Detoxification Processes from Vanadate at the Root Apoplasm Activated by Caffeic and Polygalacturonic Acids

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Abstract

In the root apoplasm, V(V) and V(IV) toxicity can be alleviated through redox and complexation reactions involving phenolic substances and the polyuronic components. In such context we report the role of polygalacturonic acid (PGA) on the reducing activity of caffeic acid (CAF) towards V(V). The redox reaction was particularly effective at pH 2.8 leading to the formation of oxidation products with redox activity towards V(V). An o-quinone was identified as the first product of the reaction which is further involved in the formation of CAF dimers. At pH 3.6 the redox activity decreased and a yield in V(IV) equal to 38, 31, 21 and 14% was found at pH 3.6, 4.0, 5.0 and 6.0 respectively compared with that obtained at pH 2.8. The redox reaction was faster in the presence of PGA and a higher yield of V(IV) was found in the 4.0–6.0 pH range with respect to the CAF-V(V) binary system. The higher efficiency of the redox reaction in the presence of PGA was related with the ability of PGA to bind V(IV). The biological significance of the redox reaction between CAF and V(V), as well as the role of PGA in such reaction, was established "in vivo" using triticale plants. Results showed that PGA reduced significantly the phytotoxic effects of the V(V)-CAF system.

Introduction

Vanadium is nowadays the object of a great attention as environmental pollutant. Its release, due to both natural erosion processes and human activities, such as the widespread use of fossil fuels [1] and phosphate fertilizers, may cause a severe pollution of the soil-water-plant system [2,3].

The mobility and bioavailability of vanadium in the environment is strictly related to its oxidation state, being V(IV) and V(V) the most stable species [4]. In soil, V(IV) is generally precipitated as hydroxide or adsorbed onto mineral surfaces or complexed by organic ligands [5]. Thus, V(IV) has relatively low mobility with respect to V(V) which is more toxic. Recent studies suggest that vanadium is essential for the living systems being an important cofactor in vanadate dependent haloperoxidases and vanadium nitrogenases [6]. Moreover, as phosphate...
analogue, vanadate can influence the behaviour of several important enzymes such as phosphorylases, mutases, phosphatases, ribonucleases, and ATPases [7]. There are firm evidence that vanadate is absorbed by plant tissues [8,9] and can inhibit the plasma membrane hydrogen (H+) -translocation ATPase. Further, V(V) was found to retard the growth of tomato plants and accumulate at higher levels in roots [10]. On the other hand, similar effects were induced by the oxovanadium(IV) cation which drastically altered soybean nutrition and reduced plant biomass when added to hydroponic solutions [11]. Morphological changes of roots and leaves of *Phaseolus vulgaris* were also reported for plants exposed to V(IV) [12].

Caffeic acid (3,4-dihydroxycinnamic acid), is recognized to have an important role in several biochemical processes associated in lowering the risks of cancer and cardiovascular disease being able to act as antioxidant and protect against free radicals and viral infection [13,14]. In addition, caffeic acid and its derivates, play an important role in the uptake of iron by plants through its reduction to Fe(II) [15–18] and on the detoxification processes of heavy metals such as Cu(II) and Cr(VI) through their reduction to Cu (I) and Cr (III) [19–21]. Similarly, CAF might be active in the reduction of V(V).

In order to determine the mechanism through which CAF could operate the reduction of V (V) and to evaluate the possible role of the root polyuronic components in this mechanism, the V(V)-CAF system was studied in the aqueous phase, as a function of time, in the presence and absence of polygalacturonic acid (PGA), in the 3.0–6.0 pH range and at different V(V)/CAF molar ratios.

Polygalacturonic acid, the main component of root apoplasm and root cape slime [22–25], plays an important role in the sorption of micro- and macronutrients by plants being able to bind them through the formation of complexes of different nature [26]. Electrostatic interactions occur for some ions such as Ca(II), Mn(II) and Zn(II) whereas inner-sphere binding through carboxylate groups occurs for ions such as Cu(II) and Pb(II) [27].

To evaluate the potential biological significance of the CAF-V(V) system, as well as the role of PGA on the V(V)-CAF interaction, triticale plants (x *Triticosecale* Wittm.) were grown at pH 6.0 in hydroponic solutions containing V(V), V(V)-CAF and V(V)-CAF-PGA. Measurements of root and shoot length were used to estimate the phytotoxicity of each system.

**Materials and Methods**

**Chemicals**

All reagents were obtained from Fluka. Sodium metavanadate and vanadyl sulfate were employed as V(V) and V(IV) source, respectively. All solutions were prepared just before the beginning of each experiment using Millipore MilliQ ultra-pure water and brought to the working pH by adding 0.001 N HClO4 or NaOH. Sodium perchlorate monohydrate was used as the supporting electrolyte at 0.01M.

