure of the roots to the metal, together with an increase in the diameter of the root tips was observed (Ryan *et al.,* 1993).

Severe phytotoxicity Al^{3+} reduces and damages the roots, making the plant susceptible to water stress and nutritional deficiencies. Among the nutritional effects, include blocking calcium and potassium channels, which cause long-term mineral deficiencies. It has been reported that phytotoxic concentrations of Al decrease the flow of oxygen in the roots; interfere with certain enzymes involved in the production and deposition of constituent polysaccharides of the cell wall, altering Donnan free space in cell wall and forms highly stable complex with amino acids and nucleic acids (Rout *et al.*, 2001).

The Al^{3+} interferes with the absorption and transport of nutrients such as Ca, Mg, K, P, B, Fe and Cu (Keltjens and Tan, 1998; Lukaszewski and Blevins, 1996; Taylor *et al.*, 1998; Lidon *et al.*, 1992; Guo *et al.,* 2004, 2007; Olivares *et al.*, 2009). The decrease in the uptake of cations may be related to the inhibition of root growth and the tolerance of cultures to Al^{3+}. Giannakoula *et al.,* 2008; studied sensitive lines of rice and tolerant to

aluminum, finding those lines sensitive to accumulate more of that the tolerant line, which largely retains the concentration of Ca, Mg and K.

Oxidative stress

Aerobic organisms are continuously exposed to the generation of reactive oxygen species (ROS). Many of the lethal processes undergone by plants under adverse conditions are mediated by ROS, which are generated in different cellular compartments as a result of malfunction of normal metabolic pathways and physiological processes (Arora *et al.,* 2002, Fu and Huang, 2001) (Table 2).

Within the group of ROS we can find free radicals (chemical species with one or more unpaired electrons in its outer orbit) and superoxide anion ($\cdot O_2^-$) and hydroxyl radical ($\cdot OH$) and generating molecules of ROS such as hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (Arora *et al.,* 2002). ROS are produced in various parts of the cell, some of the sites of ROS production and components of the antioxidant system of the cell are summarized in Table 2 (Arora *et al.,* 2002). Excessive generation of ROS occurs under stress conditions such as UV light, water and salt stress, pathogen attack, injury, cold, heavy metals (Merwin *et al.,* 1995; Ercal *et al.,* 2001). Research has shown that ROS produce damage to cell membranes by inducing lipid peroxidation, besides cause damage to the level of DNA, proteins, lipids and chlorophyll (Boscolo *et al.,* 2003; Cakmak *et al.,* 1991; Guo *et al.,* 2007; Gaballah *et al.,* 2012).

Table 2. Main sites of production of reactive oxygen species (ROS) in plants and the respective detoxification mechanisms ('scavengers').

Reactive Oxygen Species (ROS)	ROS Production	Scavenger
Hydrogen peroxide	Cellular compartments (chloroplasts, mitochondria, cytosol) Peroxisome (photorespiration)	Catalase, Ascorbate peroxidases, Glutathione
Superoxide Radical	Cellular compartments (chloroplasts, mitochondria, cytosol)	Superoxide dismutases, Ascorbic Acid, Glutathione
Singlet oxygen	Thylakoid	α-Tocopherol, Carotenoids
Hydroxyl radical	Several Sites (eg. Haber-Weiss Reaction in chloroplast)	Ascorbic Acid

Plants growing in soils with HM suffer from reduced growth affecting culture yields. At the molecular level, the oxidative stress is widely studied as a key sign of plant stress. This process is described as an imbalance between ROS and antioxidant systems (Boscolo *et al.,* 2003). Dietz *et al.,* (1999) propose a relationship between the toxicity of metals, redox homeostasis and antioxidant capacity in plants. Depending on the chemical and behavior of markers in biological systems, the toxicity may be attributed to properties of the following mechanisms: (1) interfering with the functional sites in proteins, (2) transport of essential elements, with consequent disturbance of the enzymatic functions, and (3) an onset of ROS production (Dietz *et al.,* 1999).

Metals such as Fe^{2+} and Cu^{+}, can convert the molecule H_2O_2 in · OH radical through the Fenton type reaction.

$$\mathbf{H_2O_2 + Fe^{2+}\ (Cu^{+}) \rightarrow Fe^{3+}\ (Cu^{2+}) + \cdot OH + OH^{-}\ (Eq.1)}$$

Other metals are not involved in the Fenton reaction, however; also generate ROS, as for example the Hg^{2+} it is believed that it can inhibit antioxidant enzymes, particularly the enzyme glutathione reductase, tending as a result the accumulation of ROS (Mithofer *et al.,* 2004). Cadmium does not cause directly ROS through fenton reaction, however can cause indirectly oxidative stress by activating the enzyme Lipoxygenase, which stimulates lipid peroxidation (Keunen *et al.,* 2011). Other studies suggest that the metal may act as pro-oxidant, by reducing the pool of glutathione (GSH), necessary for the synthesis of phytochelatins (Ercal *et al.*, 2001).

The Al^{3+} is a phytotoxic element for plants which also cause ROS when entering to this, various research suggests that it may induce oxidative stress, due to the high affinity of this element by the components of the Phospholipid membrane, mainly phosphate and carboxylic groups, which leads to its rigidification (Devi *et al.,* 2003; Ma *et al.,* 1998; Ryan *et al.,* 2001). This phenomenon causes an increase in the amount of reactive oxygen species (ROS), which affect the physiological functioning of plants, and may even induce cell death (Yamamoto *et al.,* 2002; Boscoso *et al.,* 2003; Corrales *et al.,* 2008). The production of ROS various damage to cellular components such as lipid peroxidation, the oxidation of proteins and DNA damage (Munné-Bosch and Alegre, 2002; Barlett and Stadtman, 1997; Ercal *et al.,* 2001).

The Malondialdehyde (MDA) is one of the resulting products of lipid peroxidation and its content accounts for the level of damage that occurs in the lipid membrane in the cell membrane. From here, it is that the MDA is an important physiological indicator of the degree of oxidative stress in the plant (Heath and Parker, 1968).

Several studies have shown that the Cd is an inducer trace element of oxidative stress, which is reflected in an increase in lipid peroxidation. Ge et al., found an increase in the content of MDA in poplar cultivars grown in hydroponic solution exposed to 50 and 100 µM of Cd (Ge *et al.,* 2012). Treatments with Cd exposure, produced lipid peroxidation in *Pisum sativum* (Lozano *et al.,* 1997; Metwally *et al.,* 2005; Pandey and Kumar, 2012; Gaballah *et al.,* 2012), in different cultivars of barley (Guo *et al.,* 2007; Wu *et al.,* 2003), in peanuts (*Arachis hypogaea*), similar results were reported in a study in tomato exposed to different concentrations of Cd (Kumar et al., 2008; Dong *et al.,* 2006).

In the case of plants subjected to treatment with Al, have reported similar behaviors to those of Cd. Studies on barley and quince determined significant increases in lipid peroxidation, in those treatments containing Al (Guo *et al.,* 2007; Wulff *et al.,* 2010). Aluminum generates oxidative damage during variable exposure time, Yamamoto *et al.,* found an increase of lipid peroxidation in pea plants after exposure for 4 hours with Al (Yamamoto *et al.,* 2001), while that Çakmak and Horst (1991), observed in soybeans plants, significant increases in the content of MDA after 48 h of exposure.

To resist oxidative stress, plants can induce a series of reactions catalyzed by detoxifying antioxidant enzymes, where are found, superoxide dismutase (SOD EC 1.15.1.1), Peroxidase (POD EC 1.11.1.7), ascorbate peroxidase (APX EC 1.11.1.11) and

catalase (CAT EC 1.11.1.6) (Garratt *et al.,* 2002), in addition to non-enzymatic low molecular weight antioxidant compounds as ascorbic acid, glutathione, tocopherol, and pigments such as carotenoids (Rama-Devi and Prasad, 1998).These three enzymes maintain the balance between the production of free radicals and eliminate these products, avoiding the effects of free radicals in cells (Gaballah and Rady, 2012). Studies have shown that the activity of these enzymes increases with stress concentration of heavy metals such as Cd and Al (Guo *et al.,* 2004; Gaballah and Rady, 2012; Ribeiro *et al.,* 2012). Therefore the tolerance of some plants, though the presence of heavy metals increases can be attributed in part to the action of the SOD, POD, CAT and other enzymes (Dixit *et al.*, 2001; Lagriffoul *et al.,* 1998; Zhang *et al.,* 2004).

Lactuca sativa exposed for two weeks at 100 µM Cd was increased enzyme activity of SOD and POD, suggesting that both enzymes act in combination to reduce the impact of Cd toxicity, especially in young leaves (Monteiro *et al.,* 2009). However, the amount of enzymes in plants is reduced, so that their role in antioxidant protection and combat the negative effects of oxidative stress induced by heavy metals appears to be limited. Several studies have shown that exposures to high concentrations of Cd decreased antioxidant capacity, mainly of CAT enzyme (Fodor, 2002; Chaoui *et al.,* 1997; Monteiro *et al.,* 2009).

The effects of Al on the activity of antioxidant enzymes have also been investigated by several authors (Meriga *et al.,* 2004; Guo *et al.,* 2007; Boscolo *et al.,* 2003). The induction of SOD activity was observed in *Zea mays* treated with Al^{3+} (36 µM) for 48 hours, while this element did not cause activation of the CAT enzyme (Boscolo *et al.,* 2003). *Artemisia annua* L plants treated with soil in the presence of 10 mM of Al^{3+}, an increase in the enzymatic activity of CAT, and SOD was observed (Aftab *et al.,* 2010).

Similar results were obtained in rice plants, an increase in antioxidant enzymes CAT, and SOD in the presence of Al^{3+} (Meriga *et al.*, 2004, Ribeiro *et al.*, 2012).

Phenolic compound and antioxidant defenses and protective role of heavy metals

The phenolic compounds are secondary metabolites synthesized by plants as a defense measure against various stresses and act as protective agents against pathogens, being secreted as a defense mechanism to stress conditions, such as infection, UV radiation, among others (Li *et al.*, 2000; Close and McArthur, 2002). These compounds are characterized by containing one or more aromatic rings (C6), together with one or more hydroxyl groups (Balasundram *et al.*, 2006; Ignat *et al.*, 2011).

The phenolic compounds have diverse functions in plants. Research has shown an increase in the amount of phenolic compounds under different environmental conditions and stresses. Research has shown that plants increase the synthesis of phenolic compounds as a strategy to control ROS. From this point, it is possible to consider that phenolic compounds play an antioxidant role or protective role when they grow in environments of stress such as the presence of HM.

Induction of biosynthesis of phenolic compounds has been observed in wheat seedlings in response to toxicity by Ni and corn in response to exposure of Al (Kováčik *et al.*, 2009, 2010). In a *Phaseolus vulgaris* study, it was exposed to Cd^{2+} accumulating phenolic compounds (Milone *et al.*, 2003). Irtelli and Navari-Izzo, (2006) found a decrease of phenolic compounds such as chlorogenic acid in *Brassica juncea*, exposed to Cd.

Studies reported on the effect of Cu in the production of phenolic compounds in *Matricaria chamomilla*, showed changes in the contents of 11 phenolic compounds such as

chlorogenic acid, caffeic acid, vallinic acid, among others, which varied depending on the metal concentration (Kováčik *et al.,* 2008).

It has been investigated that the production of phenolic compounds, help the plant to have a better tolerance to metals such as Al^{3+}, which has allowed us to develop the hypothesis that phenolic compounds have a role in detoxification when acting by complexing in a stable way Al^{3+} in plants, in addition to the antioxidant capacity that phenolic compounds have, helping Al tolerance in plants such as corn (Tolrá *et al.,* 2005, 2009; Rout *et al.,* 2001).

Concluding remarks and future trends

The trace element stress is one of the main problems affecting the agricultural productivity of soils and plants. Tolerance to these elements varies between species, which has led to different plants grow well on soils enriched by these elements. Scientific research has helped to elucidate the most important negative effects that these elements produce to cultures, showing that many of the first visible symptoms of phytotoxicity are similar between metals or in many cases similar to symptoms of nutrient deficiency. The chemical speciation of these elements in the soil solution is related to pH, organic matter, clays present, which may increase their availability to plants, increasing the toxicity of these. Elements such as Cd and Al are highly toxic due to their mobility in soil, in addition to being in the case of Al the biggest problem in acid soils system.

Different investigations have shown that the presence of trace elements in general induces ROS which negatively affects plants. The mechanisms to combat these species are highly known and studied to date, which include the enzymatic and non-enzymatic

mechanisms. It is in this sense that the study of non-enzymatic mechanisms such as the production of phenolic compounds suggests to be a promising approach for the study of the detoxification of plant with tolerant and sensitive genotypes.

Although the phenolic compounds are synthesized in normal conditions by the plant, an alteration in the conditions of growth and development, such as the presence of heavy metals, causes changes in producing these compounds. There is not a clear signal of what are the involved routes that correlate the synthesis of phenolic compounds and the presence of heavy metals.

Despite numerous studies on heavy metals or trace elements-plant interaction and synthesis of phenolic compounds, there are still steps to understand the phenomenon, due to the absence of studies on a complementary or synergistic effect of various parameters or agents that participate in periods of plant growth and development.

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Chapter 2

Effect of aluminum on antioxidant activity and phenolic compounds content in *in vitro* cultured blueberries

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Abstract

Blueberry is a popular natural food product consumed worldwide. Acid soils are found throughout the world. A significant problem of acid soils is the active aluminum content, which may result toxic to plant. The present study was undertaken to assess the toxicities of Al for Blueberry (*Vaccinium corymbosum* L.) cultivated *in vitro* and treated with 100 and 200 μM Al. The effects of Al concentration on malondialdehyde (MDA) content, antioxidant activity and phenolic compounds of blueberry after 7, 14 and 21 days of treatment were established. The analysis of the MDA accumulated in the tissues of the blueberry plantlets indicates that Al concentration increases the damage caused by lipidic peroxidation, for both treatments, after 14 days. The highest antioxidant activity in the extracts was observed at 200 μM Al after 14 days of treatment, being chlorogenic and ellagic acids the most significant metabolites involved in the antioxidant properties. Then, the content of Al in soil could be modulate the content of bioactive compounds in blueberry plants.

Keywords: Blueberry, aluminum, antioxidant capacity, phenolic compounds.

Resumen

El Arándano es un popular alimento natural consumido en todo el mundo. Los suelos ácidos se encuentran en todo el mundo. Un problema significativo de suelos ácidos es el contenido de aluminio activo, que puede resultar tóxico para la planta. Este estudio se realizó para evaluar la toxicidad del aluminio en plantas de arandano, cultivadas in vitro y tratadas con 100 y 200 mM de Al.Se establecieron los del aluminio en el contenido de malodialdehido (MDA), capacidad antioxidante y contenido de compuestos fenolicos en plantulas de arandano luego de 7, 14 y 21 dias de tratamiento. El análisis del MDA

acumulado en los tejidos de las plantulas de arándanos indica que la concentración de Al aumenta el daño causado medido como peroxidación de lípidos, para ambos tratamientos, después de 14 días. La actividad antioxidante más alta de los extractos se obseva a 200 mM de Al después de 14 días de tratamiento, siendo los ácidos clorogénico y elágico los metabolitos más importantes que participan en las propiedades antioxidantes. Entonces, el contenido de Al en el suelo podría modular el contenido de compuestos bioactivos en plantas de arándanos, alterando sus propiedades medicinales.

Palabras Clave: Arándano, aluminio, capacidad antioxidante, compuestos fenólicos.

INTRODUCTION

Aluminum (Al) phytotoxicity is one of the major agronomic problems in acid soils (Guo *et al.,* 2007; Tolrá *et al.,* 2009). Its availability and activity in the soil solution is heightened to pH less than 5.5, where concentrations in the order µM quickly may inhibit the elongation of roots and subsequently the capture of water and nutrients (Alvarez *et al.,* 2005).

A mechanism of tolerance to this element in plants is the exudation of organic acids from the roots, which trap the free Al present in soil solution (Kochian *et al.,* 2005; Pineros *et al.,* 2008; Poschenrieder *et al.,* 2008; Liu *et al.,* 2009; Giannakoula *et al.,* 2010). However, despite this defense mechanism, the Al plant uptake induces oxidative stress due to their high affinity with phosphate and carboxylic groups present in the plasma membrane. (Ma *et al.,* 2001; Ryan *et al.,* 2001; Devi-Rama *et al.,* 2003). This phenomenon increases the amount of reactive oxygen species (ROS), which affect various physiological parameters of the plant and may even induce cell death (Yamamoto *et al.,* 2002; Boscolo *et al.,* 2003; Corrales *et al.,* 2008).

Plants show efficient systems for scavenging active oxygen species that protect them from destructive oxidative reactions (Munné-Bosch *et al.,* 2001). As part of this system, antioxidative enzymes are key elements in the defense mechanisms. Garratt *et al.,* (2002) has listed some of these enzymes as catalase (CAT EC 1.11.1.6), glutathione reductase (GR EC 1.6.4.2), superoxide dismutase (SOD EC 1.15.1.1) and glutatione-S-transferase (GST EC 2.5.1.18). Superoxide dismutase, for example, metabolizes oxygen (O_2) radicals to hydrogen peroxide (H_2O_2), thus protecting cells from damage. Catalase, ascorbate peroxidase, and a variety of peroxidases catalyze the subsequent breakdown of $H_2O_2$to

water and oxygen (Garratt *et al.,* 2002). Plants with high levels of antioxidants have been reported to have greater resistance to this oxidative damage (Koca *et al.*, 2007).

It is proposed that the phenolic compounds, increase plant tolerance to Al, due to its ability to detoxify through the formation of stable complexes of Al and also for their antioxidant capacity (Tolrá *et al.,* 2009).

