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EFFECT OF THE APPLICATION OF SILVER NITRATE ON ANTIOXIDANT STATUS IN WATERMELON PLANTS

MARCELINO CABRERA-DE LA FUENTE[1], HORTENSIA ORTEGA-ORTIZ[2], ADALBERTO BENAVIDES-MENDOZA[1*], ALBERTO SANDOVAL-RANGEL[1]

[1]Departamento de Horticultura, Universidad Autónoma Agraria Antonio Narro, Blvd. Antonio Narro No. 1923, CP 25315, Saltillo, Coahuila, México;
[2]Centro de Investigación en Química Aplicada, Blvd. Enrique Reyna Hermosillo No. 140, Saltillo, Coahuila, CP 25294, México
**Correspondence author e-mail: abenmen@gmail.com; ph: +52 844 411 03 03*

Abstract

In this experiment we studied the absorption of silver by watermelon plants of the variety Jubilee, as well as the effect on the lycopene content and antioxidant status. The treatments were based on a silver nitrate solution of different concentrations: 0, 30, 60, 90 and 200 $mg \cdot L^{-1}$, applied at intervals of 8 days throughout the crop cycle. The determination of silver content was performed by atomic emission spectroscopy (AES), whereas the detection of the silver particles inside plant tissues was carried out by means of scanning electron microscopy (SEM). By analyzing the data obtained in the experiment, a statistically significant difference was detected between treatments and between different plant organs. There was a greater accumulation of silver in the roots of plants and the content was positively related to the rate of application of $AgNO_3$. The antioxidant status analyzed in the fruits increased three times in the plants exposed to 30 $mg \cdot L^{-1}$ $AgNO_3$ concentration. Meanwhile, lycopene content decreased with increased concentration of silver in solution and showed the highest content with 30 $mg \cdot L^{-1}$ $AgNO_3$.

Key words: Watermelon, Antioxidants, Licopene, Silver

Introduction

Plants respond to the presence of heavy metals through mechanisms such as chelation and sequestration with phytochelatins, the metallothioneins (MT), amino acids (Cobbett & Goldsbrough, 2002; Clemens, 2006) and organic acids that are secreted from the root towards the external environment in order to regulate the absorption and accumulation in the tissues of the cited elements (Feng *et al*., 2001).

Heavy metals in ionic form induce an oxidative cellular response resulting from the generation of free radicals by the Haber–Weiss and Fenton reaction (Clemens, 2006). The extent of oxidative response depends on the concentration of metal ions and causes accumulation of lignin and antioxidants in the affected cells (Sgherri *et al*., 2003, Haider & Azmat, 2012).

The metabolism of organic acids is related to plant tolerance to environmental stress (López-Bucio *et al*., 2000). It has been found that organic acids such as citrate (Anwer, *et al*., 2012), oxalate and malate are involved in certain processes in the plant rhizosphere zone, including nutrient uptake and detoxification of metals (Pires *et al*., 2007; Pavinato *et al*., 2008).

Another possible defense response against the ions of heavy metals is transforming the ions into uncharged particles of nanometric dimensions. This adaptive response of the cells decreases the possibility of Fenton reactions to occur and hence the formation of free radicals. Gardea Torresdey *et al*., (2003) used this response in alfalfa plants for producing silver nanoparticles. The author´s procedure used silver nitrate solutions at 0, 40, 80, 160 and 320 $mg \cdot L^{-1}$ applied to seeds cultured in agar at pH 5.8. The researchers concluded that alfalfa roots are capable of absorbing and transporting silver to the rest of the plant. These silver ions were transformed to nanoparticles and distributed to all plant organs. In the present study, the hypothesis was tested experimentally that the application of silver nitrate in aqueous solution modifies the antioxidant capacity of the plants, leading to the accumulation of antioxidant in an amount that is proportional to the accumulation of silver in the tissues. The objectives were: Verify the differences in the accumulation of silver between different plant organs and determine the impact of the application of silver nitrate on total antioxidant capacity and lycopene content of watermelon fruit.

Materials and Methods

This work was performed at the facilities of the Universidad Autónoma Agraria Antonio Narro, in the laboratories of the Centro de Investigación en Química Aplicada (CIQA) in Saltillo, Coahuila, and at the Laboratory of Scanning Electron Microscopy of the Instituto Tecnológico de Uruapan, Michoacán, México. In this experiment, we used watermelon plants of commercial variety Jubilee (Crown Seed de México).