**V(IV) and V(V) determination**

The redox reaction was studied under weak acidic aqueous condition to exclude the autoxidation of caffeic acid which is particularly active at pH ≥ 7.0 [28]. The systems were kept under stirring at room temperature for all the test time. Kinetic measurements were carried out at room temperature on systems containing 60 μM CAF and V(V) concentration ranging from 30 to 120 μM. The systems were prepared by mixing appropriate aliquots of the CAF and V(V) solutions, brought separately to pH 2.8, 3.6, 4.0, 5.0 and 6.0 by addition of HClO4 or NaOH. To determine V(V) and V(IV), aliquots of stock solutions, buffered at pH 4.0, were passed through a Chelex-100 resin (previously conditioned with sodium acetate buffer at pH 4.0) in 5 ml glass columns (4 ml of hydrated resin). In this way the V(IV), formed during the reaction,
was removed from solutions while V(V) was collected in the eluate. After rinsing the resin with water (15 ml), vanadium (IV) was eluted completely with 1N HCl (three bed volume). V(IV) and V(V) were then quantified using a Varian atomic absorption spectrophotometer with a Varian graphite furnace tube atomizer. The detection limit was 4 μg/L. V(V) was also determined in the form of V(V)-Desferri-Ferrioxamine B complex, whose absorbance was measured at 455 nm using an Agilent Technology Cary 60 UV-Vis spectrophotometer.

**CAF and oxidation products determination**

CAF and its oxidation products were determined by HPLC as previously described [21]. Briefly, a Dionex DX-300 system, equipped with an UV-Vis Merk Hitachi Diode Array detector, and an Alltech Alltima C18 5U column was used. A H₂O-acetonitrile-acetic acid (77.5%-17.5%-5.0%) mixture, brought to pH 3.5, was employed as the eluent at a flow rate of 0.5mL min⁻¹ at room temperature.

The mass of CAF and its oxidation products were achieved through their separation in LC-MS using an Agilent Technologies (Palo Alto, CA, USA) 1100 series LC/MSD equipped with a diode-array detector (DAD). A chemstation HP A.10.02 was used for data analysis. The chromatographic separation was achieved using a Luna C18 (150mm×4.6 i.d., 3 μm, Phenomenex, USA). The photodiode array detector was coupled to a mass spectrometer (quadrupole analyzer) directly to the sprayer needle where ions were generated by electrospray ionization (ESI) in both positive and negative ionization modes. Nitrogen was used as nebulizing and drying gas and different fragmentorvoltages were applied. Full scan data acquisition was performed scanning from 100 to 500 m/z using a cycle time of 2 s with step size of 0.1 μ.

**V(IV)-PGA system**

The sorption of V(IV) by (PGA) were carried out at pH 3.0, 4.0, 4.5, 5.0 and 6.0 by dipping the polysaccharidic matrix (25 mg), into 100 mL of solutions containing 12 μmol of V(IV) (120 μM). The amount of V(IV) sorbed was quantified by the difference between the initial concentration and that found at equilibrium. The FT-IR spectra of the V(IV)-PGA samples, centrifuged at 5000 rpm (5°C), washed several times with water, dehydrated and stored under vacuum, were recorded on KBr disks (2 mg of sample in 100 mg of KBr) with a Nicolet 380 spectrophotometer (Thermo Fisher Scientific).

**V(IV)-, V(V)-, PGA-, -CAF-triticale plants (x *Triticosecale* Wittm.) systems**

In order to determine the biological relevance of the redox reaction between V(V) and CAF, in the presence and absence of PGA, triticale plants (x *Triticosecale* Wittm.) were grown in the following aqueous solutions at pH 6.0: i) 120 μM V(V); ii) 120 μM V(V) + 120 μM CAF; iii) 120 μM V(V) + 120 μM CAF + 0.25 mg/mL PGA (equivalent to 56 μmol of carboxylic groups). Control plants were grown in 1 mM CaCl₂ solutions which was used as background electrolyte for all the hydroponic solutions tested. Additional control plants were grown in 120 μM V(IV) solutions. Triticale plants were selected for these assays since CAF has not any phytotoxic effects against this species and because it revealed quite sensitive to heavy metal stress [21].

The phytotoxicity of all the solutions tested was determined by assessing the root and shoot length of triticale plantlets after 7 days of growth in a controlled environment (22°C temperature, 65% relative humidity) under natural light (February-March 2015). Seeds of winter triticale (cv. Universal) were germinated in the dark at 22°C as previously described [21]. Briefly, seeds were germinated in cylindrical polystyrene containers (3.5 cm height, 3.5 cm diameter) covered by a stainless steel net and filled to the rim with 30 mL of the treatment solution. In
such a way seeds and growing roots were directly (and constantly) in contact with the treatment solutions [21]. After germination, triticale seedlings were grown under natural light and root and shoot length were measured after 7 days and their total vanadium content was determined. During the experiments, the containers were covered with foil to avoid root contact with light and photo-chemical reactions.