A plant species highly valued for its high content of phenolic compounds, is the blueberry (Kähkönen *et al.,* 2001; Dastmalchi *et al.,* 2010). Blueberries are native to North America and have a rich folklore history of medicinal uses by the native American Indians. For centuries, native American tribes have used the leaves, roots, and fruits from the blueberry plant for medicinal purposes (Sanchez-Moreno *et al.,* 2003), and blueberries continue to be used in many types of dietary health products as pharmaceutical or food supplements in modern society (Kalt and Dufour, 1997). Many of the uses, once thought to be anecdotal, are now the subject of intensive scientific research. Research on blueberries, which originally focused on antioxidant activity, has now expanded into the areas of anti-inflammation, and cell signaling (Howell, 2009). Blueberry grows well in acid soils of southern Chile, where the Al content is significantly high (Inostroza-Blancheteau *et al.*, 2012).

The free Al content in soil solution can vary by natural (rain) and anthropogenic (liming, fertilization and organic amendment) processes (Inostroza-Blancheteau *et al.*, 2012), which could modify the total content of phenolic compounds in *V. corymbosum*, affecting their antioxidant capacity. For this reason, it is important to determine which might be the ideal conditions in the management of this crop to maximize antioxidant metabolite production.

The aim of this study was to determine the effects of Al on the antioxidant capacity and profile of phenolic compounds in blueberries grown *in vitro*.

MATERIALS AND METHODS

Plant material, growth conditions and treatments

In vitro cultures of *V. corymbosum* cv. Legacy was started from shoot tips of free-pathogen certified plants and its sterilized in 10% of sodium hypochlorite solution and rinsed with sterilized and destilled water, for culture using a Lloyd-McCown media base (Lloyd and McCown, 1980) supplemented with 2.76 $mg \cdot L^{-1}$ of hormone 2-iP and 3.0 $g \cdot L^{-1}$ of agar phytagel, it mixture was place in a glass flask and was sterilizing in autoclave at 121 ºC during 15 minutes. The Al treatments ($AlCl_3$), was applied as follow: (1) pH 5.2 (control); (2) Al 100 µM pH 5.2; (3) Al 200 µM pH 5.2.

The cultures were maintained during 7, 14 and 21 days at 23 ± 2 ºC with photoperiodicity 16/8 h (day/night). *In vitro* cultivated plantlets with more than 30 days of adaptation to cultivation conditions were defined as starting control (time=0 days), and used to define the physiological base line for all the studied parameters.

Lipid peroxidation

The level of lipid peroxidation was determined in terms of MDA concentration according to the method of Heath and Packer (1968) with modifications. The concentration of MDA was calculated from the difference of the absorbance at 532 and 600 nm using the extinction coefficient of 155 $mmol \cdot L^{-1}$ cm and expressed as $nmol \cdot g^{-1}$ FW.

Extracts preparation

Fresh materials ($0.1g \cdot mL^{-1}$) were used to prepare the extract using 85% v/v of hydroethanolic solution; the samples were sonicated at 50-60 Hz of frequency during two hours at 25 ºC according to the method Rostagno *et al.,* (2002).

Antioxidant activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenger spectrophotometric assay

The removal capacity of free radicals of different extracts was evaluated using the radical DPPH technique, described by Shyu *et al., 2002.* An aliquot of the ethanol extracts was added to DPPH solution, the absorbance decrease was continuously monitored at a wavelength of 517 nm with an UV-visible spectrophotometer (Agilent 8453 UV-Vis), for 240 seconds (Brand-Williams *et al.,* 1995). Results were expressed as % of consumed DPPH.

Ferric reducing Antioxidant power (FRAP)

The FRAP assay measure the ability of the sample to reduce Fe III to Fe II (Benzie and Strain, 1996), through the formation of a blue complex with tripyridyltriazine (TPTZ) which show a maximum absorbance at 593 nm. FRAP reagent was prepared by mixing in the ratio 10:1:1 of acetate buffer (300 mM), TPTZ and $FeCl_3$ solutions. The FRAP reagent was maintained at 37 ºC. Absorbance was measured at 593 nm in a spectrophotometer (Agilent 8453 UV-Vis) to the sample containing 900 μL of FRAP reagent, 80 μL of water sample and 20 μL of ethanolic extract. The measures were expressed in Ascorbic acid equivalents.

Total phenolic content (TPC)

The total phenolic content of ethanolic extracts was determined based on the method described by Singleton and Rossi (1965). Results were expressed as Gallic acid equivalents.

Analysis of extracts HPLC-DAD

High performance liquid chromatography with diode array detector (HPLC-DAD) was used to separate, identify and determine phenolic compound in extracts ethanolic blueberry tissue. The ethanolic extract was filtered through a 0.45-µm membrane and analyzed by HPLC-DAD.

Agilent HPLC-DAD 1100 series equipped with a RP-C18 column at 25 ºC was used. The mobile phase is a gradient of acetonitrile (A) and 1% phosphoric acid (B), using the program: time = 0 minutes 10% of A, 5 minutes 25% of A, 8 minutes 35% of A, 15 minutes 60% of A, 17 minutes 35% of A and finally 20 minutes 10% of A; with 120 bar approximately pressure at start, 1 mL/min of flow and 20 µL of injection volume using a Reodyne valve, registering the signals at 254, 280, 314 and 340 nm.

Statistics analysis

The analysis was carried out using analysis of variance (ANOVA) with Fisher (F) test in all samples to determine significant difference with $n \geq 3$ in all measurements.

RESULTS

Effect of Al on malondialdehyde (MDA) content

Variations in the content of MDA, as a result of the presence of Al in the growth medium are shown in Figure 1. The results show no significant effect on the MDA content for both treatments after 7 days of exposure to the metal. The greatest accumulation of MDA over the control was observed after 14 days of treatment with 100 and 200 µM Al to decrease after 21 days of treatment, even when it remains significantly higher than control.

These results suggest that in early stages of exposure to Al (14 days), the variations in the content of MDA in *V. corymbosum* are associated with the presence of Al and not necessarily with the concentration of this element in the growth medium. When the exposure period is extended, MDA levels decreased, possibly as a result of the mechanisms of the plant to counteract the damage caused by lipid peroxidation. The effect of different concentrations of Al is appreciable when the exposure time is prolonged, where higher concentrations of Al generate a better defense against oxidative damage in blueberries, suggesting that the content of MDA in *V. corymbosum,* depends on the exposure time and subsequently the dose of Al in a first stage, showing a differential response to oxidative damage level.

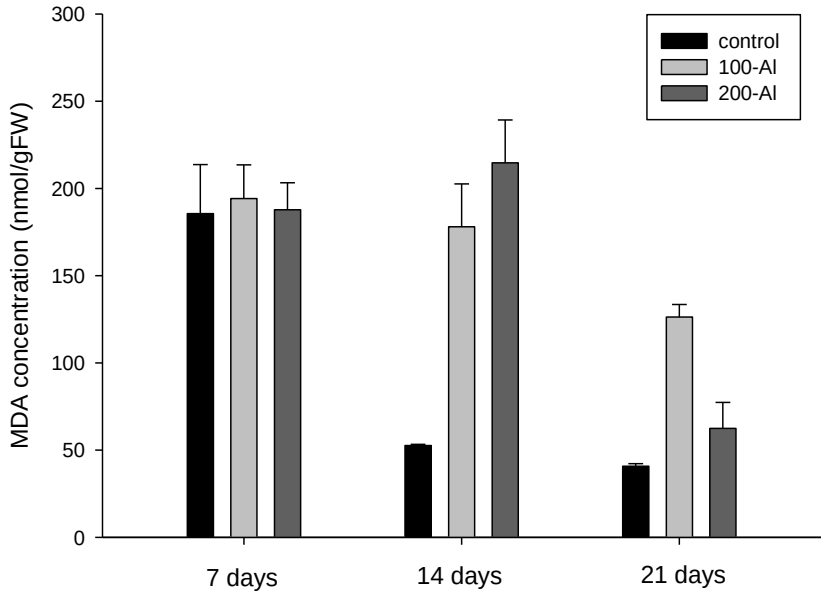

Figure 1. Effect of the Al concentration and exposure time on the content of MDA in plantlets of *V. corymbosum* L. Each value is a mean of three samples ± 1 s.d.

Antioxidant activity

The antioxidant capacity of ethanolic extracts of *V. corymbosum*, subjected to 100 and 200 uM of Al was evaluated from ethanolic extracts using DPPH and FRAP assays (Figure 2A and B). The antioxidant activity (DPPH assay) showed no significant difference between treatments and control for the first 7 days.

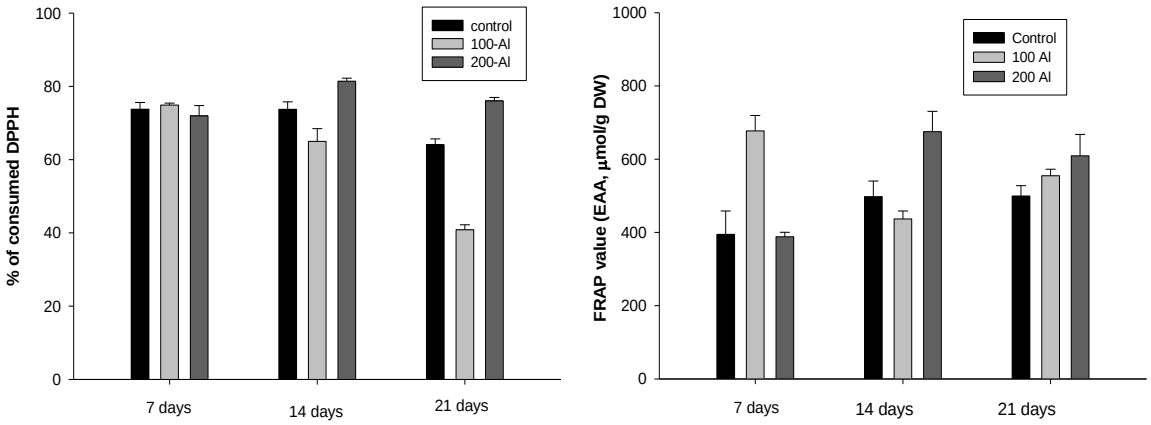

Figure 2. Antioxidant capacity of extracts of plantlets of *V. corymbosum* L. treated with Al. DPPH assay (A) and FRAP assay (B). Each value is a mean of three samples ± 1 s.d.

However, clear differences were observed at 14 and 21 days of exposure. 100 uM With Al, DPPH consumption decreased with respect to control up to 21 days, the highest antioxidant activity for the 3 conditions studied, was observed with 200 uM Al which peaked at 14 days (Figure 2A). This behavior suggests that the presence of Al generates a significant variation in consumption of DPPH in plantlets of *V. corymbosum*, which depends on the concentrations of Al present in the medium (Figure 2A).

The evaluation of the reducing power of the ethanolic extracts was performed by the FRAP assay (Figure 2B). The results obtained from this test also show a different response with respect to the Al concentration and exposure time, showing the greatest variations of

this parameter during the first two weeks of exposure. With 100 uM Al it was observed a higher FRAP value at 7 and 21 days, with a minimum at 14 days. With 200 uM Al it was observed a higher FRAP value at 14 and 21 days with a maximum at 14 days (Figure 2B).

Total phenolic content (TPC)

The content of total phenolic compounds for each extract of blueberry is shown in Figure 3. Control plants showed a slight decrease at day 21, while for the treatment at 100 µM, it was observed a maximum at day 7 (113.9 $mg \cdot g^{-1}DW$). At 21 days the total phenolic content was lower than in the control. For treatment at 200 µM of Al, it was observed a maximum at day 14 of treatment (110.7 $mg \cdot g^{-1}DW$) and a minimum at day 7, with a value below the control

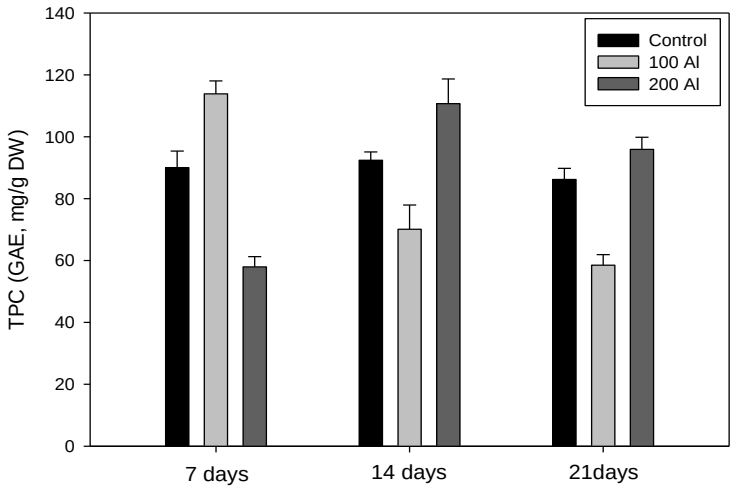

Figure 3. Variation in the content of total phenolic compounds (TPC) in plantlets of *V. corymbosum* L. treated with Al. Each value is a mean of three samples ± 1 s.d.

The different response observed in the TPC content in both treatments, may indicate that the mechanisms of antioxidant response in *V. corymbosum* are more effective when it exceeds a threshold concentration of Al, what would happen about 100 uM of Al in the growth medium.

Identification of phenolic compound by HPLC-DAD

The identification of phenolic compounds in the blueberry extract was performed using HPLC-DAD. Four compounds were identified, two of them (chlorogenic acid and ellagic acid) were also quantified (Figure 4).

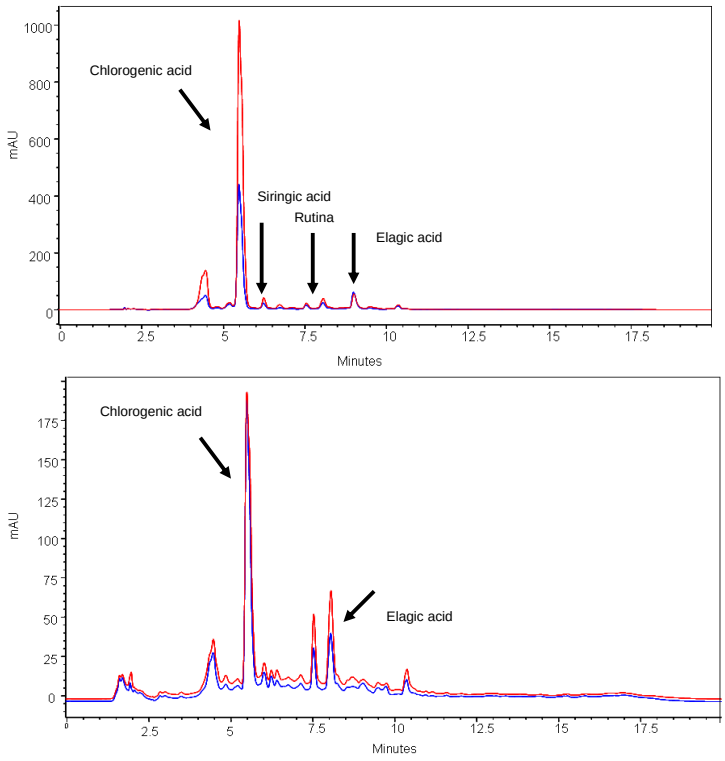

Figure 4. HPLC profiles of phenolic compound in blueberries cultivated *in vitro* at 314 nm (up) and 254 nm (bottom). Blue line: control; Red line: 200µM Al^{3+}. In both cases evaluation was done after 14 days of treatment.

Compared with the control, there was an increase in the content of chlorogenic acid for both AL levels after 7 days with a peak on day 14 (Figure 5). Similar results were reported by Zheng *et al.*, (2003), when tested blueberry plants to high oxygen content, suggesting that these phenolic compounds are biosynthesized in response to stress conditions (Zheng *et al.*, 2003).

Moreover, with 100 µM Al, ellagic acid concentration remained relatively stable over time. In contrast, with a treatment of 200 µM of Al this compound significantly increases, with a maximum at 14 days.

DISCUSSION

The phenomenon of Al toxicity in plants has been extensively studied in nutrient solutions, showing that the first symptom of Al toxicity is inhibition of root elongation, which directly impacts the absorption of nutrients into the plant (Yamamoto *et al.,* 2003; Kochian *et al.,* 2005). However, no research has been reported on the effect of Al in the nutrient medium, the antioxidant capacity of the extracts and phenolic composition of seedlings of blueberry cultured *in vitro*.

The effects of exposure to high Al levels were evaluated in a kinetics study of 21 days. The MDA, a byproduct of lipid peroxidation of membranes, it accumulates in the tissues of plants when these are subjected to stress. Treatments at 100 and 200 mM of Al, induced lipid peroxidation in blueberry cv. Legacy at 14 days of exposure, increasing the content of MDA more than three times with respect to control. This damage would be associated to the presence of the metal in the solution, since no significant differences were observed in the content of MDA between treatments, although it is highly probable that there is a threshold concentration. Research by Yamamoto *et al.,* (2002) found similar behavior in pea plants, where an increase in lipid peroxidation after exposure for 4 hours with Al was observed. Similar behavior was described by Cakmak and Horst (1991), who observed an increase in the MDA concentration in soybean, after 2 days of treatment with different doses of Al.

