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Interactive effects of aluminum and cadmium on phenolic compounds, antioxidant enzyme activity and oxidative stress in blueberry (*Vaccinium corymbosum* L.) plantlets cultivated in vitro

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ABSTRACT

To evaluate the potential role of phenolic compounds in Al and Cd stress tolerance mechanisms, *Vaccinium corymbosum* cv. Legacy plantlets were exposed to different metal concentrations. The present study used an *in vitro* plant model to test the effects of the following treatments: 100 μM Al; 100 μMAl + 50 μM Cd; and 100 μMAl + 100 μM Cd during periods of 7, 14, 21 and 30 days. The oxidative damage was determined by the accumulation of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2). The antioxidant activity values were determined using 1,1-diphenyl-2-picrylhydrazine (DPPH) and the ferric reducing antioxidant power test (FRAP). Additionally, the phenolic compound concentrations were determined using HPLC-DAD. The exposure to Al and Cd increased the MDA and H_2O_2 contents differentially, while the antioxidant capacity values showed differences between DPPH and FRAP with the largest changes in FRAP relative to Cd. SOD had the highest activity in the first 7 days, leading to a significant increase in phenolic compounds observed after 14 days, and chlorogenic acid was the major compound identified. Our results revealed that phenolic compounds seem to play an important role in the response to ROS. Therefore, the mechanisms of tolerance to Al and Cd in *V. corymbosum* will be determined by the type of metal and time of exposure.

1. Introduction

The accumulation of phenolic compounds in plants is a response to stress caused by toxic elements, either through the reduction of oxidative stress or the ability to chelate metal ions (Michalak, 2010). The antioxidant activity of phenolic compounds is determined by their chemical structure, type, position, and number of functional groups, which finally determine their electron-donating activity, deprotonation equilibrium and radical stability (Cao et al., 1997; Kanski et al., 2002; Cheng et al., 2002). Through their functional groups, both hydroxyl (-OH) and carboxylic acid (-COOH), phenolic compounds are able to bind metals, reducing their harmful effects on a plant (Winkel-Shirley, 2002; Michalak, 2010). Plants exposed to heavy metal stress have been observed to exude high levels of phenolic compounds (Tolrà et al., 2005; Kováčik et al., 2008, 2010; Mongkhonsin et al., 2016). The action of phenolic compounds complements the activity of antioxidant enzymes, the main ROS-scavenging mechanism, especially the superoxide dismutase (SOD) enzyme, which prevents cell damage by catalyzing the superoxide anion conversion to H_2O_2. Different studies have shown that the SOD activity depends on both the plant species and the type of stress to which it is subjected (Posmyka et al., 2009; Zouari et al., 2016). For example, decreases in the activity of some antioxidant enzymes in the presence of elements such as Cd have been observed (Fidalgo et al., 2011; He et al., 2011; Huang et al., 2005).

Cd and Al are among the elements that cause high toxicity in plants, Cd due to its high mobility in natural systems and Al because of its high concentrations in acid soils, limiting the productivity of crops (Sanita di Toppi and Gabbrielli, 1999; Khan et al., 2017).

As result of regular agricultural practices, both Al and Cd can be simultaneously present in the soil solution, causing oxidative stress resulting from the generation of ROS. In plants, exposure to cadmium induces phytotoxicity symptoms, such as chlorosis, reduction of biomass, inhibition of root elongation and even cell death (Lux et al., 2011; Rodojčić Redovniković et al., 2017). It has been observed that the presence of Cd indirectly generates free radicals by substituting for Fe in several proteins, increasing the free content of Fe, resulting in the

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production of hydroxyl radicals through the Fenton reaction, which finally damages plant tissues (Liu et al., 2007; Cuypers et al., 2011). A recent study from our laboratory suggests that blueberry plantlets produce phenolic compounds with reducing capacity as a protective mechanism against stress caused by Cd (Manquián-Cerda et al., 2016).

In the case of aluminum, it has been reported that micromolar concentrations in the soil solution can inhibit root elongation, a consequence that can affect the incorporation of water and nutrients (Barceló and Poschenrieder, 2002; Giannakoula et al., 2010) and increase the production of reactive oxygen species (ROS) (Bontempo et al., 2013; Feng et al., 2013; Zhao et al., 2017). It is estimated that Al^{3+} induces oxidative stress due to its high affinity for ligands with phosphate and carboxylic groups that possess donor oxygen atoms that bind with the phospholipid membrane causing its rigidification (Ryan et al., 2001). In *Rumex acetosa* L., Al induced high levels of phenolic compounds (*i.e.,* chlorogenic acid, catechin, and rutin) in shoots that may bind Al, decreasing its toxicity (Tolrá et al., 2005).