Each treatment solution was tested on a total of 30 plantlets (6 seeds per container x 5 replicates) and three independent experiments were carried out. Plant measurement data are reported as mean values ± standard errors. One-way analysis of variance was used to compare mean values and when significant $P$-values were obtained ($P<0.05$) differences between individual means were compared with the Fisher’s least significant difference test ($P<0.05$).

**Results and Discussion**

**Stoichiometry of the redox reaction**

Because of the monomer-oligomer equilibria present in the V(V) solutions [5,29], in this study we employed V(V) concentrations equal to or less than 120 μM. At concentrations as low as 120 μM, a significant amount (~15%) of trimer (V₃O₉)³⁻ is present [30]. A pronounced change in the V(V) distribution occurs if the concentration is in the millimolar or higher range as hydrolysis take place and a number of poly-nuclear species form [29,31]. In our experimental conditions, the pervanadyl ion (VO₂⁺) is the predominant species at pH < 3.0 while at higher pH, H₂VO₄⁻ and HVO₄²⁻ are the dominant species.

Tests on the redox activity of CAF towards V(V), carried out in the 2.8–6.0 pH range, evidenced a high ability of the biomolecule to reduce V(V) at pH 2.8. Thus, in order to determine the influence of the pH on the redox reaction, as a first step, we determined the stoichiometry of the reaction at pH 2.8. To this purpose, systems containing 60 μM CAF and 30, 60 and 120 μM V(V) (the V(V)/CAF molar ratio varied from 0.5 to 2.0) were studied. The amount of CAF oxidized, as a function of time and at different initial V(V) concentrations, is reported in Fig 1. The amount of V(IV) formed, at equilibrium, in the systems with a V(V)/CAF molar ratio equal to 0.5, 1.0 and 2.0 was equal to 28.5, 57.8 and 117.3 μM, respectively (Fig 1). The ratio between the amount of V(IV) produced (117.3 μM) and the CAF reacted (44.0 μM), relative to the system with V(V)/CAF molar ratio equal 2.0, indicates that the number of electrons involved in the reduction of the V(V) by one molecule of CAF is equal to 2.7, as it can be easily calculated from the ratio between the concentration in solution of VO₂⁺ produced and CAF oxidized. Thus, the following stoichiometry at pH 2.8 can be proposed:

$$\text{CAF} + 2.7 \text{VO}_2^+ + 5.4 \text{H}^+ + 2.7 e^- \rightarrow 2.7 \text{VO}^{2+} + \text{CAF oxidation products} + 2.7 \text{H}_2\text{O}$$

The proposed stoichiometry is in agreement with previous studies on the CAF electrochemical and/or chemical oxidation showing that the number of electrons released by CAF was larger than that expected by the oxidation of the OH phenolic groups to quinonic groups [32–34].

**Mechanism of the redox reaction**

The HPLC tests show that CAF oxidizes more or less quickly depending on V(V) concentration leading to the formation of three main reaction products, with a retention time lower (product A) or higher (products B and C) than that of CAF. Fig 2 reports the chromatograms relative to the system with a V(V)/CAF molar ratio equal to 2, recorded at different reaction times, and at a wavelength of 254 nm. A similar trend was observed for the V(V)/CAF systems...
with a molar ratio equal to 0.5 and 1.0. The product A forms quickly at the beginning of the reaction and increases during the first 30 min, while decreases as the reaction proceeds (Fig 2). The decrease of the concentration of product A seems correlated with the appearance of product B, suggesting that A acts as a precursor of B while this latter seems to act as a precursor of product C. The trend of the different products as a function of time is reported in Fig 3.

The A product exhibits an electronic absorption spectrum mainly characterized by an absorption band at 250 nm and other two broads band located at 315 and 410 nm (Fig 4). This spectrum is very similar to that published previously [35] and attributed to CAF $o$-quinone formed following the CAF electrochemical and/or enzymatic oxidation. This suggests that the first step of the redox reaction is consistent with the formation of a CAF $o$-quinone. The UV-Vis spectrum of product B shows an intense absorption band at 280 nm while product C presents the same band at 280 nm accompanied by a shoulder band at 315 nm (Fig 4). The lack of the bands at 295 and 325 nm, which characterize the spectrum of CAF, indicates that these products are not CAF oligomers. Therefore, we can assume that products B and C can be formed by reactions involving the side chain of a CAF semiquinone molecule and one (or more) semiquinonic radicals to form dimers, trimmers, etc.