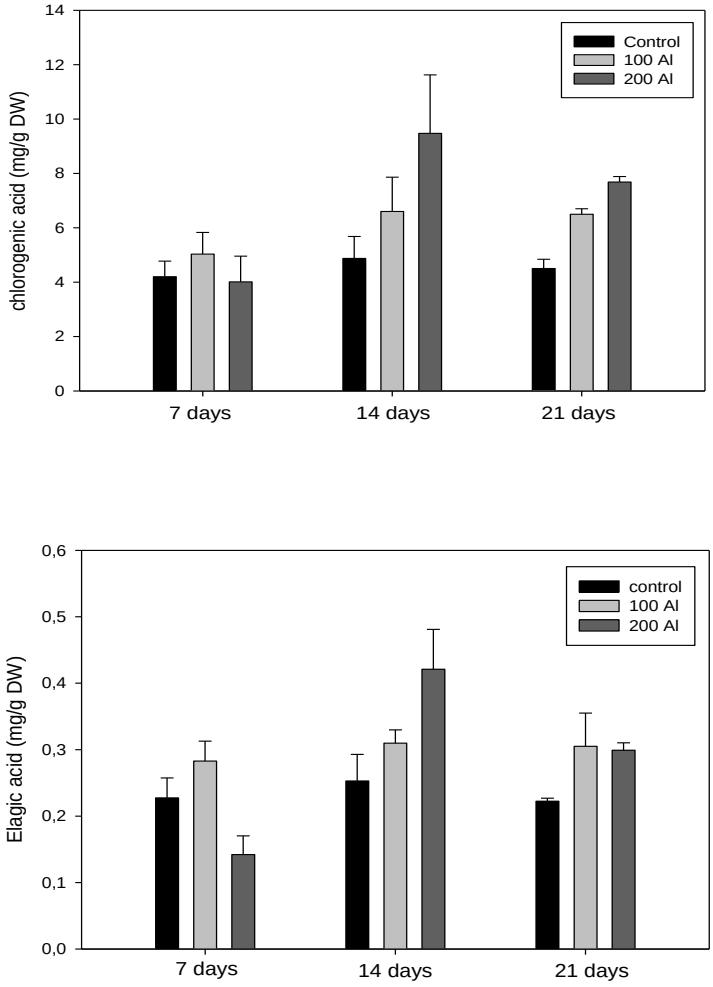

Figure 5. Variation in the content of chlorogenic acids (up) and ellagic acid (bottom), in function of exposure time and the concentration of Al in plantlets of *V. corymbosum*. Each value is a mean of three samples ± 1 s.d.

During the exposure period of blueberry plantlets to different doses of Al, a decrease in lipid peroxidation was observed, suggesting that plants were able to acclimate to the presence of Al. Studies by Reyes-Diaz *et al.*, (2010) showed an increase in the content of MDA in two blueberry varieties cv. Legacy and Bluegold growing in Hogland solution modified with Al, concluding that cv. Legacy is Al-tolerant. Our results allow us to suggest

that Al tolerance mechanisms reported by Reyes-Diaz *et al.*, (2010), for this variety of blueberry, mainly be triggered by exposure time.

The ability of a plant to enhance its capacity to remove ROS is a key factor in the mechanism of oxidative stress tolerance. It was observed that the antioxidant capacity of blueberry plantlets varied according to the Al treatment applied. The increase in antioxidant capacity of the extracts on DPPH and FRAP assays could be due to the increase in the total content of phenolic compounds. The smaller effect observed in seedlings treated with 100 µM Al, suggests that the non-enzymatic antioxidant defense mechanism is differentially activated according to Al concentration and exposure time to this element, which could be an indication that the effective antioxidant answer occur at high concentrations of Al, as was the case of 200 µM Al treatment.

By using HPLC it was possible to identify four phenolic compounds, syringic acid, chlorogenic acid, ellagic acid and rutin. We observed a significant increase in the content of chlorogenic acid at 14 days of treatment with 200 µM Al. Similar results were found by Wang *et al.,* 2009, who describe an increase in the content of chlorogenic acid in blueberry cultures exposed to UV-C radiation. The ellagic acid content increased in both treatments, but the greatest accumulation of this compound was observed at 200 µM Al. The antioxidant capacity of a molecule is reflected in the IC 50 value corresponding to the concentration of compound needed to consume 50 % of the DPPH radical. The IC 50 value for the chlorogenic acid in this study was 4.2 µg, while for the ellagic acid was 11.1 µg, indicating that the chlorogenic acid has a greater ability to remove radicals (data not shown), then, the observed variations in the antioxidant capacity of blueberry plantlets would be attributed mainly to the accumulation of chlorogenic acid (Zheng *et al.*, 2003).

In conclusion, the application of different concentrations of Al, to culture medium of blueberry produced significant effects on the content of phenolic compounds, with a greater response to a higher concentration of the metal in the culture media. Due to the importance of the levels of chlorogenic and ellagic acid in blueberry, it is very important to know factors that regulate its content, as a way to produce fruits with high antioxidant capacity. Then, soils with high levels of aluminum would help to increase the content of antioxidants in blueberry.

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Chapter 3

Effect of the availability of Cd^{2+} on the non-enzymatic antioxidant response and the phenolic compounds profile of blueberry (*Vaccinium corymbosum* L.) plantlets cultivated *in vitro*.

[†]*The chapter will be subbmited to Molecules (ISI).*

Abstract

The presence of trace elements in the soil at levels that can cause toxicity in plants is an important problem in agriculture. One of the main elements that affect the soil-plant system is cadmium, due to its great mobility in the system, in addition to the fact that it is not an essential element for the plants. With the purpose of evaluating the effects of Cd^{2+} on antioxidant response and phenolic compounds production, blueberry (*Vaccinium corymbosum* L.) plantlets were cultivated *in vitro* and exposed to 50 and 100 µM Cd^{2+} concentrations in the growth medium during periods of 7, 14 and 21 days. A significant effect was seen on the antioxidant response by means of the FRAP test, in addition to an increase in the content of MDA, showing that the Cd^{2+} present in the medium caused oxidative damage.

The profile of phenolic compounds in the treated plantlets was established by LC-MS. The main phenolic compound found in the blueberry plantlets was chlorogenic acid, whose abundance in the tissues increased due to the addition of Cd^{2+} in the medium. The presence of lower abundance of vanillic acid and quercetin was also detected. These results show that the presence of Cd^{2+} in the culture medium results in changes of phenolic compounds profile.

Key words: *Vaccinium corymbosum*, cadmium, phenolic compounds, oxidative stress.

Introduction

Mining and agriculture are important sources of incorporation of trace elements (TEs) in the environment. Mining, on the one hand, through the residues generated by its extractive and purification processes, and agriculture on the other, through the constant

application of fertilizers, biosolids and amendments, seriously modify the ecosystem's biogeochemical cycles (Basu *et al.*, 2006; Molina *et al.*, 2010; Wu *et al.*, 2010).

Among the TEs that affect the soil and water, cadmium (Cd) is often mentioned because of its high toxicity and mobility in the soil/plant system (Sanita and Gabbrielli, 1999; Hassan and Aarts, 2011). The World Health Organization (WHO) has established that the maximum concentration of this element in water for human consumption must not exceed 0.003 $mg \cdot L^{-1}$ (WHO, 2013). However, industrial and agricultural activities have enriched the soils and water courses with this element (Molina *et al.*, 2009; Lux *et al.*, 2011). In Chile, Bonomelli *et al.* (2002) analyzed the presence of Cd in phosphate fertilizers imported to Chile during 1999-2000, and found a high Cd content in fertilizers of the Triple superphosphate (TSP). Similar results were reported by Molina et al., concluding that the use of phosphate fertilizers over long periods could increase Cd levels in agricultural soils and plants uptake (Molina *et al.*, 2009; 2010, 2013).

In contrast with other TEs (e.g. Cu, Zn, Mn), Cd is not an essential element for plant growth and development, and it is considered highly toxic to them, even at trace levels (Das *et al.*, 1997; Rascio and Navari-Izzo, 2011). The symptoms associated with high Cd concentrations in plants are biomass reduction, inhibition of root elongation, in addition to perturbations in the capture and distribution of nutrients (Milone *et al.*, 2003; Chamseddine *et al.*, 2009).

It has been reported that the presence of Cd in plants generates, at the cellular level, reactive oxygen species (ROS), which are responsible of membrane damages and degradation of proteins and nucleic acids (Polge *et al.*, 2009). Depending on its concentration, Cd can inhibit or stimulate the activity of various antioxidant enzymes before the symptoms of toxicity become visible in the plant (Fu and Huang, 2001; Liu *et*

al., 2007). The oxidative stress produced by Cd has been observed as an increase of the lipid peroxidation products measured from the accumulation in the tissues of malondialdehyde (MDA), which is a by-product of the oxidation of the polyunsaturated fatty acids of the membranes, changes in the activity of antioxidant enzymes like superoxide dismutase or ascorbate peroxidase, in addition to the degradation of chlorophyl (Djebali *et al.*, 2005; Gratao *et al.*, 2008; Fidalgo *et al.*, 2011). It has been reported that plants have different mechanisms to tolerate the presence of Cd, which include the metal's exclusion from the roots and retention of the metal in the vacuole through complexes formed with organic ligands and detoxification processes (Clements, 2006; Hédiji *et al.*, 2010).

Other studies indicate that the plants have strategies that allow them to control ROS, one of which is based on the production of secondary metabolites with antioxidant properties (Mourato *et al.*, 2012). The secondary metabolites are synthesized by the plant as a defense mechanism against biotic stress, but they may also play a role in the plant's physiological functioning when it is in the presence of a high concentration of heavy metals (Close and McArthur, 2002; Fernández *et al.*, 2013).

Participation of antioxidant molecules as a response of the plant to intoxication by Cd may be a defense mechanism to the stress caused by the metal, but there are no important reports on the effects caused by changes of the levels and profiles of antioxidant molecules.

Blueberries (*V. corymbosum* L.) have become important in human nutrition in recent years, due to the high content of antioxidant molecules in their fruits and leaves (Giovanelli and Buratti, 2009; You *et al.*, 2011). In Chile the cultivation of blueberries occurs mainly in the central and southern zones (33° to 41° south latitude), developing well

in acid soils with pH between 4.8 and 5.5. Because this species has positioned itself in recent years in world agriculture, it is important to study the effect of its exposure to high Cd concentrations, managing the conditions of the plant's growth medium.

The objective of this work was to evaluate the effects on the antioxidant capacity and the profile of phenolic compounds, in the early growth stages, and the generation of MDA as an indicator of the oxidative damage in blueberry plantlets cultivated *in vitro* exposed to Cd in the nutritive solution.

2. MATERIALS AND METHODS

2.1 Plant material, growth conditions and treatments

To start the *in vitro* culture, Blueberry plantlets were obtained through the sterilized foliar segments replication (2 cm long) of two months of growth pre-cultivated plantlets. 25 mL of Woody Plant culture medium (Lloyd and McCown, 1980) were added to each flask of culture (6 cm in diameter, 10 cm high and 197.82 cm^3) containing the following components: ($mg \cdot L^{-1}$): NH_4NO_3 400 mg, $CaCl_2 \cdot 2H_2O$ 96 mg, $MgSO_4 \cdot 7H_2O$ 370 mg, K_2SO_4 990 mg, KH_2PO_4 170 mg, $Ca(NO_3)_2 \cdot 4H_2O$ 556 mg, H_3BO_3 6.2 mg, $Na_2EDTA \cdot 2H_2O$ 37.2 mg, $CuSO_4 \cdot 5\ H_2O$ 0.25 mg, $FeSO_4 \cdot 7\ H_2O$ 27.8 mg, $MnSO_4 \cdot 4\ H_2O$ 22.3 mg, $ZnSO_4 \cdot 7\ H_2O$ 8.6 mg, $Na_2MoO_4 \cdot 2\ H_2O$ 0.25 mg. The culture medium was supplemented with 2.76 $mg \cdot L^{-1}$ of 2-iP hormone (2-isopentenyl adenine), sucrose was used as carbon source (15 $g \cdot L^1$) and as a gelling agent agar Phytagel (3 $g \cdot L^{-1}$). The pH was adjusted to 5.2. The culture medium was sterilized in an autoclave for 15 minutes at 121 °C (394 K). Cultures were maintained in cultivation cabinet at 21±2 °C (294±2 K) and photoperiod of 16 hours of light/8 hours of darkness. Processes of sterilization and explants sowing were conducted in a laminar flow cabinet in aseptic conditions. Once established *in vitro* culture (care and treatment), we

used a completely randomized design, where the experimental unit corresponded to 6 clones of plantlets of blueberries (6 plantlets: 1 sample), each sample was carried out in triplicate. *In vitro* cultivated plantlets with more than 30 days of adaptation to cultivation conditions were defined as starting control (time=0 days), and used to define the physiological base line for all the studied parameters.

To calculate the chemical speciation of Cd and Al in the culture medium of the plantlets, the software GEOCHEM PC was used (Parker *et al.,* 1995).

2.2. Extracts preparation.

Fresh materials ($0.1 g \cdot mL^{-1}$) are used to prepare the extract using 85% v/v of hydroethanolic solution; the samples were sonicated at 50-60 Hz of frequency during two hours at 25±2 ºC (298±2 K) according to the method described by Adam (2009).

2.3. Antioxidant activity

2.3.1. 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenger spectrophotometric assay.

The free radicals removal capacity of each extract was evaluated using the radical DPPH technique, described by Brand-Williams *et al.* (1995) with some modifications. Briefly, 20 µL of the ethanol extract was added to 980 µL of DPPH solution ($25 mg \cdot L^{-1}$), the absorbance was continuously monitored at 517 nm using a UV-visible spectrophotometer (Agilent 8453 UV-Vis), for 240 seconds (Brand-Williams *et al.,* 1995; Huang *et al.,* 2005). Results were expressed as % of consumed DPPH.

2.3.2. Ferric reducing antioxidant power (FRAP).

The FRAP assay measures the ability of the sample to reduce Fe^{3+} to Fe^{2+} (Benzie and Strain, 1996), followed by the absorbance at 593 nm of the blue complex formed with tripyridyltriazine (TPTZ). FRAP reagent was prepared by mixing acetate buffer (300 mM), TPTZ solution (10 mM in HCl) and $FeCl_3$ solution (20 mM) at a 10:1:1 ratio.

The FRAP reagent wasmaintained at 37±2º C (310±2 K). Absorbance of samples containing 900 µL of FRAP reagent, 80 µL of water and 20 µL of ethanolic extract, was measured at 593 nm in an Agilent 8453 UV-Vis spectrophotometer. Results were expressed in ascorbic acid equivalents.

2.4. Total phenolic content (TPC)

The TPC of ethanolic extracts was determined through the method described by Singleton and Rossi (1965). Results were expressed as mg Gallic acid equivalents.

2.5 Analysis of extracts by Liquid Chromatography-Triple Quadrupole Mass Spectrometry (LC-MS).

Extracts were analyzed using a 1120 Compact LC-MS, Agilent Technologies (Santa Clara, Ca). LC was in-line with a 6400 Triple quadrupole (Agilent Technologies) with electrospray (ESI). Compounds were separated using a SUPELCOSIL LC-PAH RP-C18 column (15 cm x 4.6 mm; particle size 5µm) and a mobile phase of acetonitrile as eluent A and 0.1% formic acid as eluent B. All solutions were filtered through a 0.45 µm filter and degassed for 15 min. Elution was performed using a linear gradient from 10:90 (A: B) to 70:30 over 20 min at a flow rate of 0.3 $ml \cdot min^{-1}$. For analysis of the phenolic compounds

the MS was operated in negative ion mode, capillary temperature of 200ºC (473 K), spray voltage 4 kV, and data were acquired in MS and MS/MS scanning modes.

The multiple reactions monitoring method (MRM) was used for phenolic compounds analysis; the method involves a phenolic compounds data base considering their corresponding molecular ions and precursors.

2.6. Lipid peroxidation

The lipid peroxidation level was determined in terms of MDA concentration, according to the method of Heath and Parker (1968) with modifications. The concentration of MDA was calculated from the difference of the absorbance at 532 and 600 nm using the extinction coefficient of 155 $mmol \cdot L^{-1}$ cm and expressed as $nmol \cdot g^{-1}$ FW.

2.7. Statistics analysis.

All data shown in the study are mean ± sd. The measurement was performed in sextuple for all measured parameters. Statistical analysis was performed by two-way analysis of variance (Two-way ANOVA) and comparisons between culture medium were performed using the Tukey Averages Multiple Comparison Test.

3. RESULTS AND DISCUSSION

To achieve the objectives of this study and evaluate the behavior of blueberry plantlets when exposed to Cd, the study parameters considered were MDA content, the response mechanisms to oxidative stress estimated from the determination of the antioxidant capacity (DPPH and FRAP), and the production of phenolic compounds determined by LC-MS.

3.1 Cd-induced changes in malondialdehyde (MDA) content

The effects of oxidative stress caused by the presence of Cd in the growth medium were determined by the accumulation of MDA in the plants' tissues.

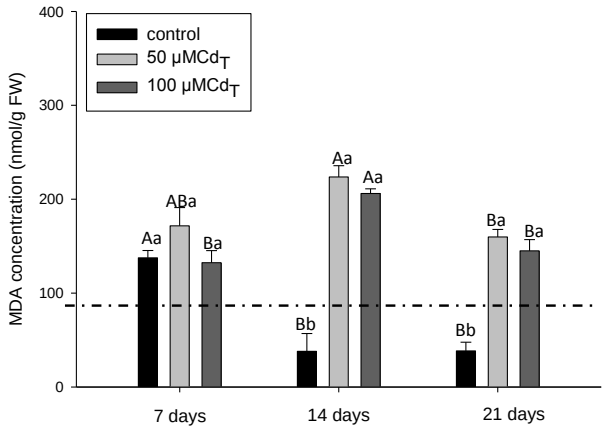

Fig. 1. Effect of Cd concentration on oxidative stress in blueberries measured as MDA. Data are mean ± sd; n = 6. Different letters (lower case) indicate statistical differences among means from treatments and time after ANOVA and Tukey HSD post hoc test ($P< 0.05$). The dashed line indicates the level of MDA measured for control when initiating treatment.

Figure 1 shows the results of the Cd treatments and the control at the different treatment times. It is seen that the control shows a decrease of MDA content with time. This behavior suggests that during the first 7 days of treatment the blueberry plantlets present a degree of stress because of the change to the *in vitro* culture medium, although no significant variation between treatments ($p < 0.05$). After the acclimation period, MDA content decreases, staying stable during the rest of the study.

