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EFFECT OF Ti (IV) ON Fe ACTIVITY IN *CAPSICUM ANNUUM*

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Key Word Index—*Capsicum annuum*; Solanaceae; catalase; Fe^{2+}; peroxidase; Ti.

Abstract—*Capsicum annuum*, L. Plants (cv Bunejo) were treated with Ti^{4+} ascorbate via soil, via leaves, and via soil plus via leaves. The Fe activity was measured through the catalase and peroxidase enzymes and the active fraction of Fe (Fe^{2+}) and Ti was determined in leaves, fruits, chloroplasts and chromoplasts. The Ti concentration increased in leaves and chloroplasts with Ti-treatments, but this did not bring about any variation in fruits and chromoplasts. The activities studied were enhanced by the application of Ti, and the Fe^{2+} concentration was higher in the isolated organelles and in the leaves and the fruits of the Ti treated plants. These results support the previous hypothesis that Ti^{3+}/Ti^{4+}, owing to its low redox potential, improves the Fe activity in plants, inducing a shift of the equilibrium Fe^{2+}/Fe^{3+} towards Fe^{2+} improving the Fe nutritional status of plants.

INTRODUCTION

It was found that Ti promotes some beneficial effects on plants [1–4]. It enhances some enzymatic activities such as peroxidase, catalase [1, 4], lipoxygenase [5, 6], nitrate reductase [1, 7]. It also improves plant growth and yields [3, 8] and induces nutrient uptake [4, 8, 9–11]. There is also evidence that Ti plays a role in photosynthetic processes of microalgae [12]. Furthermore, in previous results, titanium produced a higher concentration of fruit pigments [4, 13] in which lipoxygenase is implicated [6, 14], and in secondary metabolites such as malic acid and ascorbic acid [14]. All the evidence leads to the conclusion that Ti acts indirectly through activation of Fe. The only common key to all effects observed is Fe, because all of them depend directly or indirectly on it.

In the majority of work where titanium was used, it was applied as Ti (IV) ascorbate because most Ti compounds are insoluble in water. This compound is pH stable and non-toxic to living systems. Furthermore, it was demonstrated [5] that the enhancement of the biological activity of the Ti (IV) ascorbate sprayed on plants was induced by the cation (Ti^{4+}), whereas the anion (ascorbate) did not have any effect.

The aim of this paper is to investigate the localization and the effect of titanium in Fe activity, measuring the activity of peroxidase and catalase (iron dependent enzymes), and the Fe^{2+} concentration in leaves and fruits tissues and in isolated leaf chloroplasts and isolated fruit chromoplasts. The Ti and the Fe concentrations were also measured in some organs and organelles to determine if there is a direct or indirect relationship between them.

RESULTS AND DISCUSSION

The Ti concentration in leaves, fruits, chloroplasts and chromoplasts is shown in Table 1. Firstly it can be seen that, when Ti- was applied, the concentration of this element was only increased in leaves and chloroplasts. Furthermore, this concentration was greater with the two treatments in which the Ti-application was realized via leaves. In fruits and in chromoplasts, the amount of this trace element did not change on treatment, probably due to the low mobility of this element within the plants. This coincides with our previous results on field crops in which it was observed that the Ti-concentration was higher in roots than in leaves when it was applied via roots and the converse when it was supplied via leaves [1]. As the treatments were applied before fruit set, Ti remained in the leaves and it did not move toward fruits when they ripened.

The variation of the catalase and peroxidase activities in fruits and leaves of Ti-treated *Capsicum annuum* plants is shown in Table 2. Titanium produced an enhancement of these two enzymes and the highest response appeard when this element was applied via leaves. There was no increase of these enzymes in fruits in which the titanium is not present.

In earlier work [1, 5] it was demonstrated that the enhancement of both the biomass production and the biological activity of the Ti (IV)-ascorbate sprayed plants was induced by the cation (Ti^{4+}), whereas the anion (ascorbate) did not have any effect. Furthermore, Mozafar and Oertly [16] have demonstrated that the external application of ascorbic acid by roots and leaves did not increase the ascorbic acid concentration of the

buffer, pH 9.6. After overnight incubation, the plates were washed three times with washing buffer (PBS-T) [0.02 M Na_2PO_4, 0.15 M NaCl containing 0.05% (v/v) Tween 20]. Remaining sites were blocked by BSA (1% w/v in carbonate buffer) for 1 hr at 37°. Then, 100 μl of appropriately diluted culture supernatant were added. After 2 hr at 37°, the plate was washed as above and incubated with peroxidase-coupled rabbit anti-mouse IgG or M (depending on the isotype of the MAb under test) for 1 hr at 37°. Finally, after washing, each well was incubated for 30 min with 100 μl substrate soln (0.05 M sodium citrate, pH 5.5, containing 0.4 mg ml^{-1} *O*-phenylenediamine and 0.03% H_2O_2). The absorbance of the colour development was read at 490 nm [32].