To assess this hypothesis, our samples were analyzed by mass spectrometry. The mass analysis indicates that products B and C, with molecular masses equal to 354 and 371 respectively, can be attributed to CAF dimers. The likely structures of products B and C, and their probable mechanism of formation, are reported in Fig 5. The first step of the reaction consists in an inner-sphere electron transfer from CAF to V(IV) to give V(IV) and highly reactive phenoxy radicals ($R_1$, $R_2$, $R_3$). Previous studies showed that the formation of $o$-quinone is a key step in

**Fig 1.** CAF reacted and V(IV) formed at pH 2.8 as a function of time and at different initial V(V) concentrations. Reaction conditions: CAF 60 μM; V(V) 30 μM (a), 60 μM (b) and 120 μM (c). Reaction volume 50 mL.

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its dimerization \[36\]. Continuous disproportionation of a pair of phenoxy radicals forms CAF and \(\text{o-quinone}\). The reaction between CAF and \(\text{o-quinone}\) gave a pair of semiquinone radicals which, via random radical coupling, led to the formation of a dimer. The effect of the \(-\text{CH} = \text{CH}-\text{COOH}\) moiety in the electronic delocalization determines the energetic stability of

![Fig 2. Chromatograms recorded at 254 nm and at different reaction times relative to the V(V)-CAF system at pH 2.8. V(V) 120 \(\mu\)M, CAF 60 \(\mu\)M.](image)

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![Fig 3. Peak area vs. reaction time curves for products A, B and C. Peak areas were obtained from HPLC-UV-DAD chromatograms (absorbance at 254 nm).](image)

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the radical located on ortho position [37,38]. The product B (molecular ion: 354) likely derived from a K dimer (we were not able to detect its presence) originating by a coupling reaction (as an intermediate of one-electron oxidation) involving R₁ and R₃ radicals as reported in Fig 5. Two electrons are involved in the oxidation of the dimer K to provide the B product. The C compound (molecular ion: 371) could have originated by the breaking of the bond of the two adjacent quinonic groups of product B to give rise to the formation of a carboxylic and an aldehyde group. Such reaction can be promoted by the VO²⁺ ions following their interaction with the quinonic groups of product B.

The isolation of the B and C products to perform NMR and/or FT-IR studies to achieve a better structural characterization has proved very difficult so far, because of their transformation during storage, most likely promoted by the residual presence of vanadium ions as well as by the tendency of the dimmers to polymerize [39].

Fig 4. UV-Vis spectra of CAF (solid lines) and CAF-oxidation products (dashed lines, products A, B and C) recovered from the V(V)-CAF system at pH 2.8 after different reaction times, i.e. product A: 30 min, product B and C 24 h.

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Influence of the pH on the redox reaction

The redox reaction is strongly affected by the pH. Fig 6 reports, as a function of pH, the amount of CAF reacted and V(IV) formed after 24 hours of reaction, in the system with a V(V)/CAF molar ratio equal to 2.0.

The number of electrons released from a CAF molecule decreases as pH increases being about equal to 2.7, 1.3, 1.2, 1.1 and 1.1 at pH 2.8, 3.6, 4.0, 5.0 and 6.0, respectively.

The chromatographic analysis of the systems at pH 3.6 and 4.0 evidences the formation of the same products detected at pH 2.8 (A, B, and C), whereas at pH > 4.0 A and B are the only species detected.

The strong decrease in the redox activity may be justified by the fact that the standard redox potential (E°) for the V(V)-V(IV) couple [5] decreases with increasing pH, as it can be easily calculated by applying the Nerst equation. In particular, for the \( \text{VO}_2^+ + 2\text{H}^+ + \text{e}^- \leftrightarrow \text{VO}^{2+} + \text{H}_2\text{O} \) (E° = 1.0 V) couple the redox potential is equal to 0.76 and 0.64 V at pH 2.0 and 3.0, respectively. The main species present at pH > 3.0 is the acid-base pair \( \text{H}_2\text{VO}_4^- \leftrightarrow \text{HVO}_4^{2-} + \text{H}^+ \) (pKₐ = 8.1) being the \( \text{H}_2\text{VO}_4^- \) the predominant specie in the 3.5–6.0 pH range. By considering that the standard redox potential for the couple \( \text{H}_2\text{VO}_4^- + 4\text{H}^+ + \text{e}^- \leftrightarrow \text{VO}^{2+} \)
+ 3H₂O is 1.419 V, the redox potential at pH 4.0, 5.0 and 6.0 is equal to 0.47, 0.24 and 0.00 V, respectively. The half-peak potential (E₁/₂), relative to the couple CAF ↔ o-quinone + 2H⁺ + 2e⁻, was equal to 0.377, 0.362, 0.252, 0.162 V at pH 2.7, 3.0, 4.5 and 5.9, respectively [34].