In the case of plantlets exposed to Cd, an effect of the presence of the metal on the MDA content is seen after 14 days of exposure, with MDA levels higher than those of the control (Fig. 1) ($p < 0.05$). The presence of Cd in the growth medium seems to be more important than its concentration in the response of blueberry plantlets in the early stages of Cd exposure (14 days), since no significant differences were found between the treatments ($p < 0.05$). At longer exposure times (21 days), the MDA content decreases in the plantlets exposed to Cd, but it remains significantly higher than in the control ($p < 0.05$). This behavior agrees with the observations of Ge et al. (2012), and Chaoui et al. (1997), who found that MDA content reaches its peak after 2 week of exposure to Cd in *popolus nigra* and *Phaseolus vulgaris*. Another effect reported in the literature and observed in our study is that MDA content significantly decreases as exposure time and Cd concentration increase, indicating that blueberries develop better defense mechanisms against lipid peroxidation at higher Cd concentrations (Metwally *et al.,* 2005; Hegedus *et al.,* 2001, Liu *et al.,* 2011) These results suggest that the action of these enzymatic and non-enzymatic mechanisms strongly depends on two factors: i) exposure time (early stages) and ii) initial total Cd concentration (Cd_T) added to the culture medium (late stages), with a differentiated response at oxidative damage level. In this context it has been reported that the MDA content in the plant's tissues due to the presence of Cd in the growth medium depends on both the genotype and the mechanisms presented by the species to defend itself from the stress (Metwally *et al.,* 2005; Saidi *et al.,* 2013; Fernández *et al.,* 2013).

3.2 Antioxidant activity

The antioxidant activity of blueberries ethanol extracts was measured by the DPPH and FRAP tests, which evaluate the ability to trap the diphenylpicrylhydrazyl free radical (DPPH) and to reduce Fe^{3+} to Fe^{2+}, respectively.

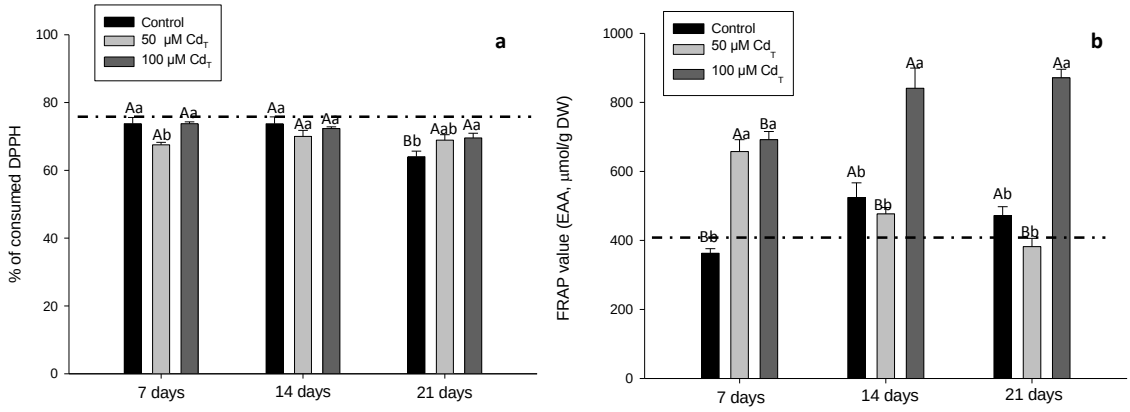

Fig. 2. Effect of Cd concentration on antioxidant activity of ethanolic extracts. (a) DPPH free radical scavenging (DPPH consumed), (b) FRAP test (ascorbic acid equivalents). Data are mean ± sd; n = 6. Different letters (lower case) indicate statistical differences among means from treatments and time after ANOVA and Tukey HSD post hoc test ($P< 0.05$). The dashed lines indicate the level of % DPPH and FRAP value respectivily measured for control when initiating treatment.

The results of the DPPH test only show changes of the antioxidant capacity with respect to the control after 7 days for 50 µM Cd_T ($p < 0.05$), where a decrease of the antioxidant capacity is seen by DPPH, and after 21 days for the 100 µM Cd_T treatment, with respect to the control, with a slight increase of the antioxidant capacity of the extract. No significant differences were found after 14 days of treatment ($p > 0.05$) for 50 and 100 µM Cd_T (Fig. 2a). These results show that the ethanol extracts have a high capacity for

trapping free radicals, and this is not largely affected by the presence and concentration of Cd.

The FRAP test showed significant differences between treatments; the reducing capacity significantly increases in the presence of Cd during the first 7 days ($p < 0.05$). As the study period got longer, the response to the oxidative damage generated by the presence of Cd is now associated to the metal's concentration. Both, the control as well as the samples exposed to 50 µM Cd_T, did not show significant differences between them ($p > 0.05$), while in the seedlings subjected to 100 µM Cd_T, the antioxidant capacity measured by the FRAP test increased 1.8 and 2.0 times after 14 and 21 days of exposure with respect to the control and the treatment with 50 µM Cd_T (Fig. 2b) ($p < 0.05$). These results suggest that Cd induces the formation of compounds with reducing ability, as a possible defense mechanism. Different studies indicate that this parameter is sensitive to the presence of Cd, with positive and negative relations in terms of exposure time and metal concentration, showing that reducing ability depends on the variety of plants studied (Das *et al.,* 1997; Cherif *et al.,* 2011; Douchiche *et al.,* 2012; Panitlertumpai *et al.,* 2013).

3.3 Total phenolic content (TPC) and LC-MS analysis

TPC in each blueberry extract is shown in Fig. 3. In the control it remains without major variations during the whole exposure time ($p > 0.05$), under the treatment with 50 µM of Cd_T there is a maximum at 7 days (113.87 $mg \cdot g^{-1}$ DW), and under the treatment with 100 µM of Cd_T the TPC exceeds the control during the whole exposure time. The response seen in TPC in the blueberry extracts, under both treatments, may indicate more than one antioxidant response mechanism. One occurs when a threshold concentration is

exceeded, in this case at 100 µM Cd_T in the growth medium, but there may also be a fast response to the presence of the metal that was seen at 7 days of exposure at both concentrations, in addition to a response over time (Fig. 3) Results shown that TPC decreases at 14 days for both treatments and increases at 21 days of treatment, suggesting that synthesis mechanism of phenolic compounds is reduced at 14 days due to their high production during the first days of treatment (early response), while at 21 days of exposure the blueberry again shows an increase of the synthesis of these metabolites in both treatments ($p < 0.05$).

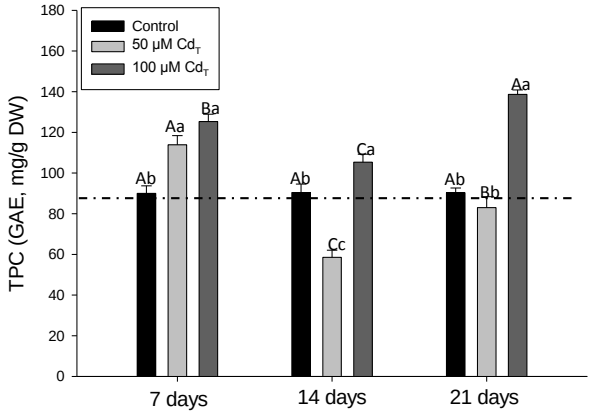

Fig. 3. Effect of Cd concentration on TPC variation in Vaccinium corymbosum L. Values are mean ± standard deviation; n = 6. Different letters (lower case) indicate statistical differences among means from treatments and time after ANOVA and Tukey HSD post hoc test ($P< 0.05$). The dashed line indicates the level of TPC measured for control when initiating treatment.

The LC-MS analysis allowed the identification of 16 different phenolic compounds (Table 1). The MRM method is extensively used as an analytical tool in these studies (Soong and Barlow, 2005; Romani *et al.*, 2006; Serrano *et al.*, 2013). The relative

abundance of the identified phenolic compounds changes with the presence of and the exposure time to Cd in the growth medium. (Table 1).

Table 1. Phenolic compounds in ethanolic extracts for control and Cd exposed blueberry plantlets.

Phenolic Compound	Molecular Formula	Molecular Weight	t_R (min)	m/z ES (-)	Main fragment ion (M-H -X)	Control			50 µM Cd_T			100 µM Cd_T		
						7	14	21	7	14	21	7	14	21
						Days								
						Relative abundance ((M^-) *m/z*)								
Gallic acid	$C_7H_6O_5$	170	1.25	169	125 [(M-H)-CO_2]	0.25	0.23	0.27	-	-	-	0.08	0.15	0.17
Vaillinic acid	$C_8H_8O_4$	168	1.25	167	123 [(M-H)- CO_2]	-	-	-	0.69	0.44	0.65	0.56	0.53	0.15
Caffeic acid hexoside	$C_{15}H_{18}O_9$	342	1.53	341	179[(M-H)-$C_6H_{10}O_5$]	1.52	1.58	1.45	-	-	-	1.12	1.55	0.87
Chlorogenic acid	$C_{15}H_{18}O_9$	354	3.91	353	191[(M-H)-$C_7H_{11}O_6$]	85.5	82.8	85.12	83.6	82.55	83.1	96.3	92.78	95.75
p-Coumaric acid	$C_9H_8O_3$	164	3.97	163	119[(M-H)- CO_2]	0.77	0.81	0.74	1.41	0.72	0.67	0.35	0.56	0.87
4-Hydroxybenzoic acid	$C_7H_6O_3$	138	4.23	137	93 [(M-H)- CO_2]	0.46	0.48	0.44	0.33	0.24	0.28	0.3	0.39	0.14
Quercetin-3-O-hexoside	$C_{21}H_{20}O_{12}$	464	4.83	463	301[(M-H)-$C_6H_{10}O_5$]	5.86	6.11	5.61	4.63	7.02	6.96	0.35	1.51	0.49
Hesperidin	$C_{28}H_{34}O_{15}$	610	5.00	609	301[(M-H)-$C_{12}H_{20}$ O_9]	2.32	2.42	2.22	4.97	5.16	4.49	0.17	0.77	0.19
Sinapic acid	$C_{11}H_{12}O_5$	224	6.80	223	179 [(M-H)- CO_2]	0.24	0.25	0.23	-	-	-	0.48	0.70	0.50
Ferulic acid	$C_{10}H_{10}O_4$	194	8.98	193	178 [(M-H)-CH_3]	0.15	0.15	0.14	0.27	0.09	0.14	-	-	-
Phloridzin	$C_{21}H_{24}O_{10}$	436	9.38	435	273 [(M-H)-$C_6H_{10}O_5$]	0.45	0.47	0.43	0.72	0.32	0.35	-	-	-
Quercetin	$C_{15}H_{10}O_7$	302	16.84	301	151 [(M-H)-$C_8H_6O_3$]	0.37	0.38	0.35	0.99	0.40	0.42	-	-	-
Hydroxybenzoic acid-O-hexoside	$C_{13}H_{15}O_8$	300	22.64	299	137 [(M-H)-$C_6H_{10}O_5$]	0.46	0.48	0.44	1.03	0.60	0.58	0.08	0.77	0.50
Methoxy carnosol	$C_{21}H_{28}O_5$	360	22.70	359	329 [(M-H)-CH_2O]	0.15	0.15	0.14	0.32	0.22	0.20	-	-	-
Luteolin	$C_{15}H_{10}O_6$	286	22.95	285	267[(M-H)-H_2O]	-	-	-	-	-	-	0.21	0.31	0.36
Catechin	$C_{15}H_{14}O_6$	290	27.00	289	109 [(M-H)-$C_9H_8O_4$]	2.53	2.64	2.42	1.03	2.23	2.16	-	-	-

For a better discussion of the effects of the presence of Cd on the production of phenolic compounds, they were divided into three groups according to their presence and relative abundance when compared to the control.

- **Group I:** Phenolic compounds whose relative abundance increases: p-coumaric acid, quercetin-3-O-hexoside, hesperidin, and hydroxybenzoic acid-O-hexoside, and chlorogenic acid, which is the most representative compound found in the blueberry plantlets (Prior *et al.*, 2001; You *et al.*, 2011).
- **Group II:** Phenolic compounds absent in the control but detected in one of the treatments: vanillic acid and luteolin.
- **Group III:** Compounds absent in one or both treatments (50 µM Cd_T and/or 100 µM Cd_T) but present in the control: gallic, ferulic, and sinapic acids, phloridzin, quercetin, methoxycarnosol, catechin, and caffeic acid hexoside**.**

In this context the relative abundance increasing of chlorogenic acid (Group I) allows to state that Cd increases its production (100 µM Cd_T), understanding that the function of chlorogenic acid is a protective response to the oxidative damage caused by the metal. Similar behaviors have been reported in *Matricaria chamomilla* treated with Cu (Kovacik and Klejdus, 2008), while in *Brassica juncea* chlorogenic acid decreases when it is exposed to Cd (Irtelli and Navari-Izzo, 2006). It was found that in blueberry plantlets Cd induces the biosynthesis of some phenolic compounds (Group II), mainly vanillic acid, which has antioxidant characteristics, although in a lower degree than chlorogenic acid. The results indicate that the presence of Cd^{2+} modifies the phenolic compound profile in blueberries. The absence of some of them (Group III) like gallic and ferulic acids, which

are known as antioxidants (Kanski *et al.*, 2002; Irtelli and Navari-Izzo, 2006), may be related to processes of consumption or inhibition of their synthetic route.

The biosynthesis of phenolic compounds in blueberries is conditioned by the exposure time to Cd and its presence in the culture medium.

The profile variation of phenolic metabolites may be a defense mechanism against induced stress, observing that during the study time the control treatment showed no significant variations in the relative abundance of detected compounds (Table 1). It cannot be established whether the presence of Cd in the metabolism of blueberries affects the different biosynthetic routes of these phenolic compounds, such as, for example, those produced as derivatives of cinnamic or benzoic acids.

3.5 Correlation between antioxidant response parameters and the content of MDA

Possible correlations between oxidative stress (MDA) and antioxidant response (FRAP, DPPH and TPC) generated by Cd exposure were evaluated (Fig. 4). From these results, it is possible to establish that the presence of Cd in the culture medium affected the MDA content and the cranberry seedlings antioxidant response.

In general the lipid peroxidation measured by changes in the content of MDA significantly correlated with antioxidant response measured by the FRAP assay ($p < 0.05$), while MDA and DPPH no significant relationship between the two variables exists ($p > 0.05$). The total phenolic content (TPC) showed a negative correlation with MDA ($p < 0.05$), whereas the values obtained from the FRAP assay, in this parameter a high correlation and significance ($p > 0.05$) was observed, which suggests that biosynthesized

phenolic compounds in studies conditions play an important role in cranberry seedlings protection with the presence of Cd in the culture medium.

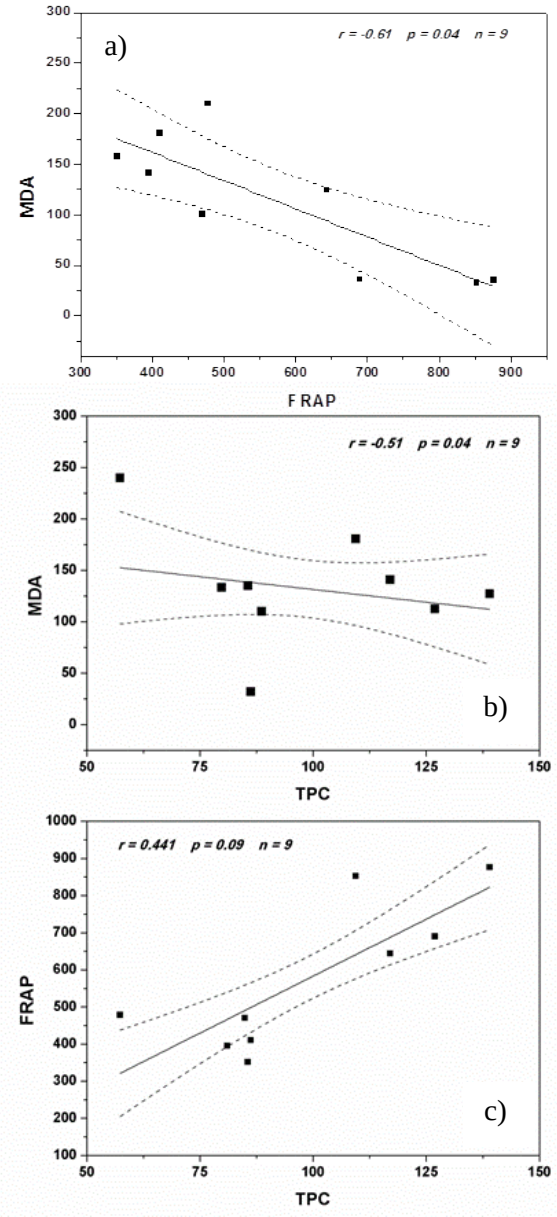

Fig. 4. Relationship between the lipid peroxidation and FRAP (a), MDA and TPC (b) and FRAP and TPC (c). Pearson's coefficients are indicated.

3.4 Chemical speciation of Lloyd-McCown nutrient medium

Every culture medium, because of its composition, has a high complexing capacity, and therefore it tends to modify significantly the distribution of the chemical forms in which the compounds present are found. To know the actual form in which the Cd added to the nutrient medium is found it is necessary to know its chemical speciation (Table 2), for which the GEOCHEM-PC program (Parker *et al.,* 1995) was used. From those results, the concentration of free Cd^{2+} was 1.168 and 16.88 µM (for a total supplied concentration of 50 and 100 µM), while it shows 0.5889 and 8.475 µM Cd activity.

Table 2. Percent distribution of Cd in the Lloyd-McCown medium culture, for a concentration of 50 and 100 µM of total Cd (Cd_T) added (Cd-anion means Cd complexed by the corresponding anion, Parker *et al*, 1995)

Species	**50 µM Cd_T**		**100 µM Cd_T**	
	Concentration µM	**Activity µM**	**Concentration µM**	**Activity µM**
Free Cd^{2+}	1.17	0.59	16.88	8.48
Cd-SO_4^{2-}	0.56	0.57	8.13	8.23
Cd-EDTA	47.86	24.1	74.13	36.7
Cd-Cl^-	0.08	0.07	1.29	1.07
Cd-NO_3^-	0.01	0.01	0.17	0.14
Cd-PO_4^{3-}	0.01	0.01	0.14	0.15

It was determined that the cadmium is found mostly complexed by EDTA and SO_4^{2-} achieving around 80% - 95% formation of highly stable complexes. Through the

GEOCHEM-PC program, no formation of any complex in which Cd and other cations present in the Lloyd-McCown solution participate simultaneously could be established. The symbol Cd_F was used to refer to the concentration of free Cd in the medium and its activity by Cd_A.