Application of silver nitrate solutions to substrate of watermelon plants: Sowing was performed in polystyrene trays using peat moss as planting substrate and the plantlets were transplanted to pots of 12 L with volcanic rock substrate and peat moss in the ratio 1:2. Plant nutrition was performed using a Douglas solution (Douglas, 1976). Silver nitrate aqueous solution was applied every week for a period of 90 days. Treatments were obtained through the application of the amount required of silver nitrate to obtain 0, 30, 60, 90 and 200 $mg \cdot L^{-1}$. The silver nitrate solutions were applied without using the irrigation system to avoid contamination. For each treatment, 30 replicates were considered and the experimental unit was one plant per pot.

Determination of silver content in plant tissues in the ICP: One gram dry weight of plant tissue was harvested at the fruit ripening stage, digested in 30 ml of concentrated nitric acid, maintaining constant volume with the acid, until all organic material was disintegrated and a yellow transparent solution was obtained. Subsequently, the sample was filtered on a Whatman #42 filter paper and diluted with 50 mL of deionized water. The analysis of the silver content in tissues was performed in an inductively-coupled plasma (ICP) optical-emission spectrometer (Thermo Jarrell Ash; Model: Irish Advantage).

Silver identification in plant tissues with scanning electron microscope (SEM): Identification of the element was performed with an SEM (JEOL JSM 6480LV), whereby complete plant body portions were placed on the plate, which is then placed inside a vacuum chamber for identification. It is noteworthy that the sample plant was analyzed without any previous preparation or coat on its surface. The analyzed portions of the sample tissues consisted of several cell layers. Once the sample was focused with the SEM beam, sample mapping was performed in order to identify the location of the silver element.

Determination of total antioxidant status of the fruits. The fruits were cut and a sample of 5 g was taken from the central part of the fruit pulp, and placed in a mortar previously cooled to 4°C with 10 mL of phosphate buffer pH 7. All was ground vigorously and the extract was then centrifuged in a Sol Bat J-12 centrifuge at 3000 rpm per 10 min.

Determination of the concentration of antioxidants was performed using a Total Antioxidant Status Assay Kit from Calbiochem (Miller *et al*, 1993) consisting of a phosphate buffer saline solution, chromogen (metmyoglobin and ABTS [radical cation 2.2 -azinobis-(3-ethylbenzothiazoline-6-sulfonate)], stabilized substrate (hydrogen peroxide), and used as vitamin E standard the analogue Trolox (6-hydroxy-2, 5, 7, 8 - tetramethyl chroman-2 -carboxylic acid) with 1.5 mM concentration.

For the analysis of samples, three reagents in the kit were prepared as follows: to the chromogen and substrate were added 10 mL buffer and 7.5 ml of pH 7, respectively, and the standard 1 ml of distilled water was added.

The spectrophotometer (Bausch & Lomb Spectronic 21) was set at 600 nm. Five minutes before use the stabilized hydrogen peroxide and chromogen were equilibrated at 37°C. The blank was prepared by adding 20 µL of distilled water and 1 mL of chromogen. The standard was prepared adding 20 µL Trolox and 1 mL chromogen. An initial absorbance reading (A_0) was taken in both cells. Then, samples of watermelon extracts were analyzed by placing 200 µL of centrifugated extract and 1 mL chromogen for each sample, they were mixed and the initial absorbance was registered. Then 200 µL of diluted stabilized hydrogen peroxide was added to each cell, mixed and initiation times were registered simultaneously. The final absorbance (A) was measured three minutes after color development. The temperature was maintained at 37°C throughout the test. To calculate the levels of antioxidants in the samples the standard Trolox concentration was used according to kit serial number (1.5 mM).

Finally, the absorbance reading at 600 nm in the spectrophotometer was registered. The gradient was determined from the absorbance (ΔA) for samples (ΔA_m), the standard (ΔA_e) and blank (ΔA_b) with the following general equation:

$$\Delta A = A - A_0 \qquad (1)$$

Afterwards, the Trolox equivalent antioxidant capacity (TEAC) in each sample was calculated using the following formula:

$$\text{TEAC (mM)} = \frac{\text{Standard Trolox concentration (1.5 mM)} \times \left[\Delta A_b - \Delta A_m\right]}{\Delta A_b - \Delta A_e} \qquad (2)$$

The result of each sample is expressed as mM Trolox equivalent·mg^{-1} fresh weight of the sample.

Determination of Lycopene in Watermelon Fruit. 3 g of watermelon pulp was taken and placed in a cooling mortar, adding 3 mL of phosphate buffer at pH 7 and ground vigorously. From this, 2 mL of sample was taken and 4 mL of mix 3:2 hexane/acetone (v/v) was added to this (Davis *et al*., 2003). This was subsequently centrifuged at 2500 rpm per 10 minutes and the supernatant solution was placed in a cell and the absorbance at 502 nm was registered. Lycopene content was calculated by using the formula (Fish *et al*., 2002):

$$\text{Lycopene } (\mu g \cdot g^{-1}) = A_{502} \cdot [1/320] \cdot 4 \qquad (3)$$

The silver content, antioxidant capacity and lycopene content were analyzed using SAS software (Anon., 1998). Test of means differences was realized by Tukey's method with a 0.05 level of significance.

