MANGANESE'S EFFECT IN THE SHEET APOPLAST *HIBISCUS SABDARIFFA* L. VAR *SABDARIFFA* FOR 10 DAYS.

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Abstract

Young shoots of sorrel leaves (*Hibiscus sabdariffa* L. var *sabdariffa*) were cultured in chamber for 10 days. They were submitted to the effect of manganese concentration 0,3µM per day for studying the accumulation and the toxicity of the ore in the apoplast of leaves. The results show that Mn is accumulated in the leaves during the 10 days of experiment; the number of brown spots and callose synthesis increase during the 10 days of experience. In the leaves, the antioxidants activities Guaiacol-POD and NADP-POD are high during exposure to Mn. They are raised from fifth to ninth days for Guaicol-POD and the fifth to the seventh day of the ninth and tenth days for the NADP-POD. The activity of Guaiacol-POD and NADP-POD is evaluated to monitor their detoxification response to Mn exposure level in the leave. This correlates with the reduction in the rate of H2O2 and NADH. The Leaves of sorrel shoots have the ability to accumulate and tolerate 0,3uM Mn during 10 days of exposure by inducing the synthesis of callose and controlling the rate of Guaiacol-POD and NADP-POD.

Keywords: *Hibiscus sabdariffa*, Guaiacol-POD and NADP-POD, Callose, Manganese, Apoplast

Introduction:

The high manganese contents, interferes with the absorption and the use of other minerals (Clark, 1982), affects the energy metabolism, decreases the rate of photosynthesis (Nable et *al*., 1988) and causes oxidative stress (Christoffers-Fecht et *al*., 2003). Growing plants in natural areas but especially in polluted areas, reactive oxygen species (ROS) such as

superoxide radicals (O2 +), singlet oxygen (1O2), hydrogen peroxide (H2O2) and the hydroxyl radical (OH +) are produced during many processes. ROS damage lipids and membrane proteins, as well as nucleic acids (Bolwell et *al*., 1997) result in a reduction in the growth and development of plants (Ogawa et *al.,* 2001). Plants contain a large integrated system in enzymatic and non-enzymatic antioxidants that regulate the level of ROS and eliminate or reduce damage caused by those below (Alscher et *al*., 1997). The non-enzymatic antioxidants include ascorbate, glutathione, tocopherol, carotenoids and phenolic compounds (Home & *al*., 1994) while the enzymatic antioxidants include, guaiacol-POD, NADP-POD, superoxide dismutase [EC. 1.15.1.1 (SOD)], catalase [EC.1.11.1.6 (CAT)], ascorbate peroxidase [EC.1.11.1.11 (APx)], glutathione peroxidase [EC. 1.11.1.9 (GSH-Px)] and glutathione transferase [EC. 2.5.1.18 (GST)].

Callose synthesis in the plasma membrane related to beta-1,3-glucan synthase, is present in all living plant cells and can be activated by a variety of abiotic and biotic (Fincher and Stone, 1980). In the sorrel leaves, induction of callose formation is a very sensitive response to high concentrations of manganese. This induction occurs well before any other symptoms of toxicity or growth.

In leaves, the apoplast is a transport path of substances; H2O2 peroxidase system in the apoplast is a sensitive marker of the toxicity of Mn (Horst et *al*. 1999 Fecht et *al*. 2001).

This study focuses on the effect of daily low Mn content in the apoplast of leaves of young shoots sorrel. The accumulation of this metal in the apoplast is obtained, as the number of dark spots and the synthesis of callose. In addition, the activity of POD and Guaiacol-NADP-POD is evaluated to ascertain the capacity of the sorrel to resist oxidative stress induced by Mn in shoots of sorrel leaves.

Methods

Plant production

The sorrel seeds are sown in pots placed in a growth chamber under controlled environmental conditions to hydroponic at 30 ° C day and 25 ° C night, with a relative humidity of 75% +/- 5%. After germination in 1 mM CaSO4, seed seedlings are transferred to a constantly aerated nutrient solution. After the pre-culture, the concentration of MnSO4 in the nutrient-rich solution is increased, while the control plants continuously receive 0,3uM Mn for 10 days. The control solution is repeated two to three times a week to avoid nutrient deficiencies.

Quantification of symptoms of toxicity

Symptoms of toxicity of Mn on leaves *Hibiscus* after 10 days of exposure are measured by counting the number of dark spots on a 1cm2 surface at the base of each leaf. Specifically, in the middle and top of the upper side of each sheet.