3.5 Evidence of the impact of Cd species on the response and damage in Vaccinium corymbosum L.

A more significant way of looking the changes associated with the total concentration of added Cd (Cd_T) and with the most significant chemical forms present in the medium (Cd_F and Cd_A) on the effects observed for blueberries. (TPC, FRAP, DPPH and MDA) is to represent the derivative in the plant's response (ΔTPC, ΔFRAP, ΔDPPH) and the damage (ΔMDA), respectively, as a function of Cd_T, Cd_F and Cd_A (Fig. 5).

Fig. 5 shows similar responses between antioxidant capacity and oxidative damage when the differences between them are analyzed *vs.* Cd_T and Cd_F, while Cd_A did not show the same behavior.Under any condition, the observed response is conditioned by the species or reagent that is in the lowest concentration. Therefore, from the results it is possible to establish that Cd_F is the Cd species responsible for its effects on blueberries, and not Cd_T, which can be explained by the size of the formation constant of the Cd-EDTA complex (K_f = $2.9x10^{16}$), Cd form which is not easily available to the plant, but represents anyway the most significant chemical form of Cd in the growth medium.

In general, the effect on the physiological parameters of blueberries will depend on the amount of Cd (or another metal) that is in its free form, and this is directly related with

the complexing agents available in the growth medium which, under natural conditions, will include the different organic compounds produced by blueberries exudation.

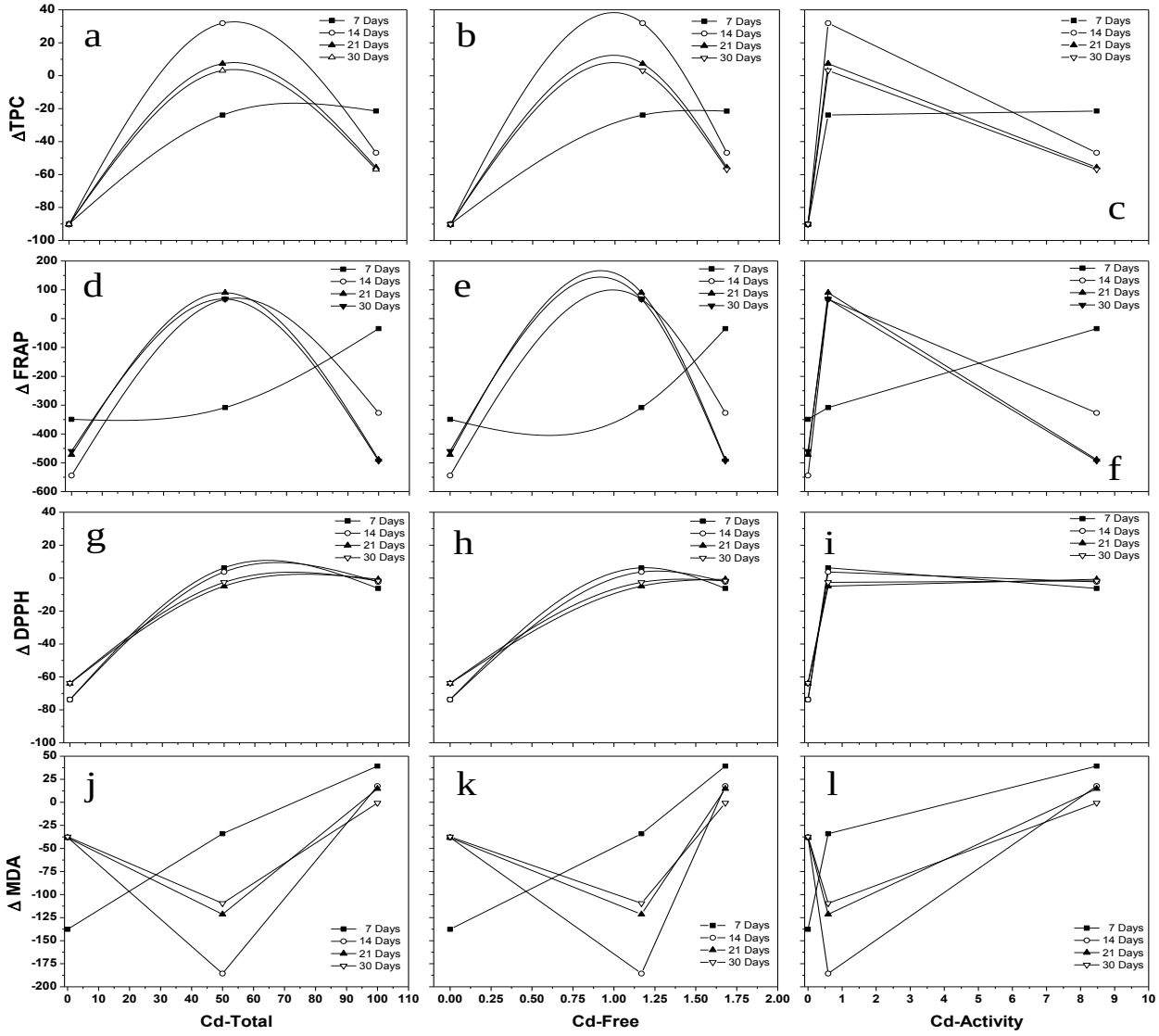

Fig. 5. Representation of derivative of the antioxidant response (ΔTPC, ΔFRAP, ΔDPPH) and the damage (ΔMDA) as a function of Cd in the growth medium.

CONCLUSIONS

The results obtained in this research suggest that phenolic compounds play an important role in response to the damage produced by the different species of Cd present in the growth medium, mainly free Cd^{3+}.

Chemical speciation allowed showing the importance of carrying out this analysis in multiple composition systems, like culture medium, in nutrient solutions and/or in *in vitro* cultivation, where complexing agents significantly reduce the free concentration of cationic elements in the system.

The presence of Cd to the growth medium resulted in a variation in the MDA content when control and the Cd treatments were compared. The greatest oxidative damage occurs at 14 days of exposure at 50 and 100 μM Cd_T. The response to oxidative damage, quantified by the DPPH and FRAP tests, suggest that one of the defense mechanisms used by the blueberry plantlets is the production of reducing molecules, and this would explain the high FRAP values, but this response was not directly proportional to the damage, showing an early response in both treatments at 7 days of exposure compared to the control, and this was not seen at 14 and 21 days for a 50 μM Cd_T concentration, where no important differences with respect to the control were observed. The high FRAP values seen in the blueberry seedlings treated with 100 μM Cd_T may correspond to a selective late response mechanism that is triggered at a higher concentration range, leading to the conclusion that blueberryhad two responses: a first one at 7 days due to the presence of the metal rather than to its concentration, and a second selective response to concentration at 14 and 21 days for 100 μM Cd_T, where the Cd_F concentration is 13 times greater than at 50 μM Cd_T.

The application of different Cd_T concentrations to the blueberry culture medium resulted in noticeable effects on the contents of phenolic compounds. The plant's response to the presence of the metal was different with respect to the concentration of Cd_T, showing a greater response at higher concentrations in the medium.

The Cd_F induces changes in the production of phenolic compounds, as shown by an increase of their proportion compared to the control medium. However, further research is proposed in this relation and concerning the presence of other metals, to see if there is synergism or antagonism with the plant's response in the production of phenolic compounds.

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Chapter 4

Oxidative stress and production of compounds of phenolic origin in blueberry plantlets cultivated in vitro under a combined cadmium and aluminum treatment

[†]The chapter will be subbmited to Environmental Experimental Botany (ISI).

ABSTRACT

Blueberries are a widely spread crop around the world, which develops in acid soil where free Al (Al^{3+}) can be found at phytotoxic levels. Their production requires constant application of fertilizers that have significant concentrations of cadmium (Cd), an element which under those pH conditions can be very highly bioavailable to the blueberries. In the present study the effect of Al and Cd combined at different concentrations on oxidative stress, antioxidant capacity, and concentration of phenolic compounds in blueberry seedlings cultivated *in vitro* during four weeks was evaluated. The results showed that the application of binary (Al + Cd) systems caused increased oxidative stress, quantified by the accumulation of MDA, with the greatest damage seen at three weeks of exposure. In the same time period an increase of the antioxidant capacity of blueberries was seen, the same as of the total concentration of phenolic compounds. The results of the HPLC study of the extracts showed increased chlorogenic and elagic acid content with all the treatments applied, suggesting that these phenolic compounds play an important role in the antioxidant response of blueberries. Our results suggest that the addition of metals to the growth medium produce changes of the profile and concentration of phenolic compounds, which play an important rolein the response of blueberries to stress by metals.

Introduction

Soil is the most important factor in the plant's growth and development, depending on its origin and physical, chemical and biological characteristics, and it conditions the availability and concentration of nutrients as well as contaminants, largely governing the

nutrient flow in the plant.(Yamamoto *et al.*, 2002; Boscolo *et al.*, 2003; Corrales *et al.*, 2008).

Soils of acid origin are found throughout the world, and it is estimated that 40% of the world's soils are used for agriculture and 12% of these have pH below 5.5. The soils in the center south zone of Chile are of volcanic origin, and they are characterized by having a high capacity to retain chemical elements of agricultural and environmental importance (nutrients, trace elements, pesticides). These kinds of soils have pH below 6.0, in addition to a low concentration of exchange bases (Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}).

Soil acidity is accelerated by the abundant rainfall in the zone and the use of acidic fertilizers (urea, ammonium phosphate), with the consequent high activity of aluminum, which can reach phytotoxic levels.

Toxicity by Al^{3+} limits the productivity of crops in acid soil, and it has been found that micromolar concentrations in the soil solution can rapidly inhibit the elongation of the root and later the absorption of water and nutrients (von Uexküll and Mutert, 1995; Giannakoula *et al.,* 2010). Research has shown that the stress generated by Al^{3+} increases the production of reactive oxygen species (ROS) that affect the physiological functioning of the plants and can even induce cell death(Yamamoto *et al.*, 2002; Boscolo *et al.*, 2003; Corrales *et al.,* 2008, Bontempo *et al.,* 2013, Feng *et al.,* 2013). The mechanisms of Al^{3+} toxicity in plants has not been completely elucidated, but it is estimated that the Al^{3+}induces oxidative stress because of its high affinity for ligands like phosphate and carboxyl groups that have donor oxygen atoms, bonding easily to the phospholipidic membrane and making it more rigid (Devi *et al,* 2003; Ma *et al,* 2001; Ryan *et al,* 2001). There are plants that have developed tolerance mechanisms to Al^{3+}, both internally and

externally, allowing them to develop in acid soil (Kochian *et al.*, 2005; Giannakoula *et al.*, 2010; Poschenrieder *et al.*, 2008). Among the tolerance to Al^{3+} mechanisms there is exudation of short chain organic acids from the roots of the plants, which is an Al^{3+} exclusion mechanism, in the soil solution before it enters the root (Pineros *et al.*, 2008; Liu *et al.*, 2009). However, the production of compounds with a phenolic origin has also been studied trying to help in the tolerance to Al^{3+}, stating the hypothesis that phenolic compounds play a role in the detoxification as stable complexing agents of the Al present in the plants (Tolrá *et al.*, 2005).

The application of fertilizers as sources of nitrogen, phosphorus and potassium, among which the most widely used are urea, triple superphosphate (TSP), and mono- and diammonium phosphate,represents also an important source of trace elements like Zn, Cd, Pb, Cr, Cu, and Ni (Chang and Page, 2000; Nicholson *et al.*, 2003; Molina *et al.*, 2009)
Among the elements that affect soil and water, cadmium (Cd) is often mentioned because of its high toxicity and its mobility in the soil/plant system (Sanita and Gabbrielli, 1999). Industrial activities, and agriculture through the application of phosphate fertilizers and biosolids, have enriched the soil with this element (Zheljazkov *et al.*, 2005).

Exposure of plants to cadmium induces various symptoms of phytotoxicity, such as chlorosis, biomass reduction, inhibition of root elongation until cell death occurs (Milone *et al.*, 2003). It has been found that the presence of Cd generates free radicals that harm the plants' tissues, and depending on its concentration and the kind of plant that is exposed to cadmium, it can inhibit or stimulate the activity of various antioxidant enzymes before the toxicity symptoms become visible (Fu and Huang, 2001; Liu *et al.*, 2007). Another effect

of the presence of cadmium is related to perturbations of the capture and distribution of nutrients in the plants (Sandalio *et al.,* 2001).

It has been described that plants have strategies that allow them to control reactive oxygen species. One of these strategies is based on the production of secondary metabolites with antioxidant properties which are synthesized by the plant as a method of defense against biotic stress, but it is possible that they also play a role in the physiological functioning of the plant when it is in the presenceof a high concentration of heavy metals (Close and McArthur, 2002).

Studies made with blueberry plantlets cultivated *in vitro* showed higher concentrations of phenolic compounds, mainly chlorogenic acid, when they were subjected to different concentrations of Al^{3+} (Manquián *et al.,* 2013). On the other hand, studies made with blueberry plants subjected to the presence of Cd^{2+} in the growth medium have shown that this element causes damage by oxidative stress which has been quantified by means of the increased content of malondialdehyde (MDA), which is a by-product of the degradation by oxidation of the polyunsaturated fatty acids in the membranes, and it has also been shown that the presence of Cd causes a change in the profile of phenolic compounds generated by the plant (Ge *et al.,* 2012, Guo *et al.,* 2007, Irtelli and Navari-Izzo, 2006). However, most of the research is centered on the physiological effects of the plants when exposed to heavy metals, and there are not many studies that evaluate the effect of the metals on the production of phenolic compounds and the generated oxidative stress.

Therefore, it is important to carry out a study to evaluate the effects of exposure to both metals when they are present in the growth medium on the production of phenolic compounds and their implicationin oxidative stress. For that purpose, blueberry plantlets

cultivated *in vitro* were exposed to the combined action of Al and Cd to determine the effects of the presence of both metals on the damage by oxidative stress, on the plant's response by means of its antioxidant activity, and on the concentration of phenolic compounds as a consequence of their presence in the growth medium.

2. MATERIALS AND METHODS

2.1 Plant material, growth conditions and treatments

To start the in vitro culture, blueberry plantlets were obtained by replication of sterilized leaf segments (2 cm long) from two-month old precultivated plantlets. To each culture flask (6 cm diameter, 10 cm tall, and 200 cm^3) was added 25 mL of Woody Plant culture medium (Lloyd and McCown, 1980) which contains the following components ($mg \cdot L^{-1}$): NH_4NO_3 400, $CaCl_2 \cdot 2H_2O$ 96, $MgSO_4 \cdot 7H_2O$, K_2SO_4 990, KH_2PO_4 170, $Ca(NO_3)_2 \cdot 4H_2O$ 556, H_3BO_3 6.2, $Na_2EDTA \cdot 2H_2O$ 37.2, $CuSO_4 \cdot 5H_2O$ 0.25, $FeSO_4 \cdot 7H_2O$ 27.8, $MnSO_4 \cdot 4H_2O$ 22.3, $ZnSO_4 \cdot 7H_2O$ 8.6, $Na_2MoO_4 \cdot 2H_2O$ 0.25. The culture medium was supplemented with 2.76 $mg \cdot L^{-1}$of hormone 2-iP (2-isopentenyl adenine) purine, using sucrose as a source of carbon (15 $g \cdot L^{-1}$) and Phytagel agar (3 $g \cdot L^{-1}$) as gelling agent.The pH was adjusted to 5.2. The media were sterilized in an autoclave during 15 minutes at 121 °C. and the culture medium was modified by adding Al and/or Cd, setting the following conditions: (1) Control (original culture medium); (2) 100 µM of total Al (designated as100Al), (3) Al + Cd (100 µM total Al + 50 µM total Cd, designated as 100Al/50Cd), (4) and Al + Cd (100 µM total Al + 100 µM total Cd, designated as 100Al/100Cd). The

concentration of Cd and Al was adjusted using the corresponding 0.01 M stock solutions of $CdCl_2 \cdot 2H_2O$ and $AlCl_3$.

The cultures were kept in culture chambers at 21 ± 2 ºC and photoperiods of 16 hours light/8 hours darkness. The sterilization and planting procedures were carried out in a laminar flow chamber under aseptic conditions. Once the *in vitro* culture had become established (control and treatments), a completely random design was used in which the experimental unit included six blueberry plantlets clones (6 plantlets: 1 sample), and every sample was ran in triplicate. *In vitro* cultivated plantlets with more than 30 days of adaptation to cultivation conditions were defined as starting control (time=0 days), and used to define the physiological base line for all the studied parameters.

The computer program GEOCHEM-PC (Parker *et al.,* 1995) was used to calculate the chemical speciation of Cd and Al in the growth medium of the plantlets.

2.2. Extracts preparation.

Fresh materials ($0.1 g \cdot mL^{-1}$) are used to prepare the extract using 85% v/v of hydroethanolic solution; the samples were sonicated at 50-60 Hz of frequency during two hours at 25 ºC (298 K) according to the method described by Adam (2009).

2.3. Antioxidant activity

2.3.1. 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenger spectrophotometric assay.

The free radicals removal capacity of each extract was evaluated using the radical DPPH technique, described by Brand-Williams *et al.* (1995) with some modifications. Briefly, 20 µL of the ethanol extract was added to 980 µL of DPPH solution ($25 mg \cdot L^{-1}$), the absorbance was continuously monitored at 517 nm using a UV-visible

spectrophotometer (Agilent 8453 UV-Vis), for 240 seconds (Brand-Williams *et al.*, 1995; Huang *et al.*, 2005). Results were expressed as % of consumed DPPH.

2.3.2. Ferric reducing antioxidant power (FRAP).

The FRAP assay measures the ability of the sample to reduce Fe^{3+} to Fe^{2+} (Benzie and Strain, 1996), followed by the absorbance at 593 nm of the blue complex formed with tripyridyltriazine (TPTZ). FRAP reagent was prepared by mixing acetate buffer (300 mM), TPTZ solution (10 mM in HCl) and $FeCl_3$ solution (20 mM) at a 10:1:1 ratio. The FRAP reagent was maintained at 37±2º C (310±2 K). Absorbance of samples containing 900 μL of FRAP reagent, 80 μL of water and 20 μL of ethanolic extract,was measured at 593 nm in an Agilent 8453 UV-Vis spectrophotometer. The measures were expressed in ascorbic acid equivalents.

