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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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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 defensive mechanism, 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), glutathione reductase (GR), superoxide dismutase (SOD) and glutatione-S-transferase (GST). 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/L of hormone 2-iP and 3.0 g/L 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).

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/L cm and expressed as nmol/ g FW.

Extracts preparation

Fresh materials (0.1g/mL) 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 realized using analysis of variance (ANOVA) with Fisher (F) test in all samples to determine significant difference with $n \geq 3$ in all measurements.

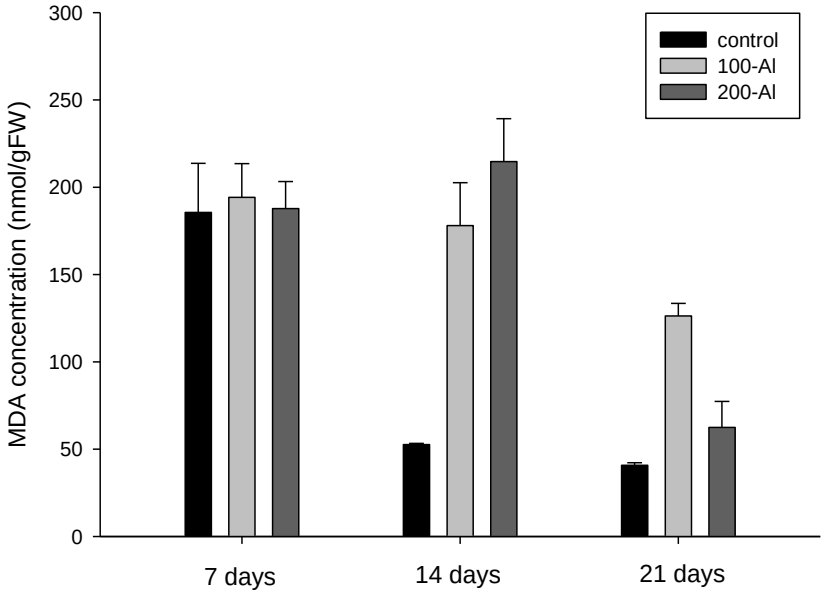
RESULTS

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* L, 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 seedlings of *V. corymbosum* L. Each value is a mean of three samples ± 1 s.e



Antioxidant activity

The antioxidant capacity of ethanolic extracts of V. corymbosum L., 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. 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 seedlings of *V. corymbosum* L., 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/g 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/g DW) and a minimum at day 7, with a value below the control