Results and Discussion

For the case of silver accumulation in the roots of watermelon plants a statistical difference was detected between treatments. The control treatment showed a low content compared to the others treatments under study. The application of 200 mg·L^{-1} $AgNO_3$ showed a greater accumulation of silver in the roots (Fig. 1). The SEM analysis of the root tissue of that sample determined the apparently homogeneous distribution of the metal in the root tissue (Fig. 2). This homogeneous distribution may be due, on the one hand, to the SEM sample consisting of an undetermined number of cell layers of the different tissues of the root and, on the other hand, to the known tendency of vegetal cells to accumulate metal ions in the vacuole (Clemens, 2006), which could explain the pattern groups (clumps) of detection by the SEM. It is known that the root is the main structure of uptake and accumulation of heavy metals by plants (Hirsch, 1998; Mukherjee *et al*., 2001; Shankar *et al*., 2004; Nazir *et al*., 2011), apparently due to poor mobility of these from the external cortex to the inner vascular tissues (Uda, 1995; Ouzounidou & Constantinidou, 1999).

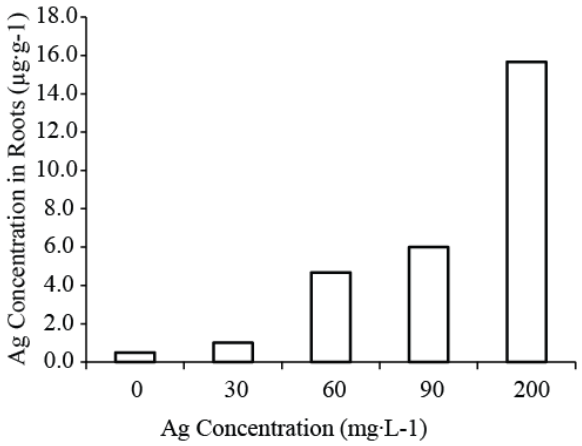

Fig. 1. Silver concentration in the roots of watermelon. The plants were treated with different concentrations of silver nitrate. The roots were collected during the harvesting stage and analyzed with ICP.

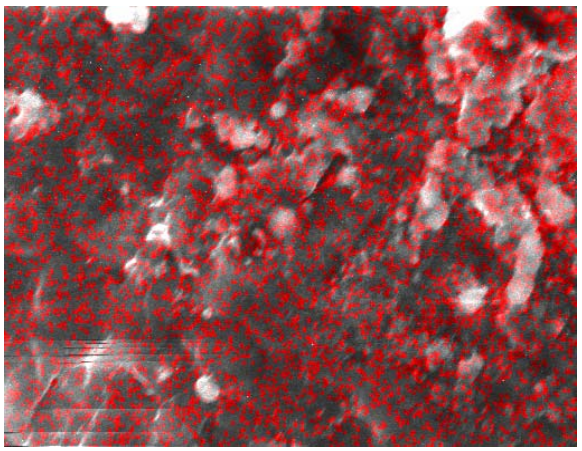

Fig. 2. Image of SEM for the detection of silver in the root tissue of watermelon treated with applications of silver nitrate (200 mg·L^{-1}). The red dots indicate the highest density of silver accumulation.

With respect to the silver content in the stems of watermelon plants there were statistical differences between treatments (Fig. 3). The SEM analysis of the stem sample of the treatment with 200 mg·L^{-1} showed an apparently homogeneous distribution of the silver throughout the tissue (Fig. 4) as was observed in the root. It is well known that metal ions are strictly regulated in terms of their concentration in the cytoplasm by a number of mechanisms such as proton pump, activation of channels, complex formation with organic acids or specialized proteins, sequestration in vacuoles or cell wall, extrusion by roots and leaves and the formation of uncharged metallic nanoparticles (Cobbet, 2000, Cobbet & Goldsbrough, 2002, Mittler, 2002). The activation of these mechanisms occurs in response to exposure to ions of toxic elements (Sharma *et al*., 2004; Clemens, 2006), and allow the exclusion or absorption, accumulation, remobilization and sequestering of the element in the entire plant (Tangahu *et al*., 2011).