2.4. Total phenolic content (TPC)

The total phenolic content of ethanolic extracts was determined based on the method described by Singleton and Rossi (1965). Results were expressed as mg Gallic acid equivalents.

2.5 HPLC-DAD analysis of extracts

High performance liquid chromatography with diode array detector (HPLC-DAD) was used to separate and determine the phenolic compounds in the ethanolic extracts of blueberry tissues. The ethanolic extract was passed through a 0.45-μm membrane filter and analyzed by HPLC-DAD. An Agilent HPLC-DAD 1100 series chromatograph equipped with a RP-C18 column at 25 ºC, was used. The mobile phase is a gradient of acetonitrile

(A) and 1% phosphoric acid (B), using these program: time=0 minutes 10% of A, 5 minutes 25% of A, 8 minutes 35% of A, 15 minutes 60% of A, 17 minutes 35% of A and finally 20 minutes 10% of A; with an initial pressure of about 120 bar, 1 mL/min of flow and 20 μL of injection volume using a Reodyne valve, the register was carried out at 254, 280, 314 and 340 nm.

2.6. Lipid peroxidation

The level of lipid peroxidation was determined in terms of MDA concentration according to the method proposed by Heath and Parker (1968) slightly modified. The concentration of MDA was calculated from the difference of the absorbance at 532 and 600 nm using the extinction coefficient of 155 $mmol \cdot L^{-1}$ cm^{-1} and expressed as $nmol \cdot g^{-1}$ FW.

2.7. Statistics analysis.

All the data shown in the study correspond to X±1sd. The tests were made three times for the HPLC measurements and six times for the other measurements. The statistical analysis was made by two-way Anova and the comparisons of averages were made by means of the Tukey Multiple Comparison of Means test. To determine the correlation between physiological variables, Pearson's test was performed. Statistical significance was set at $p< 0.05$.

3. RESULTS

3.1 Chemical speciation of the Woody-Plant nutrient medium

The availability and mobility of Cd and Al in environmental systems like soil-soil solutions is not only related to total concentration, but to the concentration of the different Cd and Al species present in the solution, forming either inorganic complexes, organic

complexes, and precipitates, or as free species. Using computer programs like GEOCHEM-PC it was possible to determine the chemical forms in which Cd and Al are found in the culture medium.

Table 1. Percent distribution of Al and Cd in Lloyd-McCown culture medium for the different conditions studied ("complexed with" means Al and Cd complexed by the respective anion, Parker *et al.,* 1995).

Condition	**Speciation (%)**				
	100Al	**100Al/50Cd**		**100Al/100Cd**	
Species	**Al**	**Al**	**Cd**	**Al**	**Cd**
Free metal	0.66	1.05	8.63	1.26	20.81
Complexed with					
Sulfate	2.05	3.24	4.19	3.88	10.09
Borate	0.12	0.19	0	0.22	0
EDTA	51.5	23.46	86.23	9.06	66.82
Phosphate	44.8	70.83	0.07	84.09	0.17
Cloride	0.00	0.00	0.79	0.00	1.9
Nitrate	0.00	0.00	0.08	0.00	0.2
OH (Hydroxides)	0.79	1.24	0.00	1.49	0.00

According to the chemical speciation obtained from GEOCHEM-PC (Table 1), it was found that free Al^{3+}increases with the addition of Cd to the culture medium. The Cd is found in greater proportion as free Cd^{2+} in both binary treatments (8.63% for 100Al/50Cd and 20.81% for 100Al/100Cd), with a lower free Al^{3+}proportion in solution (<1.5% of the total in both binary treatments).

In Table 1 it is seen that Al in the absence of Cd is found mainly forming complexes with EDTA and to a smaller degree with phosphate. In the presence of cadmium, Al is

found mostly complexed by PO_4^{3-} (70-90%), while Cd is forming complexes mostly with EDTA (> 60%).

Table 2 shows the free concentration and the activity of each metal in solution. For both Cd and Al the GEOCHEM-PC program showed that the free concentration as well as the activity in solution increased in the binary system.

Table 2. Molar concentration and activity of Al and Cd in Lloyd-McCown culture medium for the different treatments applied.

Tratamiento	Specie	Concentration (M)	Activity (M)
100Al	Free Al^{3+}	$5.01x10^{-7}$	$1.48x10^{-7}$
50Cd	Free Cd^{2+}	$1.17x10^{-6}$	$5.90x10^{-7}$
100Cd	Free Cd^{2+}	$1.69x10^{-5}$	$8.48x10^{-6}$
100Al/50Cd	Free Al^{3+}	$1.04x10^{-6}$	$2.23x10^{-7}$
	Free Cd^{2+}	$4.32x10^{-6}$	$2.18x10^{-6}$
100Al/100Cd	Free Al^{3+}	$1.26x10^{-6}$	$2.68x10^{-7}$
	Free Cd^{2+}	$2.08x10^{-5}$	$1.05x10^{-5}$

3.2 Antioxidant activity

The antioxidant activity of the ethanol extracts of blueberries was measured by means of the diphenylpicrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays, which evaluate the capacity of the extract for trapping the DPPH free radical and for reducing Fe(III) to Fe(II), respectively.

The results obtained from the DPPH showed that the response to the application of the binary Al + Cd system (in its different concentrations) in the growth solution did not affect significantly the antioxidant capacity of blueberries in the first three weeks of

treatment with respect to the control (Fig. 1), but after 30 days of exposure significant differences were seen in the DPPH values between the control and the Al + Cd treatments applied. However, a significant decrease of the DPPH values was seen in the blueberry seedlings treated only with aluminum (100Al) compared tothe other treatments and at different times during the study (Fig. 1)

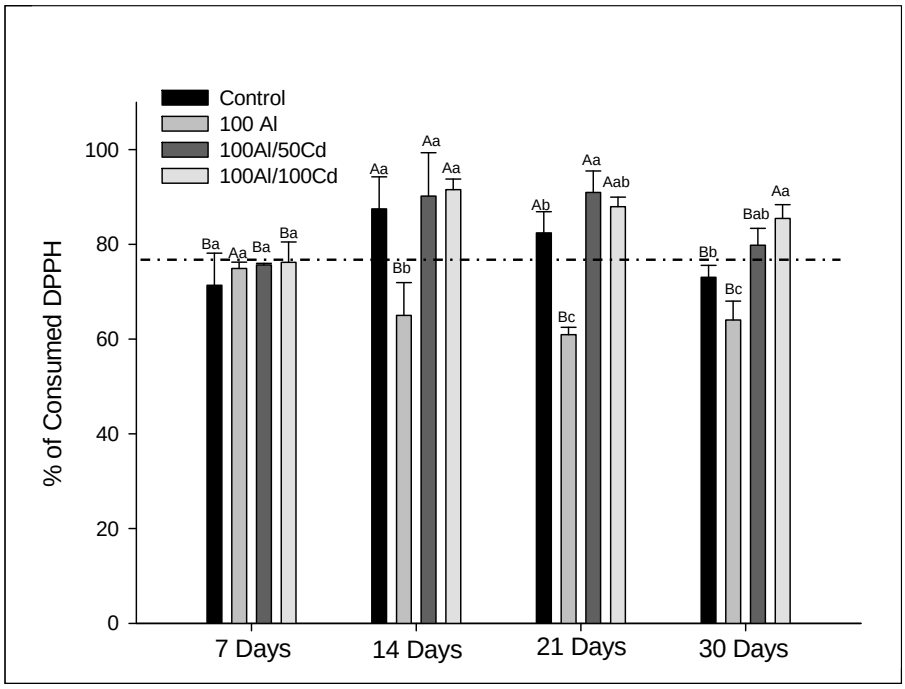

Fig. 1. DPPH scavenging, the result is expressed in % of consumed DPPH (mean ± standard deviation n= 6). A different lower case letters indicate statistically significant differences (Tukey, $P < 0.05$) between same day and different treatment. A different upper case letters indicate differences (Tukey, $P \leq 0.05$) between different day and same treatment. The segmented line shows the % of DPPH consumed for the t=0 days control.

The measurement of the reducing capacity of the ethanol extracts was made with the FRAP test (Fig. 2). The data obtained from this assay show a trend similar to that obtained from the DPPH test. The 100Al/50Cd treatment did not show significant differences with respect to the control after the first 7 and 14 days de exposure, while the 100Al/100Cd

treatment showed a significant increase of the FRAP value during the whole study ($p < 0.05$) with respect to the values obtained for the control and 100Al. Similar results have been reported for plantlets exposed to only 100 µM Cd, in which the FRAP value increased at 7, 14 and 21 days (Manquián et al., 2013).

In the case of the 100Al treatment the FRAP values were smaller over the whole exposure time compared to the Al + Cd systems, and this is consistent with what was seen with the DPPH assay (Fig. 1).

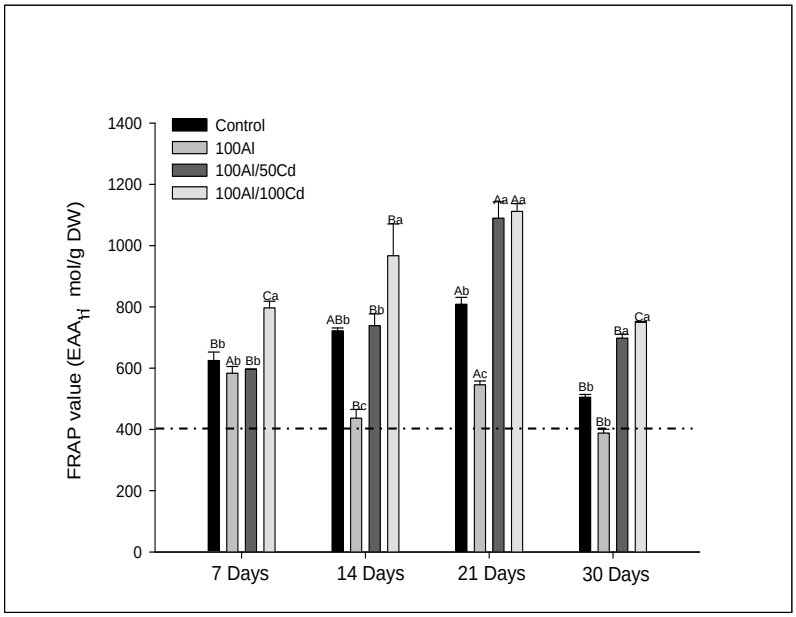

Fig. 2. Ferric reducing/Antioxidant power (FRAP) the results as expressed in ascorbic acid equivalents (mean ± standard deviation, n= 6). A different lower case letters indicate statistically significant differences (Tukey, $P < 0.05$) between same day and different treatment. A different upper case letters indicate differences (Tukey, $P \leq 0.05$) between different day and same treatment. The segmented line shows the FRAP value for the t=0 days control.

3.3 Cd-induced changes in malondialdehyde (MDA) content

The effects of oxidative stress due to the presence of heavy metals can be quantified from the content of MDA, which is a by-product of the degradation of polyunsaturated fatty acids in the membranes (Fig. 3). In the first week of exposure the highest MDA content was found in the control, while no significant differences in the MDA content between the binary systems (Al + Cd) and the 100Al treatment ($p < 0.05$) (Fig. 3).

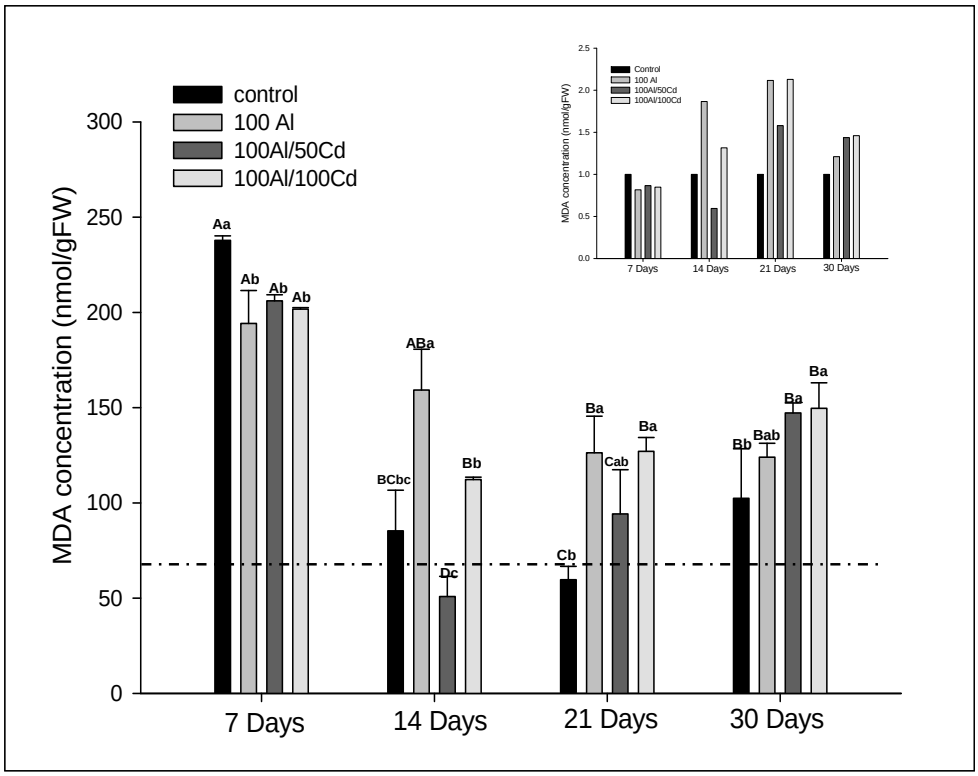

Fig. 3. Effects of the different stress treatments on MDA contents (mean ± standard deviation, n= 6). A different lower case letter indicate statistically significant differences (Tukey, $P < 0.05$) between same day and different treatment. A different upper case letters indicate differences (Tukey, $P \leq 0.05$) between different day and same treatment. Inset: Normalized data with respect to control ($MDA_{sample}/MDA_{control}$). The segmented line shows the MDA level for the t=0 days control.

This behavior would account for the degree of stress generated by transferring the plantlets to the *in vitro* culture medium (high damage in the control) and the kind of response of the plant as a defense mechanism when it is under stress by metals, finding that in those culture media where there are heavy metals (Al + Cd and 100Al) the blueberries increase their antioxidant defense system in order to reduce the damage from the presence of the metals, but the plants are unable to respond quickly to the stress generated by the culture medium change. At 14 days of treatment a decrease of the MDA content was seen in both binary systems, with the lowest point obtained with the 100Al/50Cd treatment ($p < 0.05$), while the largest MDA values were seen with the 100Al treatment. In the last two weeks of the study (21 and 30 days), the damage by stress was significantly greater for all the treatments with respect to the control ($p < 0.05$).

3.4 Total phenolic content (TPC) and HPLC-DAD phenolic compounds identification.

Total phenolic compound content is shown in Fig. 4. Compared to the control, during the first seven days a significant increaseof TPC is seen in the 100Al treatment, which decreases with increasing exposure time (14, 21 and 30 days) ($p < 0.005$).

No significant changes are seen with both binary Al + Cd treatments during the first 7 days of exposure. In the following 14, 21 and 30 days of the study a significant increase of the TPC is seen for the binary treatments compared to the control and 100Al treatments.

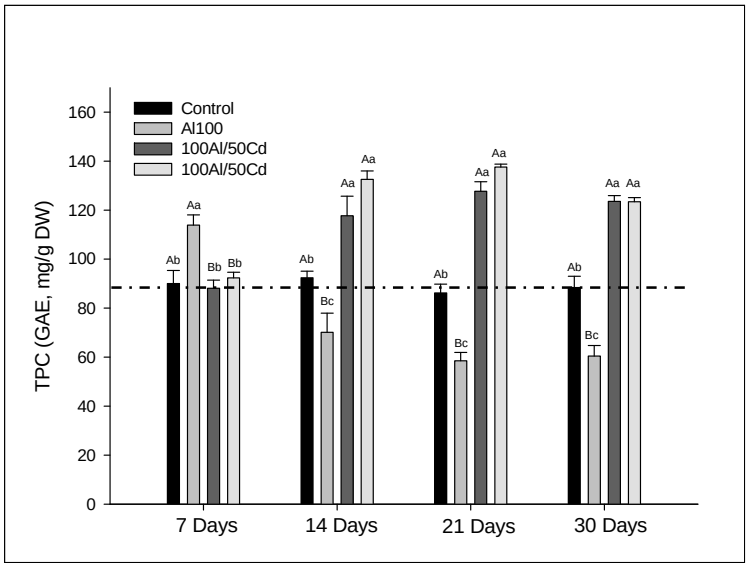

Fig. 4. Effect of different stress treatments on the total content of phenolic (TPC) compounds (mean ± standard deviation).A different lower case letter indicates statistically significant differences (Tukey, $P < 0.05$) between same day and different treatment. A different upper case letters indicate differences (Tukey, $P \leq 0.05$) between different day and same treatment. The segmented line shows the TPC for the t=0 days control.

The HPLC analysis of the ethanol extracts showed that the predominant compounds in the controls are chlorogenic and elagic acid, compounds that have been identified in previous studies in blueberry fruits at different ripening stages (Rivera *et al.*, 2011). It was also possible to identify compounds such as rutin and quercetin, which are found in significantly lower concentrations compared to the two most important ones (Fig. 5).

Fig. 5 shows the HPLC chromatograms of the blueberry ethanol extracts. In the seedlings subjected to the two Al + Cd treatments it was seen that the areas corresponding to chlorogenic and elagic acid increase with increasing Cd concentration in each binary combination.