As can be deduced by the trend in redox potentials, the redox reaction is favored in the 2.8–4.0 pH range, while is disadvantaged at pH values higher than 4.5. However, the redox activity recorded on the systems equilibrated at pH 5.0 and 5.8 can be due to the ability of CAF to bind V(IV) ions.

The UV-Vis spectra of solutions containing CAF/V(IV) in a 2:1 molar ratio, recorded in the 3.0–6.0 pH range, are characterized by the presence of two absorption bands located at 775 and 570 nm (Fig 7A). The sorption band at 775 nm is attributable to the VO(H₂O)₅²⁺ ion, while that at 570 nm is indicative of the involvement of carboxylic and/or catecholate groups of CAF in the metal coordination. The band at 775 nm disappears as pH increases and a concomitant increase of the band at 570 nm was observed. The band at 570 nm belongs to PhO⁻ → V (dπ) ligand-to-metal charge transfer (LMCT) transition [40,41]. Previous studies [42] report that V(IV) interact with caffeic acid leading to the formation of several complexes in which both the carboxylate and catechol or catecholate groups are involved. The ML is the main species in the 4.0–5.0 pH range. Such mode of binding (catecholic type) has been also suggested in the reaction between CAF and Cr(III) in weak acidic solution [43] and CAF and Al(III) ions [44].
Redox activity of the V(V)-CAF-PGA system

In order to evaluate the role of the polygalacturonic acid (PGA) on the redox reaction, as a first step, we studied the interaction between V(IV) and PGA in the 3.0–6.0 pH range. Fig 8 reports, as a function of pH, the amount of V(IV) sorbed by 25 mg of PGA with an initial concentration of V(IV) equal to 120 μM. The amount of V(IV) sorbed increases as the pH increases from 3.0 to 4.0 and decreases from 4.5 to 6.0. This behaviour can be due to the VO\(^{2+}\) hydrolysis which leads to the formation of VOOH\(^+\) and (VOOH)\(^{2+}\) species with lower affinity for the carboxylate groups of PGA.

To verify such hypothesis, V(IV)-PGA samples were investigated by FT-IR spectroscopy. Fig 9 reports the FT-IR spectra of the V(IV)-PGA systems, at different pH values, polygalacturonic acid (HPGA) and Na-PGA.

The FT-IR spectra of the (HPGA) (Fig 9; spectrum a) is characterized by an absorption band at 1740 cm\(^{-1}\), assigned to the \(\nu(C = O)\) stretching frequency of the carboxylic group. This band progressively decreases following the replacement of the carboxylic hydrogen ion, as pH increases, and two strong bands became visible in the 1610–1635 and 1414–1421 cm\(^{-1}\) regions (Fig 9; spectra b-e). These latter bands can be assigned to the asymmetric (\(\nu_{as}\)) and symmetric (\(\nu_s\)) stretching mode of the carboxylate group, respectively. The magnitude of the difference between the \(\nu_{as}\) and \(\nu_s\) stretching (\(\Delta\nu\)) gives information about the type of interaction of the carboxylate groups with the metal ion [45,46]. The \(\Delta\nu\) value of the V(IV)-PGA system in the pH range 3.0–4.0 (\(\Delta\nu = 214\); Fig 9, spectra b-c) is higher than that recorded for Na(I)-PGA (\(\Delta\nu = 195\); Fig 9, spectrum f). This would suggest that carboxylate groups are directly involved in the coordination of the metal ion. As pH increases from 4.0 to 6.0, the band at 1628 cm\(^{-1}\) splitted into two new bands at 1635 and 1610 cm\(^{-1}\) (Fig 9; spectra d-e) suggesting that two V(IV)-PGA complexes coexist. In addition to the \(\Delta\nu = 214\) cm\(^{-1}\) found for the systems equilibrated at pH 3.0 and 4.0, a \(\Delta\nu = 189\) is found for the V(IV)-PGA samples equilibrated at pH 5.0 and 6.0. A similar value was found for Na-polygalacturonates (Fig 9; spectrum f) suggesting that also an electrostatic interaction regulates the V(IV) sorption by PGA. This can be due to the fact that at pH > 4.0, the V(IV) in solution is present manly as hydrolysed mononuclear (VOOH\(^+\)) and polynuclear ((VOOH)\(^{2+}\)) species, where the positive charge is lower or more delocalized than for VO\(^{2+}\) [30]. The band at 1018 cm\(^{-1}\), which characterizes the HPGA and Na-PGA systems, is not shifted to lower frequencies in the presence of VO\(^{2+}\) as found for Cu (II)- and Pb-PGA systems by [27] and [47] respectively. This suggests that the -OH groups and the oxygen ring of PGA are not involved in the coordination of the metallic ion to form

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**Fig 8.** Sorption of V(IV) by 25 mg PGA at different pH values. Reaction volume 100 mL, V(IV) 120 μM. Mean values ± standard deviations.