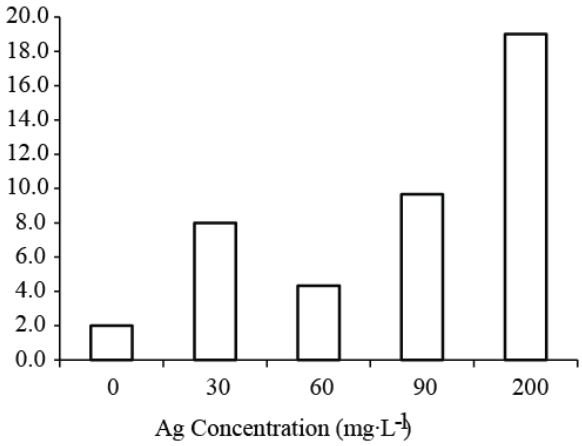

Fig. 3. Silver content in the stems of watermelon plants. The plants were treated with different concentrations of silver nitrate. The roots were collected during the harvesting stage and analyzed with ICP.

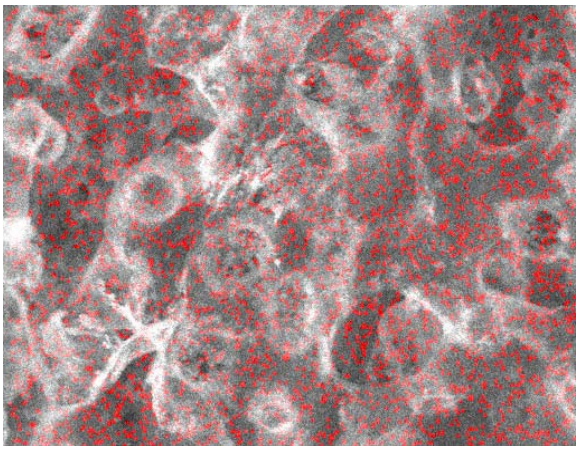

Fig. 4. Silver content in the stems of watermelon plants observed by SEM. The red dots indicate the highest density of silver accumulation.

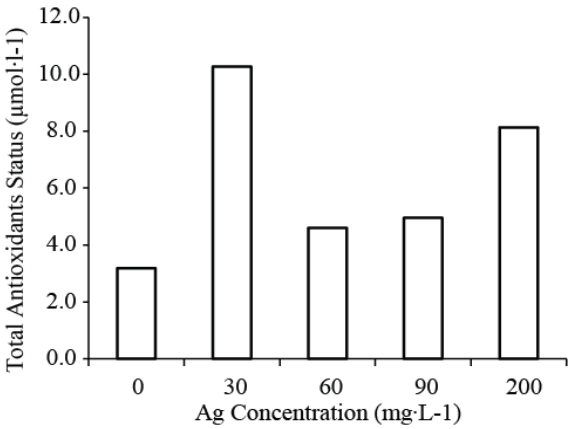

Fig. 5. Total antioxidant status of watermelon fruit treated with silver nitrate. The plants were treated with different concentrations of silver nitrate. The roots were collected during the harvesting stage and analyzed with ICP.

Silver was also found in the leaf tissues of the plant with average concentrations of 1-5 $mg \cdot kg^{-1}$, however, there were no statistical differences between treatments. The content of silver in fruits, according to analysis by ICP, were not detectable. There was a highly significant difference between plant organs with respect to the silver content, being the highest concentration found in roots followed by stems and the lowest concentration in leaves.

Total antioxidant status of the fruits: The plants obtained with applications of 30 $mg \cdot L^{-1}$ silver nitrate showed the greatest total amount of antioxidants in fruits (Tukey, $p \leq 0.05$) (Fig. 5). Our results differ from those found by Sahandi *et al*., (2011), where the contents of polyphenols and tannins in vegetal tissue showed an increase proportional to the concentration of silver nitrate in the range of 100 to 300 $mg \cdot L^{-1}$.

Lycopene content: There were no statistically significant differences between treatments for lycopene content. The maximum content of lycopene was 2.73 $\mu g \cdot g^{-1}$ and this value is very low compared with the 47.8 $\mu g \cdot g^{-1}$ reported by Perkins *et al*., (2001). A partial explanation of this result was given by Davies *et al*., (1990) who found that the exogenous application of $AgNO_3$ decreases the synthesis of lycopene. Another factor that probably affected the synthesis of lycopene, without effect on total antioxidant status, was the temperature above 30°C which was presented during the fruit development. Thermal ranges above this level negatively affect the synthesis of lycopene (Lurie *et al*., 1996).

Conclusions

Most of the silver absorbed by plants was found to be concentrated in the roots, with smaller quantities of this element in the stems and leaves.

By adding silver nitrate to watermelon plants it was possible to modify the total antioxidant status in fruits. The antioxidant status increased threefold in the plants exposed to a 30 $mg \cdot L^{-1}$ $AgNO_3$ concentration. Meanwhile, lycopene content showed no response to $AgNO_3$ application.

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