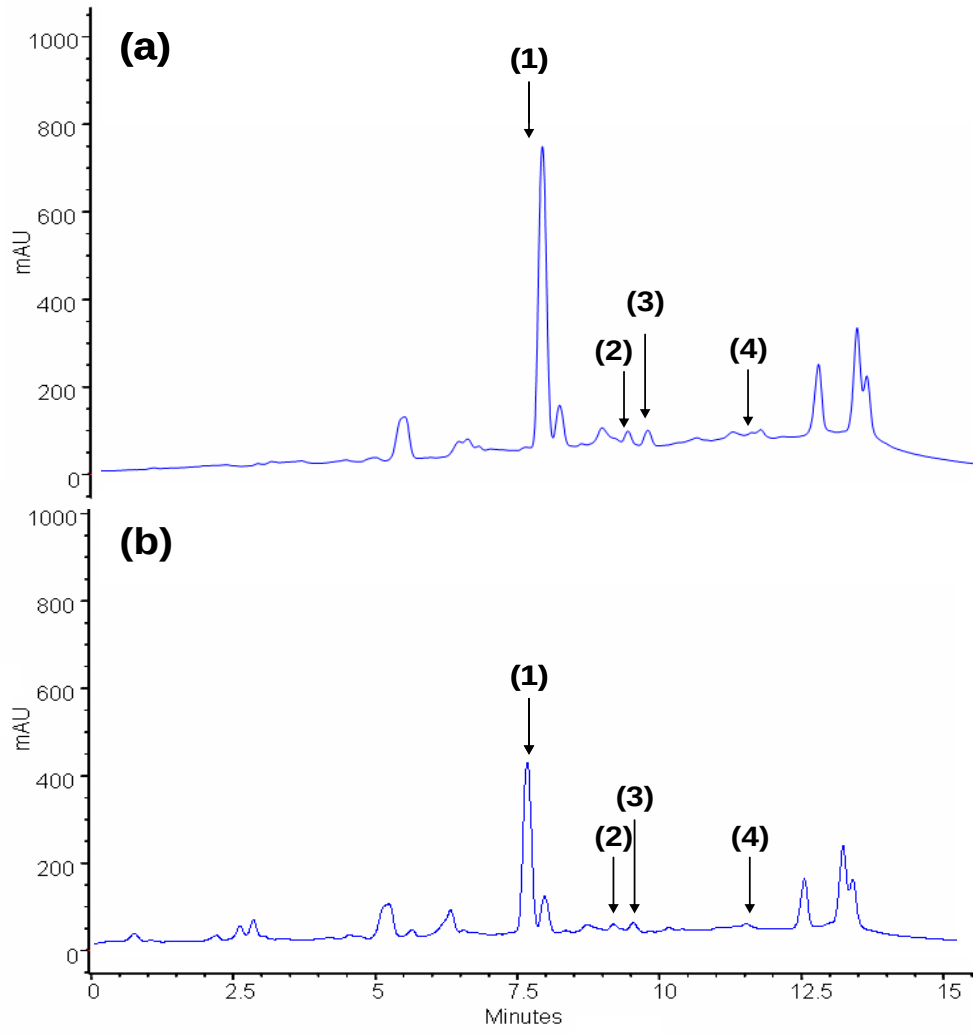

Fig. 5. Chromatogram of blueberry extracts of 14 days measured at 314 nm, for the treatment 100Al/100Cd (a) and control (b). The numbers correspond to identified phenolic compounds (1) chlorogenic acid, (2) Rutin, (3) ellagic acid and (4) Quercetin.

The Fig. 6 shows the concentration of chlorogenic and elagic acids obtained with the applied treatments. It is seen that no significant changes occurred in the control over the whole study period ($p < 0.05$). At 14 days of exposure the chlorogenic acid concentration increased with the binary treatments and a significant increase only with the 100Al/100Cd treatment compared with 100Al. At 21 days there was a significant increase in the

chlorogenic acid concentration with the binary (Al + Cd) mixture. It should be stressed that between 14 and 21 days of exposure there were higher chlorogenic acid concentrations with all the treatments except with the control. For the 100Al/50Cd and 100Al/100Cd conditions an important decrease of the chlorogenic acid concentration ($p < 0.005$) was seen during the fourth week of the study (Fig.6a).

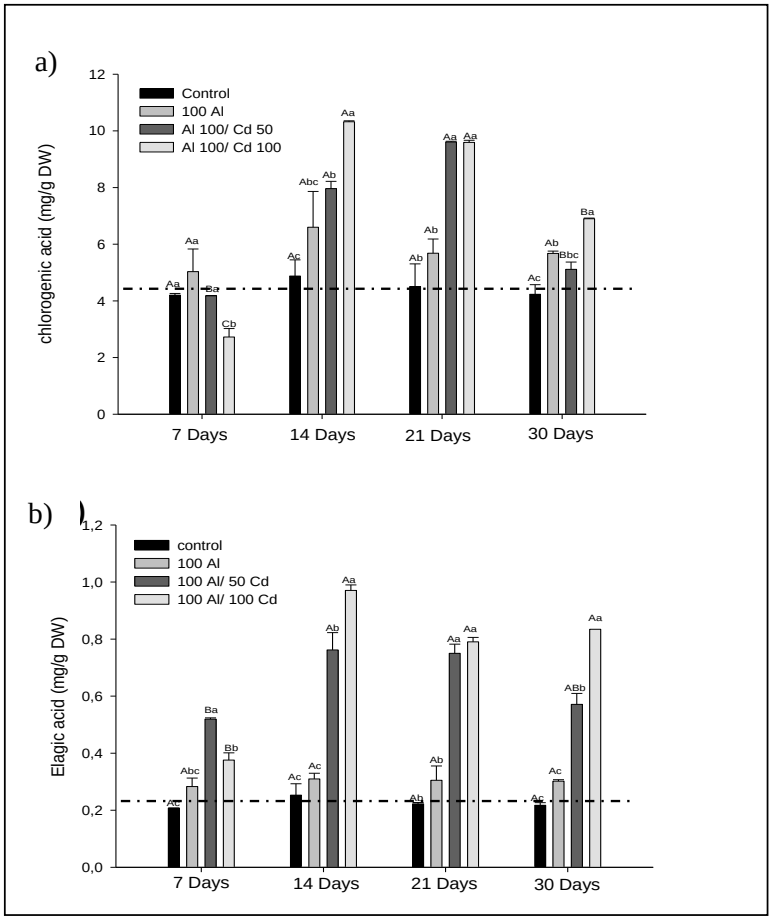

Fig. 6. Variation of (a) chlorogenic and (b) elagic acid content (mean ± standard deviation) as a function of time and the concentration of the different treatments in blueberries (thegraphs have different scales. A different lower case letter indicates statistically significant differences (Tukey, $P < 0.05$) between same day and different treatment. A different upper case letter indicates differences (Tukey, $P \leq 0.05$) between different day and same treatment. The segmented line shows the elagic and chlorogenic acids concentration for the t=0 days control.

Similar results were seen in the elagic acid concentration over time, with the peak concentration occurring between 14 and 21 days of exposure to the binary treatments ($p < 0.005$). The differences with respect to the control and the 100Al treatment remained until the end of the study (Fig. 6b).

3.5 Correlation between the antioxidant response parameters and MDA content

The combined presence of Al and Cd causes oxidative stress and changes in the profile of phenolic compounds biosynthesized by blueberries; this is shown by the correlation between the MDA, DPPH and chlorogenic acid content during exposure of the plantlets to the different conditions considered (Fig. 7). From these results it can be suggested that MDA content decreases when the capacity to capture free radicals (expressed as a higher DPPH content) increases (Fig. 7c, $r = -0.56$, $p < 0.05$). A significant negative correlation (Fig. 8b, $r = -0.626$, $p = 0.099$) between lipid peroxidation and chlorogenic acid content indicates that the latter plays a critical role in the decrease of MDA, which is justified by the high correlation (Fig. 7e, $r = 0.70$, $p < 0.05$) found between the chlorogenic acid content and DPPH. Similar results were reported by Manquián et al., for a study made with blueberry plantlets subjected to different concentrations of Al (Manquián *et al.*, 2013).

The reducing power of the extracts determined by means of FRAP essays and by the elagic acid content did not correlate significantly with MDA (Fig.7a, d), indicating that this protection mechanism is not very effective to reduce the lipid peroxidation undergone by blueberries under the studied conditions.

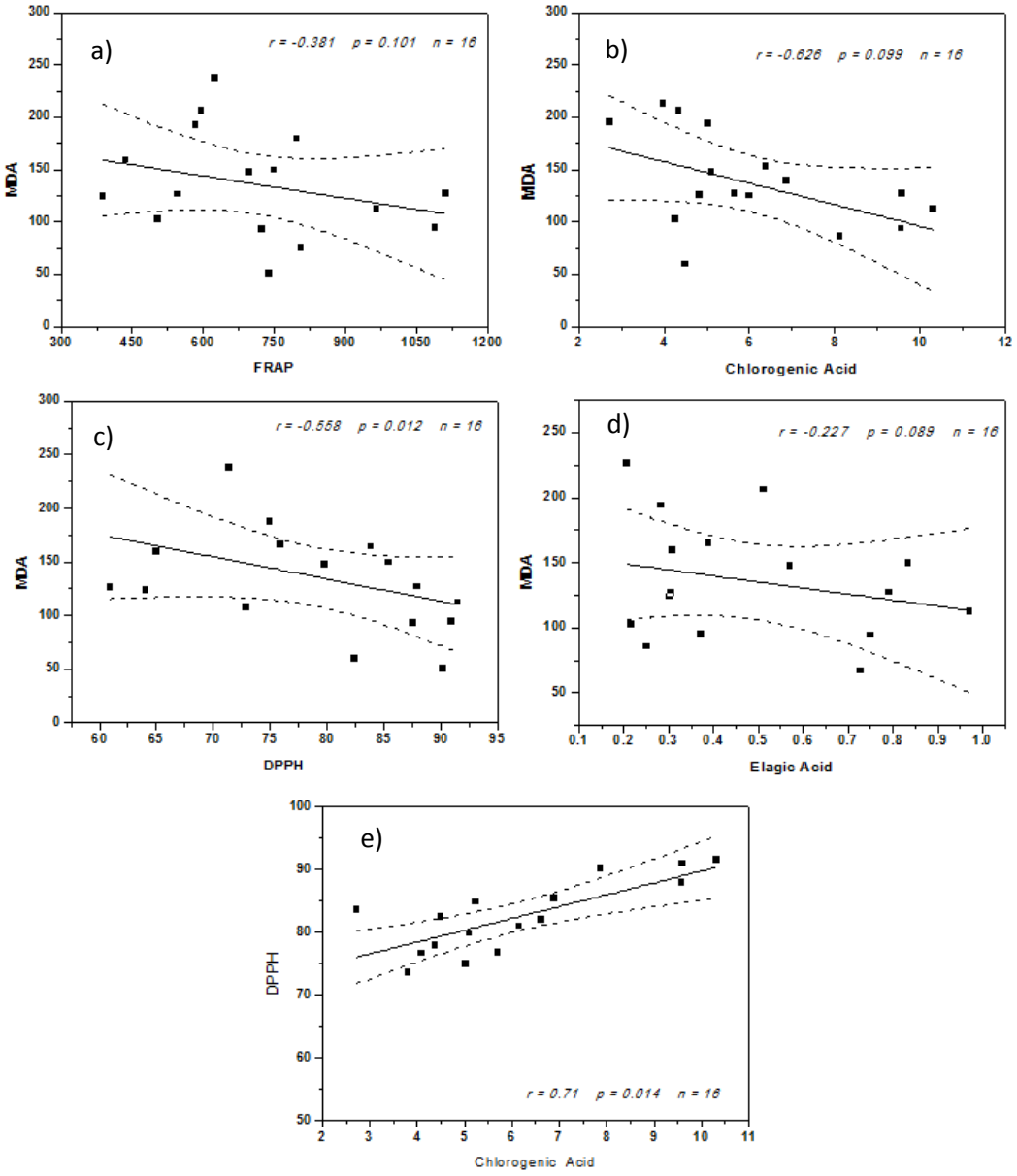

Fig. 7. Relations between lipid peroxidation (MDA) and the antioxidant response measured by FRAP, DPPH, Chlorogenic acid, and ellagic acid addition ratio DPPH and chlorogenic acid. Pearson's coefficients are indicated.

4.-DISCUSSION

Aluminum is one of the most limiting factors of cultivation in acid soils, and furthermore the use of phosphate fertilizers, particularly thoseof natural origin like triple superphosphate (TSP),increase the concentration of trace elements (Cd, Cu, Ni, etc.) in the soil. The acidity of these soils favors the mobility of the metals in the soil solution, which results ingreater activityof metals like Al and Cd. While at the usual pH of these acid soils (4.5-5.5) 29-15% of the total Al and more than 99.8% of the total Cd are found in solution (as free species or forming some soluble complex), in a neutral or alkaline soil (pH 7.0-8.0) more than 98% of the total Al and Cd are found as precipitates. Therefore, in view of this greater availability, the presence of trace elements in soil derived from volcanic materials will have a greater impactonthe behavior of plants compared to what can happen in neutral or alkaline soils.

The increased concentration of trace elements or heavy metals in the growth solution has as a consequence a saturation of the complexing capacity of the medium, and this results in increase of the free concentration and the total activityof the trace elements, in turn increasing the availability of these elements to the plant. An equivalent situation can occur in real terms, with an excessive increase of the total concentration of trace elements in the soil solution, with the result of the inability of the plant to generate short chain organic acids which can complex elements, resulting in increased availability that can finally reach phytotoxic levels.

The application of metals to the culture medium produced effects in the antioxidant response of the plant, showing an increase of the antioxidant capacity of the blueberry extracts treated with the binary combination.

Similar results were found in a study in which only the effectof Cd on the antioxidant activity of blueberry extracts was determined (Manquián *et al.*, 2013). These results suggest that the joint presence of both metals in the culture medium produces a greater response of the blueberry non-enzymatic antioxidant mechanisms (FRAP and DPPH).

The presence of trace elements like Al and Cd can induce oxidative stress, whose effects are determined by meansof the analysis of lipid peroxidation (MDA). The increased MDA content in the first seven days, for all the treatments, including the control, is a consequence of the stress to which the plant is subjected at the time of being transplanted to the *in vitro* medium, but no differences are seen between the treatments and the control. However, once the plantlets have become climatized, after 14 days, a significant decrease in MDA content was seen in the binary treatments. At 21 and 30 days of exposure to the binary treatments no significant differences were seen in the accumulation of MDA with respect tothe 100Al treatment, but they did appear with respect to the control, where the presence of the metals causes increased lipid peroxidation. These results may indicate that there was no synergism in the damage produced by the presence of both metals in solution, as could have been expected, because through chemical speciation there was increased activity of the metals in solution in the binary composition for both treatments. But the results of the MDA content in the combined treatments can be reflected in an increased FRAP value in the combined treatments and in the values of the concentration of phenolic compounds, which increases at days 14 and 21 of combined treatment. Tolerance to abiotic stress is often related to increased antioxidant activity, so our study would be indicating that aluminum tolerant plants would increase their antioxidant components to counteract

the damage by the metal (Fig. 7). The induction of phenolic compounds due to the presence of metals in the growth medium has been observedin different studies (Michalak *et al.*, 2006). In the phenolic compounds found in blueberry seedlings, chlorogenic and elagic acids were quantified, both of them well known for their antioxidant power (Niggeweg *et al.*, 2004). The results showed similarities between antioxidant capacity and phenolic compounds, whose concentration increased after 14 days. The concentration of chlorogenic acid was stimulated at 14 days of treatment mainly in the 100Al/100Cd binary mixture, while at 21 days both combinations, 100Al/50Cd, and 100Al/100Cd, increased the concentration of chlorogenic acid. These results agree with those obtained for MDA and decreased damage by lipid peroxidation at 14 and 21 days, showing that chlorogenic acid has a protective role in the damage caused by lipid peroxidation (Niggeweg *et al.*, 2004).

The quantification of chlorogenic acid in the presence of metals has been studied by various authors (Kováčic, 2009; Irtelli and Navari-Izzo, 2006; Manquián *et al.*, 2013), and the trend has always beenincreased chlorogenic acid concentration in the presenceof the metal in the growth medium.

Elagic acid is a polyphenol found in berries like strawberries, raspberries, blackberries and blueberries (Jiménez-García *et al.*, 2013; Da Silva *et al.*, 2008, Rivera *et al.*, 2010). In strawberries it has been found that elagic acid content represents 51% of the total acids of phenolic origin (Aiyer *et al.*, 2008). In our study we found a significant increase of elagic acid content after 14 days with the binary treatments, and yet this amount is significantly low compared to the chlorogenic acid content. Similar results have been obtained in studies on phenolic compounds, where the main compound found in blueberries

is chlorogenic acid and to a smaller extent other compounds like elagic acid (Ribera *et al.*, 2010; Može *et al.*, 2011).

The binary study also allowed us to detec to ther compounds that were only identified but not quantified, mainly rutin and quercetin. Rivera *et al.,* (2010), determined the presence of rutin and quercetin in blueberries at different ripening stages. Studies made by Kovacik *et al.,* (2010), on chamomile plants exposed to 60 and 120 µM Al showed increased content of different phenolic compounds. Our results show that there is an variation in the profile of the chromatograms, indicating that there is an change of these compounds when the plantlet grows in the presenceof Al + Cd, indicating that phenolic compounds may be affected by the amount and kind of metal to which the plants are exposed during growth.

5.- CONCLUSION

In general, the results of this study showed the stress-causing effect of trace elements in the growth medium, which was reflected in increased damage by lipid peroxidation, finding that the greatest oxidative damage occurs after 14 days of exposure to both treatments. The antioxidant response increased in the binary treatments, where the largest responses were seen after 14 days, and this increase is related to an increase in the content of quantified phenolic compounds, with chlorogenic acid as the main component, concluding that antioxidant activity plays an important role as a defense mechanism against damage by trace elements.

In conclusion, the Al + Cd mixture induces changes in the production of phenolic compounds, evidenced by an increase of their proportion compared to those in the control, indicating that it would actually be possible to modulate plantlets to favor the production of

compounds of interest, in this case phenolic compounds, but the impact on the composition and incorporation of trace elements at later growth stages, when the leaves of the plants can be used as infusions, must be evaluated.

Acknowledgements

Financial support of this work by the Center for the Development of Nanoscience and Nanotechnolgy (CEDENNA). K. Manquián-Cerda acknowledges a scholarship from UFRO (Chile) and DICYT-DGT (Universidad de Santiago de Chile, GE. Zúñiga).