Mineral Analysis

The amount of manganese in the tissue mass of leaves after treatment is determined after drying at 480 ° C for 8 hours in a dehydrator. Dried and ground leaves are dissolved in 6 moles of hydrochloric acid with 1.5% by hydroxylammonium chloride. The obtained solution is diluted to 1/10 th with water. The measurements of the amount of Mn present in the dilute solution are obtained by optical emission spectroscopy.

Detection and removal of callose

Four leafs discs of sorrel 150mg in total are cut and fixed in ethanol to measure callose formation. After 3 days, the ethanol was replaced by demineralized water. The leaf discs are incubated in deionized water overnight. These discs are homogenized in 1 mL of sodium hydroxide (NaOH, 1M). The homogenate is incubated for 15 min at 80 ° C in a water bath. After centrifugation for 5 minutes 13,00g, 100ul of supernatant is mixed with 600uL mix aniline (0.59 M Gly buffer (pH = 9.5), 0,21M HCl and 0.04% (w / v) blue aniline). The obtained solution is incubated in water at 50 ° C for 20 minutes. After incubation, the concentration of callose is measured by detecting the fluorescence excitation (400nm / 30nm) and emission (485 nm / 40 nm) with a microplate reader. Witnesses measurements are made from the Gly-HCl buffer solution without aniline blue. For calculations, the molar ratio of excitation is used 11,32mgL e = -1.

POD activity in the protein solution of leaves apoplast

Guaiacol-POD in the protein solution to the apoplast of hibiscus leaves is determined spectrophotometrically with lambda = 470 nm by following the evolution of the H2O2-dependent oxidation of guaiacol. The samples were mixed with 20 mM solution of guaiacol in 10 mM Na2HPO4 buffer (pH = 6) with 0.03% H2O2).

The activities of NADH-POD in the leaves of protein solution are determined by spectrophotometry *Hibiscus* with lambda = 340nm following the decrease of NADH, 1.6mm p-coumaric acid and 16mm MnCl2 in 100 mM NaAc buffer (pH = 5).

Results

Assimilation Mn

Leaves of *Hibiscus* were treated daily 0,3uM Mn for 10 days. The Mn content in the leaves of seedlings *Hibiscus* evolves in four phases. A first stationary phase from day 0 to 1 (Cmn = 0,3uM), a second heating phase from day 1 to 3 (* Cmn = 0,9uM), a third phase of decreasing day 3 to 4 (Cmn = 0,1uM) and a fourth exponential phase after the fourth day to the 10th day (Cmn = 1.8 pM) (Fig. 1).

* Cmn: Mn content in the leaves of young shoots of Hibiscus.

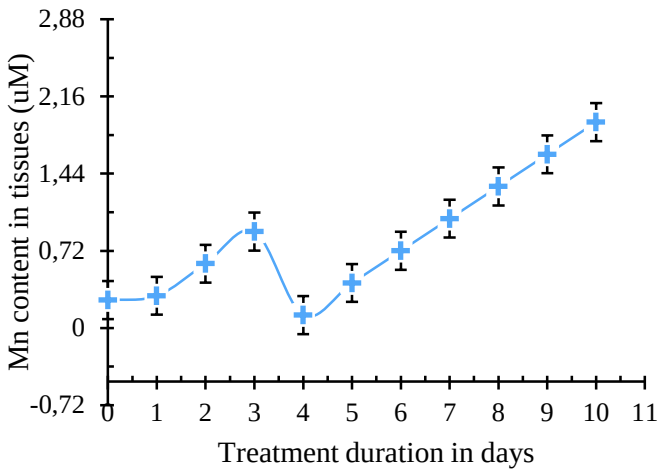

Figure 1: Daily assimilation 0,3uM Mn in young shoots of leaves of Hibiscus for 10 days under controlled environmental conditions. The bar represents the average of the different parameters and the values shown are the mean +/- standard deviation of n = 3. The Mn content in the tissues of leaves of young shoots sorrel is correlated with the duration of treatment with R2 = 0.7273 and y = 0.0827 + 0,1507x.