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multidentate complexes. The band at about 960 cm\(^{-1}\) (Fig 9; spectra b-e) can be assigned to the \(V = O\) stretching. This value is lower than that of \(\text{VOSO}_4 \cdot 5\text{H}_2\text{O}\) which is usually around 975–985 cm\(^{-1}\) [48]. The shift of the \(V = O\) bond to lower frequencies is indicative of a \(\sigma\) electron donation of the polysaccharidic matrix to antibonding orbitals of the \(V = O\) group.

When PGA was added to the \(V(\text{V})\)-CAF system, caffeic acid revealed more reactive particularly at pH 4.0, 5.0 and 6.0. This can be easily appreciated looking at the amounts of CAF oxidized by vanadate in 24 h in the presence and absence of PGA (Fig 10). Moreover, the concentration of the \(V(\text{IV})\) formed at pH 2.8, 4.0, 5.0 and 6.0, in the \(V(\text{V})\)-CAF-PGA systems was 0.6, 30, 36 and 58% higher compared to the respective binary \(V(\text{V})\)-CAF systems. This behaviour can be explained by taking into account the higher affinity of the polysaccharidic matrix towards \(V(\text{IV})\) with respect to CAF as can be deduced by the comparison of the UV-Vis spectra of the binary \(V(\text{IV})\)-CAF and ternary \(V(\text{IV})\)-CAF-PGA systems recorded in the 3.0–6.0 pH range (Fig 7A and 7B). The ratio between the absorbance values recorded at 570 nm, in the absence and presence of PGA, was equal to 2.8 in the whole pH range studied. This is consistent with Islam et al. [49] which highlighted how chelating ligands which stabilize a metal complex in a lower oxidation state can promote the reduction of the metal with a higher oxidation state. Similarly, [50] showed that NADPH reduces \(V(\text{V})\) to \(V(\text{IV})\) in the absence of enzyme catalysis when EDTA is present in the reaction mixture. In contrast, NADPH can only partially reduce \(V(\text{V})\) in the absence of EDTA.

This result can be of great importance from a biochemical point of view as pectic substances, acting as complexing agent towards \(V(\text{IV})\), can cooperate with reducing agents to buffer the vanadium toxicity at the soil root-interface where pectic polymers and reducing agents are abundant. For instance, PGA could avoid the reaction of the reduced metal with hydrogen peroxide, which is readily converted by a Fenton-type reaction into hydroxyl radicals which can induce a multitude of effects on many cellular functions such as apoptosis [51].
Biological relevance of the redox reaction between V(V) and CAF in the presence and absence of PGA

To evaluate the biological significance of the redox reaction between CAF and V(V), as well as the role of PGA in such reaction, triticale plants (x Triticosecale Wittm.) were grown at pH 6.0 in solutions containing V(V), V(V)+CAF and V(V)+CAF+PGA. The presence of 120 μM V (V) in the solution induced significant phytotoxicity effects on triticale plants. In particular,
after 7 days of growth, root and shoot length was significantly reduced with respect to control plants (Fig 11A and 11C). This is consistent with the results reported by [52] which recorded a significant reduction of root length and shoot dry matter in cuphea plants (family Lythraceae) when V(V) was added to hydroponic solutions in the $25\text{–}150\mu M$ range. Similar phytotoxic effects were also recorded for the V(V)-CAF system (Fig 11A and 11C). Since it was recently demonstrated that CAF has no phytotoxic effects against triticale [21], the observed results can be attributed to the presence in solution of V(V) and V(IV), this latter species originating from the redox reaction between CAF and V(V). The presence of V(IV), in the V(V)-CAF system, was also supported by the visual observation of typical symptoms induced by V(IV) such as dark roots (not observed in the V(V) system; Fig 11B) [11]. Such symptoms were apparent when triticale plants were grown in the presence of V(IV) (S1 Fig) but were also present in the V(V)-CAF-PGA system which, however, showed the least phytotoxicity (Fig 11A–11C). Root elongation was reduced by 35, 51 and 60%, with respect to control plants, for V(V)-CAF-PGA, V(V) and V(V)-CAF system respectively. On the other hand, shoot length in the V(V)-CAF-PGA system was not significantly different from that of control plants while it was
approximately reduced by 26% in V(V) and V(V)-CAF systems (Fig 11 and S2 Fig). The lowest phytotoxicity recorded in the V(V)-CAF-PGA system can be explained by the ability of PGA to bind the V(IV) which originates from the redox reaction between CAF and V(V) (Figs 7–9), which ultimately leads to a lower concentration of V(V) in solution. The presence of PGA induced a significant reduction in the total vanadium accumulated in roots (33 μg/g d.m.) and shoots (3.7 μg/g d.m.) compared to plants grown in the V(V) and V(V)-CAF systems (44.5 and 5.5 μg/g d.m. for roots and shoots respectively, average values). Moreover, these latter concentrations were approximately 6-fold higher than those recorded in control plants.