Sandwich ELISA. Rabbit anti-legumin IgG coated plates were incubated with legumin (1 μg ml^{-1}) and then treated as described above.

Competitive ELISA. Microtiter plates were coated with antigen and washed as previously described for the indirect ELISA. In separate test tubes, appropriately diluted MAb was preincubated with an equal vol. of serial 10-fold dilutions of competitor antigens (11S proteins from 1 to 100 μg ml^{-1}) for 2 hr at 37°. Subsequently, 100 μl of the antibody–antigen mixture were transferred to legumin coated plates. The microplates were then incubated for 2 hr at 37°. All subsequent steps were the same as in the indirect ELISA.

Immunoblotting. Proteins and polypeptide fragments were separated by SDS–PAGE [33]. Immunoblotting was performed as previously described [34]. The proteins were applied to nitrocellulose in 0.1 M Tris–glycine buffer, pH 8.7. The blot was saturated with 1% (w/v) BSA to block remaining protein-binding sites and then incubated with appropriately diluted culture supernatant for 2 hr at room temperature. The polypeptides reacting with the MAbs were detected using peroxidase-coupled rabbit anti-mouse IgG or M (depending on the isotype of the MAb under test) and 4-chloro-1-naphthol as substrate.

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and the Fe deficiency is probably due to smaller amounts of Fe^{2+}.

It has been proposed that, in the Ti (IV) sprayed plants, the low Ti^{3+}/Ti^{4+} redox potential induces the activity of other metals in chloroplasts and cytoplasm in which Ti accumulates [1]. This higher iron activity is responsible for the enhancement of the plant biological activity which titanium produces. In fruits and in chromoplasts, where Ti concentration was not changed by the treatments, the same effect was observed. So, the fruits which import nutrients from the leaves probably also receive this active fraction of Fe, producing the same effect as observed in leaves.

EXPERIMENTAL

Red pepper plants (*Capsicum annuum* L. cv Bunejo) obtained by J. C. Costa (CRIA, Murcia, Spain) were selected for the experiments.

The assays were carried out in a crop chamber at 25° ± 3°, 65% humidity and under a maximum level of 560 μmol m^2 sec^{-1} PAR (Sylvania F86-WIGRO and Philips TDL36 w/83) at midday with 12 hr of light and 12 hr of darkness. The plants were grown on polyurethane trays (25 plants/tray) containing inert medium (acid washed sand). Five trays of each treatment were placed in the chamber.

The treatments were: (i) reference: Hoagland nutrient solution (HNS): 1.18 g l^{-1} Ca $(NO_3)\cdot H_2O$, 0.505 g l^{-1} KNO_3, 0.492 g l^{-1} $MgSO_4\cdot 7H_2O$, 0.136 g l^{-1} KH_2PO_4, 2.86 mg l^{-1} H_3BO_3, 0.22 mg l^{-1} $ZnSO_4$, 0.015 mg l^{-1} $(NH_4)_6MO_7O_{24}\cdot 4H_2O$, 0.08 mg l^{-1} $CuSO_4\cdot 5H_2O$, 1.53 mg l^{-1} $MnSO_4\cdot H_2O$, 16.67 mg l^{-1} Fe-EDDHA (6%Fe). (ii) Ti_R: HNS contained 2 mg Ti l^{-1}, pH 6, via soil [Titavit, Ti (IV)-ascorbate complex, 1 g Ti l^{-1}, donated by chemolimpex, Budapest]. (iii) Ti_L: HNS and one application 2 mg Ti l^{-1}, pH 6, leaf spray treatment. 50 ml $plant^{-1}$, 3.5 months after sowing (before the set fruit). (iv) $Ti_{R\&L}$: both above together. The plants of each tray formed a sample from which aliquots for analytical determination were taken.