Concentration of Mn in the apoplastic liquid leaves of young shoots of sorrel

In the treated leaves of Hibiscus, the concentration of Mn in the apoplastic liquid increases gradually until the tenth day. The increasing evolution of Mn content reveals two phases of activity in the cytoplasm and in the apoplast. At the apoplast activity Mn content is passive (of 0-1 uM Mn) and is active in the cytoplasm (Cmn> 1)

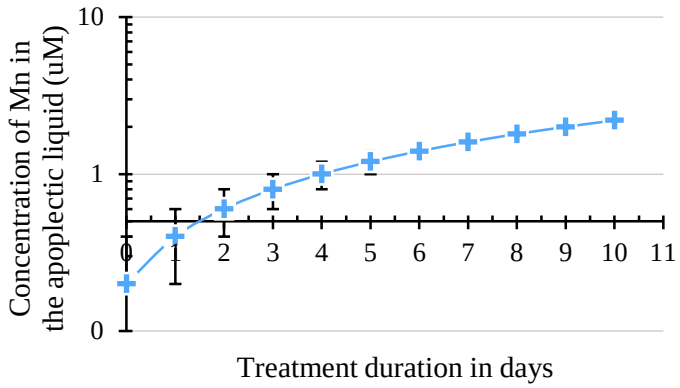

Figure 2: Evolution of the Mn content in the apoplast of leaves of young shoots *Hibiscus* for 10 days under controlled environmental conditions. The bar represents the average of the different parameters and the values shown are the mean +/- standard deviation of n = 3. The concentration of Mn in the apoplastic fluid is correlated with the duration of treatment with R2 = 1 and y = 0.2 x + 0.2.

III. 3. Ratio of Mn content and apoplastic liquid:

The ratio of Mn in the apoplastic liquid and the Mn content in the tissue is 0.2% at t = 0. After one day of treatment, he believes sawtooth (0.9%). And then decreases until the initial report: 0.2%. Increasing the concentration of Mn provides a triphasic curve with specific optimal. The optimum first appears after the first day of treatment, the second after the third day, with an increase of Mn in the tissues and the third to sixth optimum days. The shape of the curve shows that after the 10th day that the distribution of Mn level found between the two compartments (cytoplasm and apoplast).

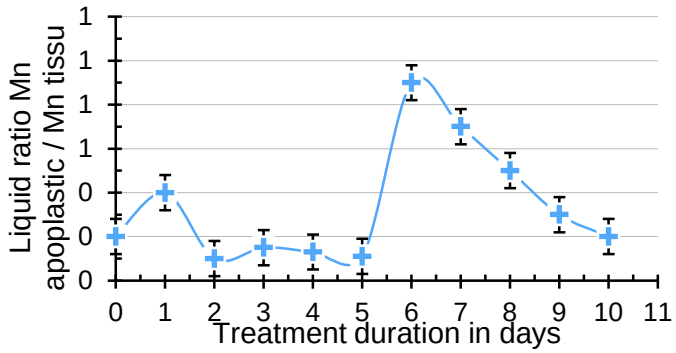

Figure 3: The ratio between the manganese content in the apoplastic fluid and that present in the tissues of young leaves of sorrel exposed for 10 days. The bar represents the average of the different parameters and the values shown are the mean +/- standard deviation of n = 3.

Evaluation of the number of brown spots in the tissues of the leaves of young shoots of sorrel

The number of brown spot is missing in the tissues of the leaves of young shoots of sorrel at baseline (day = 0) to the first day of treatment. Spots appear from the 1st to 3rd days in accordance with the increase in Mn in tissues. The decrease in Mn of the third and fourth days does not cause the decrease brown spots which have a concave trend of the number of brown spots in the tissues. Fourth to seventh days growth in the number of spots is changing significantly with the increase of Mn in tissues. From 4th to 5th day, specifically, the Mn content increases again, the environmental stress caused appears to induce the increase in the number of spots in the tissues of young leaves sorrel. The increase of the 7th to 10th spots days continues to increase proportionally to the increase in Mn content.

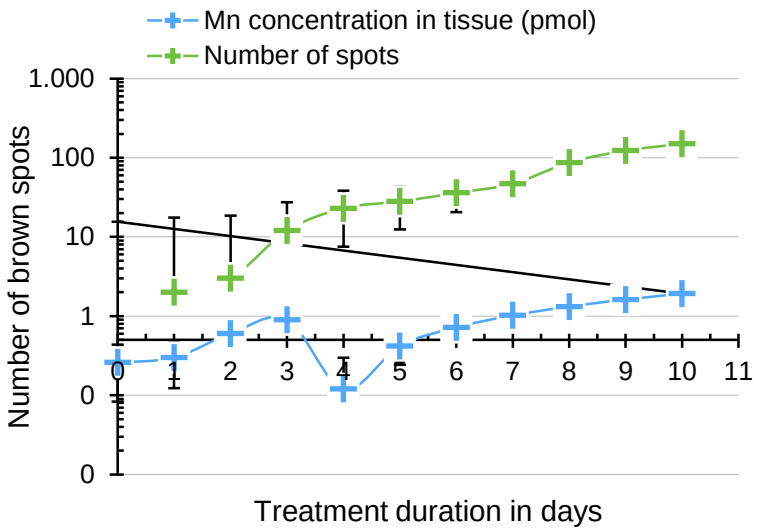

Figure 4: Number of brown spots according to the processing time for 10 days. The bar represents the average of the different parameters and the values shown are the mean +/- standard deviation of n = 3. The number of brown spots is correlated with the concentration of Mn in the leaf tissues of seedlings sorrel with R2 = 0.8468 and y = 14,264x-24,864.