These results highlight significant phytotoxic effects of the V(V)-CAF system at pH 6.0 which were comparable with those induced by V(V) alone. In the presence of PGA, however, the phytotoxicity of the V(V)-CAF system was significantly reduced confirming the high affinity of the polysaccharidic matrix towards the V(IV) formed and highlighting the in vivo relevance of PGA in the detoxification processes of V(IV) occurring at the soil-root interface.

Conclusions

The results reported show that CAF can promote the reduction of V(V), highly toxic for living systems (e.g. V(V) acts as a phosphate competitor), to V(IV). Polygalacturonates, beside promoting the redox reaction, also act as effective buffering agents against the phytotoxicity of the V(IV) formed following CAF oxidation. On the basis of the results obtained we can retain that pectic substances, well represented in the apoplasm and at the soil-root interface, have an important role in the processes that regulate the reduction of V(V), not only by CAF, but also by other phenolic compounds and biological reducing agents such as NAD(P)H, ascorbate or cysteine.

Supporting Information

S1 Fig. Root appearance of triticale plants grown in the presence of 120 μM V(V) (a) and 120 μM V(IV) (b). CaCl₂ (1 mM) was the supporting electrolyte in both solutions. Note typical dark roots of plants grown in the presence of V(IV).
(TIF)

S2 Fig. Shoot biomass of representative triticale plants grown for 7 days in 1 mM CaCl₂ (control), 120 μM V(V), 120 μM V(V)+120 μM CAF and 120 μM V(V)+120 μM CAF+0.25 mg/mL PGA.
(TIF)

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Author Contributions

Conceived and designed the experiments: SD GG. Performed the experiments: AP GG GPL EM CS BM. Analyzed the data: SD AP GG BM. Contributed reagents/materials/analysis tools: GPL CS. Wrote the paper: SD GG.
References

1. Kabata-Pendias A (2011) Trace elements in soils and plants 4th Ed. Boca Raton FL: CRC Press.

2. McBride MB, Spiers G (2001) Trace element content of selected fertilizers and dairy manures as determined by ICP-MS. Communications in Soil Science and Plant Analysis 32: 139–156.

3. Vachirapatama N, Dicinoski GW, Townsend AT, Haddad PR (2002) Determination of vanadium as 4-(2-pyridylazo)resorcinol-hydrogen peroxide ternary complexes by ion-interaction reversed-phase liquid chromatography. Journal of Chromatography A 956: 221–227. PMID: 12108654

4. Greenwood NN, Earnshaw A (1997) Chemistry of the Elements, second edition. Madras, India: Elsevier Butterworth-Heinemann.

5. Evans LJ, Barabash SJ (2010) Molybdenum, Silver, Thallium and Vanadium. In: Hooda PS, editor. Trace Elements in Soils. Chippenham, United Kingdom: Wiley. pp. 515–549.

6. Crans DC, Smee JJ, Gaidamauskas E, Yang LQ (2004) The chemistry and biochemistry of vanadium and the biological activities exerted by vanadium compounds. Chemical Reviews 104: 849–902. PMID:14871144

7. Tracey AS, Willisky GR, Takeuchi ES (2007) Vanadium—Chemistry, Biochemistry, Pharmacology and Practical Applications. Boca Raton, FL: CRS Press.

8. Ulrich-Eberius CI, Sanz A, Novacky AJ (1989) Evaluation of arsenate-associated and vanadate-associated changes of electrical membrane potential and phosphate transport in Lemna gibba G1. Journal of Experimental Botany 40: 119–128.

9. Link A, Balaguer F, Goel A (2010) Cancer chemoprevention by dietary polyphenols: Promising role for epigenetics. Biochemical Pharmacology 80: 1771–1792. doi:10.1016/j.bcp.2010.06.036 PMID: 20599773

10. Deiana S, Premoli A, Senette C (2008) Oxidation of caffeic acid by Fe(III) trapped in a Ca-polygalacturonate network. Plant Physiology and Biochemistry 46: 435–443. doi:10.1016/j.plaphy.2008.02.003

11. Garau G, Mele E, Castaldi P, Lauro GP, Deiana S (2015) Role of polygalacturonic acid and the cooperative effect of caffeic and malic acids on the toxicity of Cu(II) towards triticate plants (× Triticosecale Wittm). Biology and Fertility of Soils. 53: 341–343.
24. Gessa C, Deiana S (1992) Ca-polygalacturonate as a model for a soil-root interface. Fibrillar structure and comparison with natural root mucilage. Plant and Soil 140: 1–13.