Crude homogenate. The leaves were washed and cut into short segments. This fresh material 3 *g* was cold homogenized in 50 mM K-Pi buffer, pH 7, containing 0.2% Triton X-100 (1:3, w:v), filtered through 4 layers of nylon cloth and centrifuged at 8000 *g* for 15 min at 4°. Peroxidase activity was measured in crude extracts. 0.1 ml of the homogenate was added to 2.5 ml pyrogallol reagent (10 ml 0.5 M pyrogallol and 12.5 ml 0.1 M KPi buffer, pH 6, diluted up to 100 ml with H_2O) in a colorimetric tube, and then 0.02 ml of 1% H_2O_2 were added. The time in sec which was required for the change of the *A* from 0 to 0.4, owing to pyrogallol oxidation, was measured [25]. The enzyme activity is expressed in relative peroxidase units (PU). Catalase activity was determined from the change in *A* at 240 nm when 0.1 ml of crude extract was added to a 10.6 mM H_2O_2 sol during 2 min, which gave an *A* of *ca* 0.5 in 0.1 M K-Pi buffer, pH 7 [26]. The decomposition of H_2O_2 can be followed directly by the decrease in *A* at 240 nm (ε_{240} = 0.00394 ± 0.0002 1 $mmol^{-1}$ mm^{-1}). The difference in *A* per unit time is a measure of catalase activity (CAU).

Chloroplasts isolation. Chloroplasts were prepared as described in ref. [28]. For the mechanical preparation of chloroplast, 20 g leaves, cut into 2 cm segments, were homogenized in 100 ml of a semi frozen grinding medium containing 0.33 M sorbitol, 10 mM EDTA, 5 mM $MgCl_2$, 0.1% (w/v) BSA, 0.2% (w/v) Na D-isoascorbate and 20 mM Hepes (pH 7.6) for 4 sec in a polytron blender. The homogenate was squeezed through four layers of Miracloth and placed in centrifuge tubes. The cellular debris was eliminated by a 5 min centrifugation at 150 *g* and then spun at 7000 *g* for 50 sec. The pellet was resuspended in *ca* 15 ml of a soln containing 0.14 M sorbitol, 10 mM EDTA, 5 mM $MgCl_2$, 0.1% (w/v) BSA and 20 mM Hepes (pH 7.3) and centrifuged at 7000 *g* for 30 sec. The pellet was resuspended in 3 ml of the above sol.

Chromoplast isolation. Intact chromoplasts were isolated from the red fruits of peppers using the method described in ref. [29]. The fruit pericarp (50 g) was cut into small segments and incubated in 100 ml of a medium containing 0.4 M sucrose, 1 mM EDTA, 1 mM 2-mercaptoethanol, 50 mM Tris–HCl buffer (pH 8). The material was homogenized in a blender for 3 × 3 sec and then filtered through four layers of Miracloth. The cellular debris was eliminated after a 5 min centrifugation at 150 *g*. The chromoplast fraction was pelleted by a 10 min centrifugation at 2000 *g*, and then resuspended in extraction medium. The crude chromoplast suspension was laid on the top of a tube containing a discontinuous sucrose gradient: 0.45 M, 0.84 M, 1.45 M sucrose buffered with 50 mM Tris–HCl (pH 7.6). After 1 hr centrifugation at 62 000 *g* the intact chromoplasts were found in the 0.84–1.45 M interface [28]. Chromoplasts were removed with a pasteur pipette. The sucrose concn was slowly reduced to 0.4 M with 50 mM Tris–HCl (pH 7.6), the intact chromoplasts were pelleted after a centrifugation at 2000 *g* for 10 min and resuspended in 50 mM Tris–HCl (pH 7.6) with 10 mM dithiothreitol.

Fe^{2+} *determination.* Leaves and fruits (1 g) were cut into small segments and the suspension of chromoplasts and chromoplasts (1 ml) were homogenized with 10 ml *o*-phenanthroline 1.5% (pH 3 with M HCl) and filtered through 2 layers of whatman N° 2 filter. The Fe^{2+} concn was estimated directly in the filtrate by measuring the *A* at 510 nm [18].

Ti determination. Leaves, fruits, choloroplasts and chromoplasts were dissolved in 5 ml 6 M HNO_3, heated at 90° for 30–60 min and the vol was made up to 25 ml with H_2O. The measurement was carried out according to ref. [30]

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