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

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

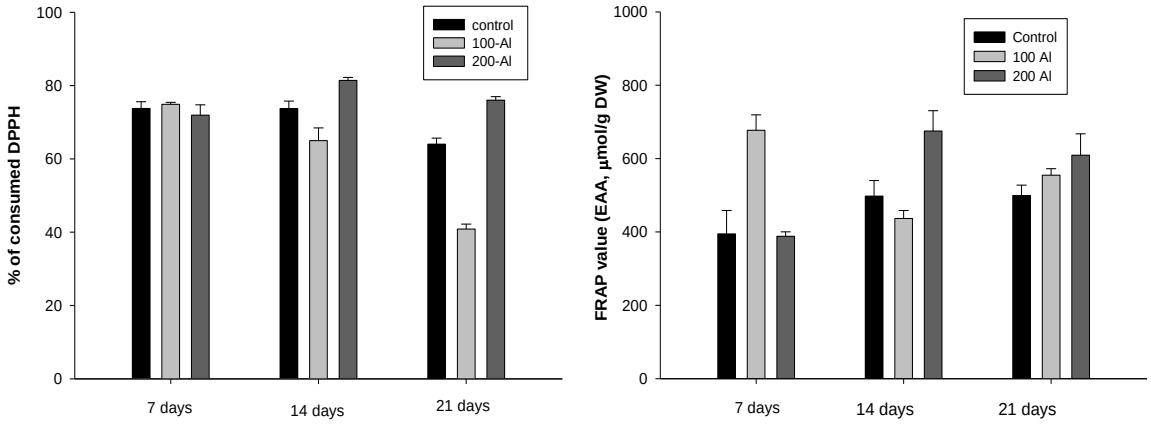
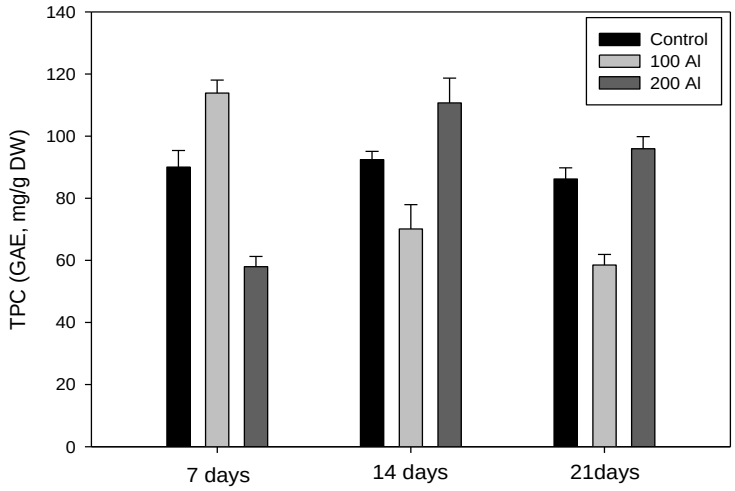


Figure 3
Variation in the content of total phenolic compounds (TPC) in seedlings of *V.corymbosum* L. treated with Al. Each value is a mean of three samples ± 1 s.e