Estimated callose content in the leaves of young shoots of sorrel

The evolution of the concentration of Mn in the tissues of leaves of young shoots of sorrel and content in this callose, similarly, a three-phase development. From the first to the fourth day, the seventh day of the fourth and seventh to the tenth days of treatment, content callose increases with the Mn content in the tissues of sorrel leaves. The fourth and the seventh day

have a concave changing content in callose. The toxicity of Mn appears as the inducer of the synthesis of callose in sorrel.

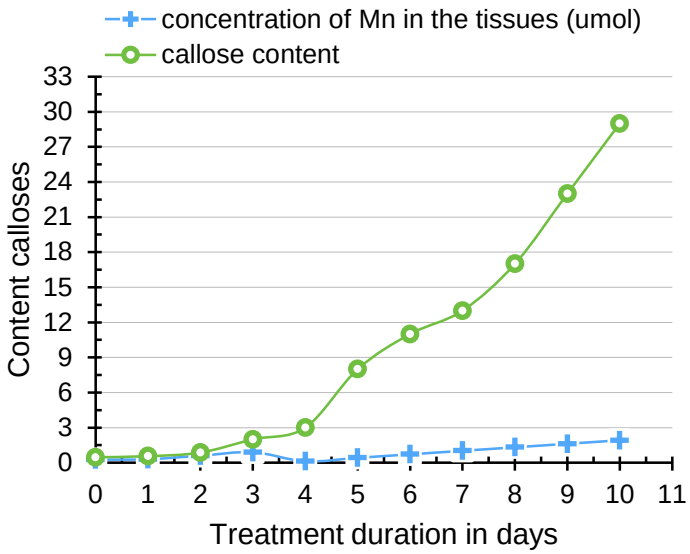

Figure 5: Relationship in callose content of the Mn concentration in the tissues of leaves of young shoots sorrel daily for 10 days of treatment Mn. Callose content is correlated with the concentration of Mn in the leaf tissues of seedlings sorrel with R2 = 0.9083 y = 2.8255 to 4.3182 with.

Guaicol-POD activity in the leaves of young shoots of sorrel

The Guiaicol-POD activity grows during treatment of Mn sorrel plants. It proportionally increases from the first to fifth day with a proportionality range of 0.3 uMmin-1. By cons, growth from fifth to sixth day is of order 0.6 uMmin-1. From the sixth to the seventh day Guaicol-POD activity decreases of 0.1 uMmin-1. And it is of order 0.9 uMmin-1 from the seventh to ninth days. The Guaiacol-POD activity decreases the ninth to the last day of treatment.

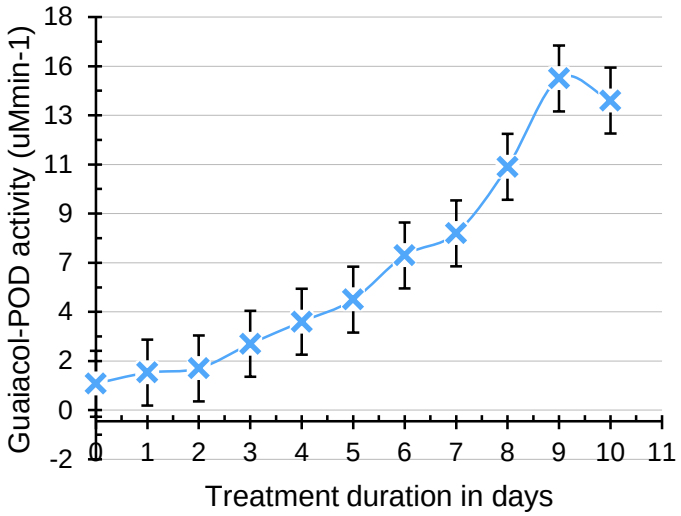

Figure 6: Guaicol-POD activity according to the duration of treatment per day for 10 days. The bar represents the average of the different parameters and the values shown are the mean +/- standard deviation of n = 3. The Guaicol-POD activity correlates with the Mn content in the young shoots of sorrel leaves with R2 = 0.9232 and y = 0.6341-1,4321x.