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DISCUSIÓN GENERAL

La deficiencia o exceso de nutrientes produce en las plantas diferentes desórdenes metabólicos (Liu *et al.,* 2007; Benavides *et al.,* 2005; Gaballah *et al.,* 2012). En general las plantas son más resistentes al incremento de la concentración de nutrientes, que a la insuficiencia de los elementos esenciales. En el desarrollo de esta tesis se describió (*Capítulo I*), que elementos como Zn, Mn, Fe, Cu, Mg entre otros, son esenciales para el crecimiento y desarrollo normal de la planta, sin embargo elementos como Cd y Al que se encuentran por diferentes fuentes presentes en los suelos, pueden resultar tóxicos para las plantas (Guo *et al.,* 2007; Wulff *et al.,* 2010).

Los sistemas de cultivo alternativos como solución nutritiva, hidropónica o cultivo *in vitro* pueden ser considerados modelos del sistema suelo o más específicamente de la solución suelo. La ventaja principal de los medios de cultivo es que la composición de la solución de cultivo se puede definir, manipular y diseñar con un alto nivel de precisión (Wulff *et al.,* 2010).

Los sistemas de cultivo en solución son una simplificación del sistema suelo, donde las raíces de las plantas obtienen de manera ideal los nutrientes, mientras que en el suelo las raíces deben explorar continuamente para obtener los nutrientes y el agua. Sin embargo, este sistema ideal puede ser alterado cuando se desea investigar los efectos de elementos que no son componentes de estas soluciones. Cada medio de cultivo, debido a su composición, tiene una alta capacidad complejante, y por lo tanto tiende a modificar de manera significativa la distribución de las formas químicas en las que se encuentran los compuestos presentes cuando se adiciona un elemento que pueda interaccionar con los

agentes complejantes. De esta manera la aplicación de metales como Cd y Al al medio de crecimiento altera la composición química de la solución, dejando en algunos casos mayor disponibilidad de elementos esenciales para la planta, la cual puede ver alterado su metabolismo por el aumento de la concentración de nutrientes en el medio.

El arándano, tanto sus frutos como sus hojas poseen una alta concentración de compuestos activos antioxidante de lo hace de este cultivo importante desde el punto de vista nutricional y económico (Castrejón *et al.,* 2008). Esta especie en los últimos años ha aumentado su cultivo en las zonas central y sur de Chile, donde se caracterizan los suelos por tener un pH ácido donde se presenta una alta concentración de Al libre (Mora *et al.,* 2009), además de aumentar la actividad fitotóxica del Cd presente en los suelos por diferentes factores como la fertilización (Molina *et al.,* 2009).

La aplicación de Al y Cd al medio de cultivo (*Capítulo II y III*) produjo efectos similares en las plántulas de arándano, daño oxidativo medido mediante el contenido de MDA. Diversas investigaciones han demostrados que ambos metales producen peroxidación lipídica en diversas especies (Ribeiro *et al.,* 2012; Ge *et al.,* 2012; Cavusoglu *et al.,* 2010). Sin embargo el estrés oxidativo es generados por un aumento de las especies reactivas de oxigeno (ROS) en el sistema, las cuales pueden verse aumentada por diversos factores abióticos, como estrés por metales, cambio en la disponibilidad de nutrientes en el sistema, estrés hídrico y salino (Arora *et al.,* 2002). Esta información nos da cuenta que el daño observado por la aplicación de Al y Cd al medio podría ser una resultante de los estreses producidospor al cambio en disponibilidad de nutrientes debidola adición de metales al medio. Para determinar las especies químicas de los componentes que

constituyen la solución nutritiva, se realizó una especiación química utilizando el programa GEOCHEM (Parker *et al.*, 1995). Se determinó que entre un 40-70 % de los metales aplicados (Al y Cd), formaron complejos con EDTA, modificando la disponibilidad nutrientes como Fe, Zn y Cu disponibles para la planta.

Esta tesis además contempló analizar el efecto combinado de Al+Cd aplicados en medios de cultivos, donde una de las principales diferencias con los estudios individuales fue la mayor respuesta antioxidantes, el cual se vio reflejado en una disminución en el daño por peroxidación lipídica (*Capítulo IV*). La aplicación de ambos metales al medio de modificó la disponibilidad de nutrientes, observándose una variación de Zn libre, aumentando 7 veces su disponibilidad en el tratamiento binario de Al+Cd con respecto al medio enriquecido con solo un metal. El Zn es un micronutriente esencial, el cual forma parte diferentes enzimas, jugando un rol crítico en los sistemas de defensa en contra las especies reactivas de oxígeno que inducen peroxidación lipidica (Cakmak, 2000; Smeets *et al.*, 2005). Se ha observado que desbalances en el contenido de Al y Cd de manera independiente provocan un aumento en la peroxidación lipídica. En el caso Cd este puede ingresar a la planta, donde genera un incremento en la actividad de lipoxigenasa (LOXs), enzima encargada de oxidación de los ácidos grasos, (Smeets *et al.*, 2008), mientras que el Al puede producir peroxidación lipidica debido a la rigidificación de la membrana al unirse este a los fosfolopídos presentes en ella (Devi *et al.*, 2003).La aplicación binaria de metales aumento la disponibilidad de Zn en el medio de crecimiento, lo cual sugiere que este elemento, ayudaría a disminuir los posibles daños asociados a la presencia de elementos como Cd y Al en el medio de crecimiento, aumentando la respuesta antioxidante del arándano. Resultados similares se han encontrado en plantas tratada con Cd, donde al

aumentar la disponibilidad de Zn en el medio, se observa una reducción del daño por peroxidación lipidica (Cherif *et al.*, 2011).

La acumulación de compuestos fenólicos en plantas puede ser inducida por estreses bióticos y abióticos. Diversos estudios indican que estrés abiótico producido por metales pesados promueven la producción de ROS. La capacidad antioxidante de los compuestos fenólicos se encuentra estrechamente ligada al número de grupos hidróxilos que posea la molécula (donde la actividad antioxidante se incrementa en aquellos compuestos con una alta sustitución de grupos -OH), de su estructura (grado de plimerización) y los sustituyentes (Sgherii *et al.*, 2003; Caillet *et al.*, 2006). Los posibles mecanismos involucrados establecen donación de electrones o átomos de hidrógeno a los radicales libres, generándose un radical fenoxilo que es menos reactivo ya que se estabiliza por resonancia en el anillo aromatico (Procházková *et al.*, 2011). Además de la función antioxidantes que poseen estos compuestos, estos también participan como quelantes de metales (Vasconcelos *et al.*, 1999). Sin embargo existe poca información que dé cuenta de los cambios en el perfil de compuestos fenólicos y su rol en la desintoxicación de ROS y el impacto de los metales pesados. Una investigación realizada por Tolra *et al.*, (2005) detectaron cambios en el contenido de ácido ferúlico y p-cumárico en plantas de *Rumex acetosela* tratadas con aluminio, sin embargo no fue posible establecer si estos compuestos están implicados en la desintoxicación de aluminio.

En nuestra investigación la presencia de Al y Cd provocó cambios en la biosíntesis de compuestos fenólicos en arándano, principalmente ácido clorogénico y elágico, los cuales son reconocidos por ser metabolitos con propiedades antioxidantes e inhibidores de la

actividad en los tumores (Charurin *et al.*, 2002; Yen *et al.*, 2005; Tavani and Vecchia, 2004, Koshiro *et al.*, 2006). Estos cambios pueden ser consecuencia de una serie de procesos desencadenados por los contenidos de Cd y Al, tales como:

1. La enzima Fenilalanina Amonio-Liaza (PAL por sus siglas en inglés), una de las enzimas que forma parte de la vía Fenilpropanoide, principal ruta de biosíntesis de compuesto fenólicos, ve incrementada su actividad como consecuencia del aumento de especies reactivas de oxígeno (ROS), los cuales juegan un rol de señalizadores para esta enzima, frente a condiciones de peroxidación lipídica. Estudios han encontrado una correlación positiva entre la actividad de la PAL y la acumulación de compuestos fenólicos y flavonoides solubles, en hojas tratadas con Cd de *Azolla imbricata* (Dai *et al.*, 2006) y en las raíces de *Panax ginseng* sometidas a la presencia de Cu (Ali *et al.*, 2006).

2. La competencia que se genera entre los micronutrientes y elementos trazas por agentes complejantes, los cuales pueden estar presente en los medios de crecimiento (EDTA) o ser exudados radicales, ocasionan cambios en las disponibilidades de micronutrientes. En esta tesis se determinó que la adición de Al y Cd al medio de cultivo modificó la disponibilidad de nutrientes. Estas modificaciones alterarían el funcionamiento de la enzima PAL, debido a que esta enzima responde de manera diferenciada a desequilibrios en el contenido de micronutrientes,incrementando su actividad, lo que explicaría el incremento en los contenidos de los ácidos clorogénico y elágico, en las plántulas tratadas (Luo *et al.*, 2010).

El alto contenido de compuestos fenólicos que contiene el arándano posee una marcada respuesta antioxidante, lo cual se vio reflejado en los valores de DPPH y FRAP medidos a los extractos etanólicos. Nosotros sugerimos que la respuesta de las plántulas de arándano frente a la aplicación de elementos trazas en la actividad antioxidante, puede estar directamente relacionada con el aumento de estrés oxidativo generado por los cambios en el medio de crecimiento.

La adición de Cd y Al en sistemas mono y multicomponentes en el medio de cultivo *in vitro*, han permitido correlacionar positivamente los compuesto fenólicos con la respuesta antioxidante y negativamente con el daño por peroxidación lipídica, siendo estos resultados la base para establecer posibles modulaciones de compuestos fenólicos, principalmente ácido clorogénico y elágico, cuyas importancias nutricionales y médicas son relevantes para las proyecciones del arándano.

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CONCLUSIONES GENERALES

En este estudio se logró desarrollar e implementar un sistema de propagación *in vitro* de arándano y aplicar diferentes tratamientos destinados a generar estrés por metales (Al^{3+}y Cd^{2+}), modificando la composición de las soluciones de crecimiento y evaluando los cambios en diferentes parámetros fisiológicos, ocasionados por la presencia de agentes inductores de estrés, con el fin de estudiar la producción de compuestos fenolicos producidos bajo condiciones de estrés.

Mediante un estudio de especiación química realizada a las soluciones del medio de cultivo, fue posible establecer que especie(s) de los diferentes metales aplicados como tratamientos (Al^{3+} y Cd^{2+}), tienen un rol importante en los efectos observados en arándano. Adicionalmente fue posible determinar que la concentración libre del metal es más importante que su actividad, en relación con el comportamiento a nivel de daño oxidativo yrespuesta antioxidante en arándano. Este procedimiento demostró la importancia de los estudios de especiación química en matrices complejas, ya sea en solución nutritiva y/o cultivos *in vitro*, en donde existen componentes acomplejantes, que modifican la distribución de las especies presentes de cada elemento en el sistema.

Se investigó la aplicación de Al en plántulas de arándanos cultivados *in vitro (capitulo II).* El estudio mostró que los extractos de arándano poseen un alto contenido de compuestos fenólicos, además de una alta actividad antioxidante. La aplicación de Al^{3+} al medio de cultivo produjo efectos en la capacidad antioxidante y un aumento en el daño por peroxidación lipídica donde los mayores efectos se observaron a los 14 días de tratamiento. El contenido de compuestos fenólicos aumento, principalmente ácido clorogénico y ácido

elagico, este comportamiento se observó independiente de la concentración de aluminio aplicado, sugiriendo que estos compuestos fenólicos juegan un rol importante en la respuesta ante el estrés producido por este metal.

Las plántulas de arándano que crecieron en presencia de Cd mostraron un daño significativo por peroxidación lipídica en todas las concentraciones aplicadas, siendo el mayor daño a los 14 días de exposición. En términos de la respuesta antioxidante, si bien se observó un aumento respecto del tratamiento, no fue suficiente para controlar el daño oxidativio generado, pues para ambas concentraciones de Cd, los niveles de MDA fueron mayores que en el control respectivo. Esto sugiere que las plantas de arandano cultivadas in vitro, presentan una alta sensibildiad al Cd.

La aplicación de diferentes concentraciones de Cd^{2+}, al medio de cultivo de plantulas de arándano produjo efectos notorios en los contenidos de compuestos fenólicos. La respuesta de la planta frente a la presencia del metal fue diferenciada con respecto a la concentración de Cd, observándose una mayor respuesta a una mayor concentración presente en el medio.

Los efectos de la presencia de Cd + Al en el medio de cultivo de plantulas de arandano se estudiaron en el capitulo IV. Los resultados obtenidos mostraron que se induce daño oxidativo en las etapas iniciales del tratamiento, sin embargo, luego a partir de los 14 días, se produce un aumento en la respuesta antioxidante, lo que estaría relacionado con un aumento en el contenido de compuestos fenólicos cuantificados, donde el componente principal es el ácido clorogénico, lo que permite concluir que la actividad antioxidante juega un rol importante como mecanismo de defensa frente al daño por elementos trazas.

Por lo tanto nosotros podemos concluir luego del desarrollo de esta tesis, que la disponibilidad de nutrientes y elementos trazas como Cd y Al, en arándano se encuentra estrechamente relacionada con los componentes totales de la solución de crecimiento, los cuales pueden aumentar su toxicidad o disminuirla, dependiendo de los agentes complejantes y el pH en el que se encuentra el medio de crecimiento (solución nutritiva, cultivos *in vitro* o suelos). De esta manera la aplicación de elementos trazas como Cd y Al producen efectos importantes en elperfil de compuestos fenolicos, aumentando en algunos casos su concentración, como por ejemplo el ácido clorogénico y ácido elégico, concluyendo que si es posible manipular el medio de crecimiento de manera de modular a la planta para la generación de compuestos bioactivos de interés.

Futuras Proyecciones

Las proyecciones que surgen de este trabajo son:

- El desarrollo de una metodología de estudios basados en la producción *in vitro* de arándano u otras especies vegetales, donde es posible modificar y simular factores medioambientales, permitiendo investigar la respuesta de las plantas desde un punto de vista metabolómico, conociendo como posibles alteraciones en los factores ambientales modifican la regulación de la biosíntesis de compuestos bioactivos antioxidantes.
- El sistema de cultivo *in vitro* de arándano o cualquier especie a la cual se propague y desarrolle en óptimas condiciones, permitirá desarrollar estudios de parámetros fisiológicos, bioquímicos y moleculares, orientados a identificar mecanismos de tolerancia o resistencia a condiciones externas como la aplicación de diferentes estreses medioambientales.
- Considerando que las plantulas de arándano cultivados *in vitro* poseen un alto contenido de compuestos fenólicos, los cuales pueden ser en algún grado modificados por la presencia de metales pesados. Entre los principales compuestos fenólicos se destaca el ácido clorogénico, el cual es un compuesto de gran interés comercial debido a su alto poder antioxidante. De esta manera el uso de los extractos de arándano a nivel comercial podría significar un aporte para la generación individuos *in vitro* con un alto poder antioxidante.
- El poder reductor de los extractos de arándano obtenidos en cultivos *in vitro*, sometidos a diferentes tratamientos con metales pesados, están siendo evaluados como agentes reductores para la síntesis verde de diferentes nanopartículas

metálicas de valencia cero (nMVC), lo que abre perspectivas en la revalorzación de los residuos generados en el proceso de cosecha y poda de arándano, permitiendo el cruce entre diferentes áreas de la ciencia. Este trabajo está siendo desarrollado bajo el marco del proyecto CORFO 12IDL2-16251 y el Centro de Nanociencia y Nanotecnología (CEDENNA)

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Effect of aluminum on antioxidant activity and phenolic compounds content in *in vitro* cultured blueberries

[Efectos de aluminio en la capacidad antioxidante y en el contenido de compuestos fenólicos en plántulas de arándano cultivadas *in vitro*]

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Abstract
Blueberry is a popular natural food product consumed worldwide. Acid soils are found throughout the world. A significant problem of acid soils is the active aluminum content, which may result toxic to plant. The present study was undertaken to assess the toxicities of Al for Blueberry (*Vaccinium corymbosum* L.) cultivated *in vitro* and treated with 100 and 200 µM Al. The effects of Al concentration on malondialdehyde (MDA) content, antioxidant activity and phenolic compounds of blueberry after 7, 14 and 21 days of treatment were established. The analysis of the MDA accumulated in the tissues of the blueberry seedlings indicates that Al concentration increases the damage caused by lipid peroxidation, for both treatments, after 14 days. The highest antioxidant activity in the extracts was observed at 200 µM Al after 14 days of treatment, being chlorogenic and ellagic acids the most significant metabolites involved in the antioxidant properties. Then, the content of Al in soil could be modulate the content of bioactive compounds in blueberry plants.

Keywords: Blueberry, aluminum, antioxidant capacity, phenolic compounds.

Resumen
El Arándano es un popular alimento natural consumido en todo el mundo. Los suelos ácidos se encuentran en todo el mundo. Un problema significativo de suelos ácidos es el contenido de aluminio activo, que puede resultar tóxico para la planta. Este estudio se realizó para evaluar la toxicidad del aluminio en plantas de arandano, cultivadas in vitro y tratadas con 100 y 200 mM de Al. Se establecieron los del aluminio en el contenido de malodialdehido (MDA), capacidad antioxidante y contenido de compuestos fenolicos en plantulas de arandano luego de 7, 14 y 21 dias de tratamiento. El análisis del MDA acumulado en los tejidos de las plantulas de arándanos indica que la concentración de Al aumenta el daño causado medido como peroxidación de lípidos, para ambos tratamientos, después de 14 días. La actividad antioxidante más alta de los extractos se obseva a 200 mM de Al después de 14 días de tratamiento, siendo los ácidos clorogénico y elágico los metabolitos más importantes que participan en las propiedades antioxidantes. Entonces, el contenido de Al en el suelo podría modular el contenido de compuestos bioactivos en plantas de arándanos, alterando sus propiedades medicinales.

Palabras Clave: Arándano, aluminio, capacidad antioxidante, compuestos fenólicos.

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