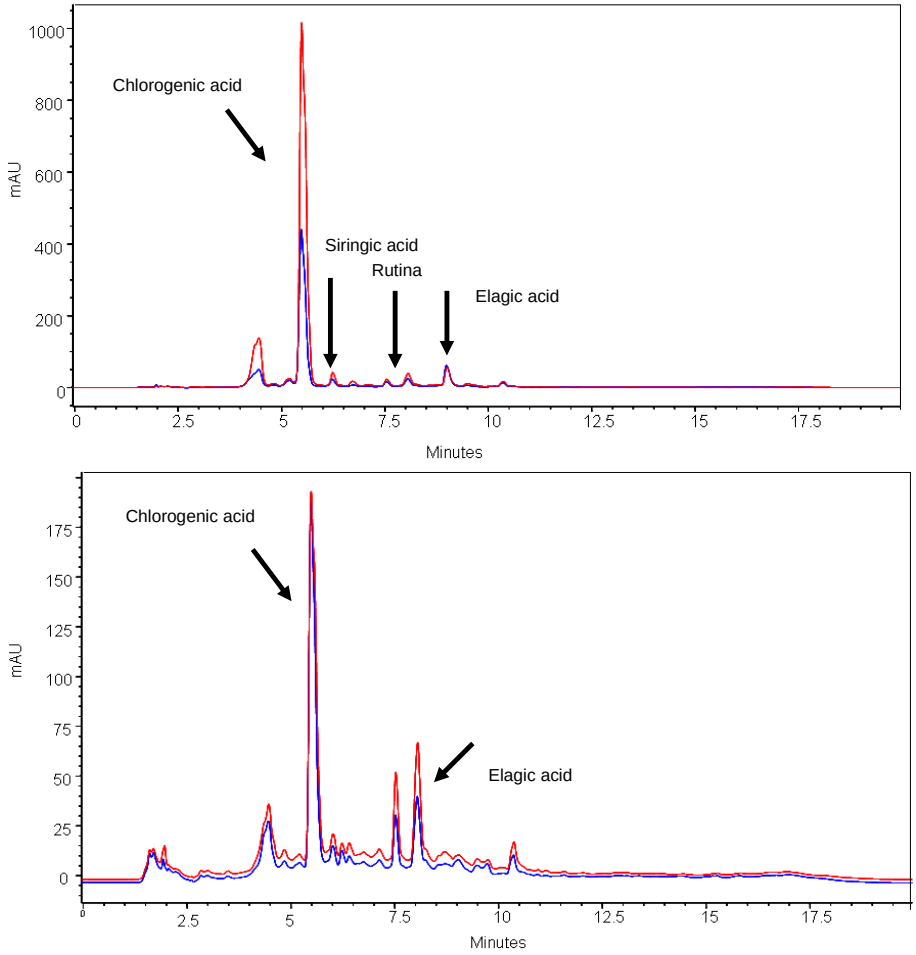


Identification of phenolic compound by HPLC-DAD
The identification of phenolic compounds in the blueberry extract was performed using HPLC-DAD. Four compounds were identified, two of them (chlorogenic acid and ellagic acid) were also quantified (Figure 4).

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.

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



DISCUSSION

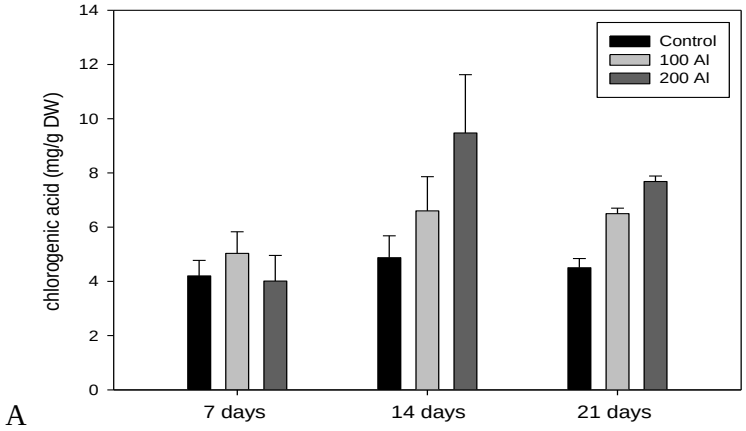
The phenomenon of Al toxicity in plants has been extensively studied in nutrient solutions, showing that the first symptom of Al toxicity is inhibition of root elongation, which directly impacts the absorption of nutrients into the plant (Yamamoto *et al.*, 2003; Kochian *et al.*, 2005). However, no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

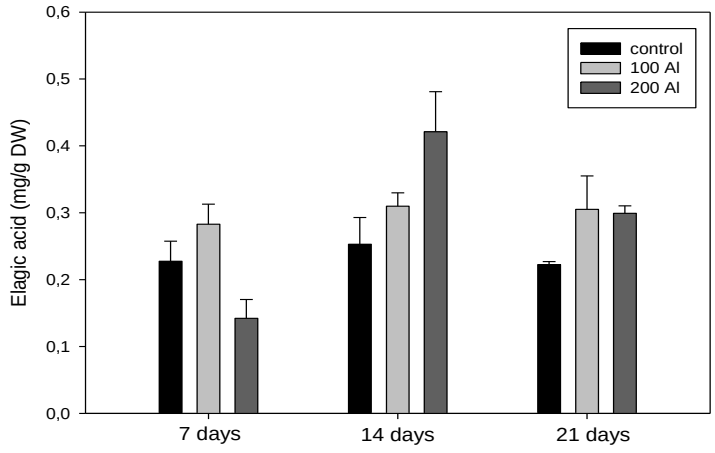
an increase in the MDA concentration in soybean, after 2 days of treatment with different doses of Al.

During the exposure period of blueberry seedlings 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.

Figure 5
Variation in the content of chlorogenic acids (A) and ellagic acid (B), in function of exposure time and the concentration of Al in seedlings of *V. corymbosum* L. Each value is a mean of three samples ± 1 s.e.



A



B

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 seedlings 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 seedlings 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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