NADP-POD activity in the apoplast of the leaves of young shoots of sorrel

The NADP-POD activity increases gradually until the 10th day. Unlike the previous curves NADP-POD activity reveals a stationary phase the seventh to the ninth day and a strong NADP-POD rate of the ninth to the last day of treatment Mn. Nevertheless, the evolution of NADP-POD remains the same from the first to the fourth day of the fourth and seventh days as in previous curves.

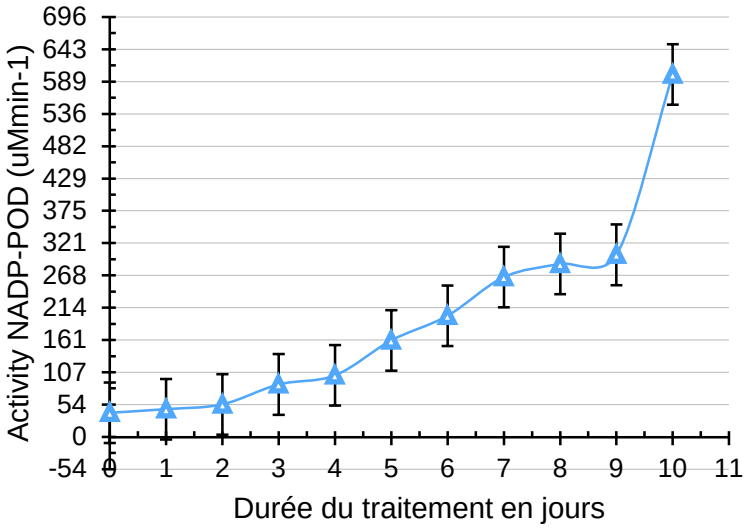

Figure 7: NADP-POD activity according to the duration of treatment per day for 10 days. The bar represents the average of the different parameters and the values shown are the mean +/- standard deviation of n = 3. The NADP-POD activity is correlated with the Mn content in the young shoots of sorrel leaves with R2 = 0.8139 and y = 45,3x-31.5.

Discussion

Young leaves sorrel submitted to the experimental conditions 30 ° C day and 25 ° C night, the stomata open when the sheets are exposed to light. The stomatal aperture triggers the activity of Mn in the apoplast sorrel cells and closing results in an increase of the activity of Mn, followed later by a decline. Epidermal cells release Mn in an acid medium due to extrusion conditions protons by sorrel cells. Acidification of the apoplast of plant cells and accumulation of Mn have been linked to explain the theory of chemiosmotic accumulation of Mn in plant cells (Zeiger 1983).

In the leaf epidermis sorrel a part of the manganese content is absorbed by the plant as a nutrient. The gradual addition of the Mn content causes the appearance of chlorosis at the leaf surface. In the cytoplasm (active area of the flow Mn) Manganese is abundantly accumulating preventing chloroplasts capture light that will be responsible for photosynthesis and thus the chlorophyll contained in chloroplasts can transform light energy chemical energy in due to the growth of sorrel. The essential role of Mn in the photosystem II (Wydrzynski, 1991) may explain the high demand in Mn for the growth of plant chlorophyll.

The disruption of the plasma membrane is a factor trigger the synthesis of callose. Poovaiah and Leopold in 1976 showed that callose formation is stimulated at very low concentrations of Mn which indicates a

function of Mn in the stabilization of the plasma membrane. Furthermore, an altered composition of the plasma membrane could be causally related to the induction of callose. Several modes of action illustrate the induction of synthesis of callose to high concentrations of Mn. Manganese can directly stimulate the (1,3) glucan synthase -f3, although Ca is more efficient (Lucas Morrow, 1986). In addition to toxic concentrations of Mn efflux of K can be increased (Waldren et *al*., 1987) indicating a disruption of the membrane properties. Ohana et *al*., 1992 have shown that endogenous activator callose synthase has been identified in different species of plants. This activator is inactive at non-stressful conditions due to the compartmentalization in the vacuole.

Conclusion:

In the apoplast of shoots sorrel the efflux of water facilitates the transport of manganese (Mn). The Mn content gradually increases in the leaves during the treatment. From a toxicity threshold Mn, sorrel leaves brown spots which marks the absence of chlorophyll and thus of the photosynthesis process. It follows a proportional induction callose due to Mn. The antioxidant activity of Guaiacol-POD and NADP-POD during treatment Mn respectively reduced the rate of hydrogen peroxide (H2O2) and NADH.

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