Vaccinium corymbosum L. is a species with nutraceutical properties due to its high content of antioxidant molecules in its fruits and leaves (Giovanelli and Buratti, 2009; Dragović-Uzelac et al., 2010). It grows mainly in acid soils with a pH between 4.8 and 5.5, which are usually found in Chilean volcanic ash-derived soils. However, among the problems presented by growing this crop in acidic soils, such as those of volcanic origin, is the low concentration of exchange bases (Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}), high rainfall and the use of fertilizers (urea and ammonium phosphate), causing soil acidification, which increases the phytotoxicity of aluminum (Mora et al., 2009). On the other hand, phosphate rock and triple super phosphate are intensively used as fertilizers abd provide Cd (up to 288 mg per kg of P) (Bonomelli et al., 2002; Molina et al., 2009). Cadmium accumulates in agricultural soils because, in general, the inputs (fertilization, irrigation water and atmospheric deposition) to the soil are larger than the outputs (plant uptake and leaching) from this medium to the biosphere. For example, studies in highly intensive maize (*Zea mays* L.) production systems indicate that the use of phosphate fertilizers over extended periods of time could increase the Cd levels in maize-cultivated soils (Molina et al., 2010). This manifests itself in the first half of the growing season during which the Cd absorption rate is higher than that during the rest of the season (Milone et al., 2003). In the case of blueberries, it has been observed that although they have a lower requirement than most crops, the use of phosphate fertilizers is commonly employed (Bryla and Strik, 2015).

Thus, plants growing in the presence of elements such as Al and Cd should regulate the synthesis, accumulation, and profile of secondary metabolites that are produced as a result of the stress induced by these elements (Hawrylak et al., 2007; Nasim and Dhir, 2010, Okem et al., 2015).

Therefore, to determine the effects of Al and Cd, i.e., two metals present in acid soils, *V. corymbosum* cv. Legacy was used as a test subject. The oxidative stress induced in the plants by the metals was measured by the accumulation of malondialdehyde and hydrogen peroxide. Along with the measurement of the antioxidant activity using the DPPH and FRAP assays, the changes in the profiles of phenolic compounds were determined.

The purpose of this work was to identify the importance and possible detoxification role of phenolic compounds in the management of oxidative stress in *V. corymbosum* cultivated *in vitro* and exposed to Al and Cd for up to a maximum of 30 days.

2. Materials and methods

2.1. Growth conditions and treatments

The growth of the blueberry plantlets followed the procedure described by Manquián-Cerda et al. (2016). These treatments were considered: (1) pH = 5.2 (Control); (2) pH = 5.2 and 100 μM of Al (Al_{100}); (3) pH = 5.2 and 100 μM of Al and 50 μM Cd (Al_{100}/Cd_{50}); and (4) pH = 5.2 and 100 μM of Al and 100 μM Cd (Al_{100}/Cd_{100}). The Al and Cd concentrations were supplied from 0.01 M $AlCl_3$ and $CdCl_2{\cdot}2H_2O$ stock solutions. The effects of Al and Cd in *V. corymbosum* were measured at 7, 14, 21 and 30 days. We used a randomized design with three replicates, and an experimental unit corresponded to 6 clones of blueberry plantlets (6 plantlets: 1 sample).

2.2. Chemical speciation of the Lloyd-McCown nutrient medium

The chemical speciation of the Lloyd-McCown nutrient medium (Lloyd and McCown, 1980) was carried out with the computer program Geochem-PC (Parker et al., 1995). The computed speciation considered not only the nutrient medium composition but also the different levels of Al and Cd used in the study and the potential presence of short chain organic acids exuded by plants roots, specifically citric and oxalic acids, which have been reported as the most significant species in *in vitro* cultures of *V. corymbosum* (Suzuki et al., 1999).

2.3. Determination of lipid peroxidation and hydrogen peroxide (H_2O_2)

Fresh materials were used in the determinations of thiobarbituric acid reacting substance (TBARS) and H_2O_2. The lipid peroxidation level was determined in terms of the malondialdehyde (MDA) concentration according to the Heath and Packer (1968) method as modified by Du and Bramlage (1992). The content of MDA was determined using an extinction coefficient of 155 nmol L^{-1} cm and was expressed as nmol g^{-1} DW.

The hydrogen peroxide content was determined using an RQflex 10 Plus Reflectoquant analyzer (Merck) and applying a sensitivity range between 0.2–20 mg L^{-1}. The results were expressed in mg g^{-1} of Dry Weight (DW).

2.4. Superoxide dismutase (SOD) activity

To determine the enzymatic activity, 0.1 g of fresh leaves were homogenized with 2 mL of 50 mM pH 7.0 potassium phosphate buffer. The samples were centrifuged at 4 °C for 15 min at 12,000 g, and the supernatant was used for the determination of this activity. The activity of superoxide dismutase (SOD; EC. 1.15.1.11) was determined by measuring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. One SOD unit was defined as the amount of enzyme corresponding to 50% inhibition of the NBT reduction, and the SOD activity was calculated on a protein basis (Donahue et al., 1997). The protein content of the enzyme extracts was determined using the method described by Bradford (1976). The protein concentration was calculated using a calibration curve made with bovine serum albumin (BSA).

2.5. Extract preparation

The preparation of the extracts for the determination of the antioxidant activity, total phenol content and HPLC analyses was performed as described by Adam et al. (2009) and as modified by Manquián-Cerda et al. (2016).

2.6. Antioxidant activity

2.6.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenger spectrophotometric assay

The free radical scavenger capacity of each extract was evaluated using the radical DPPH assay (Brand-Williams et al., 1995) as modified by Manquián-Cerda et al. (2016). The radical DPPH• has an absorption band at 517 nm. The results are expressed as % of consumed DPPH.