25. Leppard GG (1974) Rhizoplane fibrils in wheat: demonstration and derivation. Science 185: 1066–1067. PMID: 17738249

26. Deiana S, Manunza B, Palma A, Premoli A, Gessa C (2001) Interactions and mobilization of metal ions at the soil-root interface. In: Gobran GR, Wenzel WW, Lombi E, editors. Trace elements in the rhizosphere. Boca Raton, FL: CRS Press. pp. 127–148.

27. Castaldi P, Lauro G, Senette C, Deiana S (2010) Role of the Ca-pectates on the accumulation of heavy metals in the root apoplasm. Plant Physiology and Biochemistry 48: 1008–1014. doi: 10.1016/j.plaphy.2010.09.017 PMID: 20951053

28. Borraccino R, Kharoune M, Giot R, Agathos SN, Nyns EJ, et al. (2001) Abiotic transformation of catechol and 1-naphthol in aqueous solution—Influence of environmental factors. Water Research 35: 3729–3737. PMID: 11561636

29. Gharib F, Sayadian M, Shame A, Mobasher-Moghaddam M (2008) Formation equilibria of vanadium (V) species in different ionic media: Salt effect and protonation constant. Journal of Molecular Liquids 138: 9–13.

30. Chasteen ND (1983) The biochemistry of vanadium. Copper, Molybdenum, and Vanadium in Biological Systems: Springer Berlin Heidelberg. pp. 105–138.

31. Pettersson L, Andersson I, Gorzsas A (2003) Speciation in peroxovanadate systems. Coordination Chemistry Reviews 237: 77–87.

32. Fulcrand H, Cheminat A, Brouillard R, Cheynier V (1994) Characterization of compounds obtained by chemical oxidation of caffeic acid in acidic conditions. Phytochemistry 35: 499–505.

33. Hotta H, Sakamoto H, Nagano S, Osakai T, Tsujino Y (2001) Unusually large numbers of electrons for the oxidation of polyphenolic antioxidants. Biochimica Et Biophysica Acta-General Subjects 1526: 159–167.

34. Deiana S, Piolo M, Premoli A, Senette C, Solinas V, et al. (2003) Interaction of oxidation products from caffeic acid with Fe(III) and Fe(II). Journal of Plant Nutrition 26: 1909–1926.

35. Cheynier V, Mououtouet M (1992) Oxidative reactions of caffeic acid in model systems containing polyphenol oxidase. Journal of Agricultural and Food Chemistry 40: 2038–2044.

36. Tazaki H, Taguchi D, Hayashida T, Nabetani K (2001) Stable isotope-labeling studies on the oxidative coupling of caffeic acid via o-quinone. Bioscience Biotechnology and Biochemistry 65: 2613–2621.

37. Leopoldini M, Marino T, Russo N, Toscano M (2004) Antioxidant properties of phenolic compounds: H-atom versus electron transfer mechanism. Journal of Physical Chemistry A 108: 4916–4922.

38. Leopoldini M, Russo N, Toscano M (2011) The molecular basis of working mechanism of natural polyphenolic antioxidants. Food Chemistry 125: 288–306.

39. Hotta H, Nagano S, Ueda M, Tsujino Y, Koyama J, et al. (2002) Higher radical scavenging activities of polyphenolic antioxidants can be ascribed to chemical reactions following their oxidation. Biochimica Et Biophysica Acta-General Subjects 1572: 123–132.

40. Karpishin TB, Dewey TM, Raymond KN (1993) Coordination chemistry of microbial iron transport. 49. The vanadium(IV) enterobactin complex—structural, spectroscopic, and electrochemical characterization. Journal of the American Chemical Society 115: 1842–1851.

41. Karpishin TB, Stack TDP, Raymond KN (1993) Octahedral vs trigonal prismatic geometry in a series of catechol macrobicyclic ligand metal-complexes. Journal of the American Chemical Society 115: 182–192.

42. Williams PAM, Baro ACG, Ferrer EG (2002) Study of the interaction of oxovanadium(IV) with a plant component (caffeic acid). Synthesis and characterization of a solid compound. Polyhedron 21: 1979–1984.

43. Thoma V, Tampouris K, Petrou AL (2008) Kinetics and mechanism of the reaction between chromium (III) and 3,4-dihydroxy-phenyl-propenoic acid (caffeic acid) in weak acidic aqueous solutions. Bioinorganic Chemistry and Applications.

44. Lapouge C, Cornard JP (2007) Reaction pathways involved in the mechanism of Al-III chelation with caffeic acid: Catechol and carboxylic functions competition. Chemphyschem 8: 473–479. PMID: 17183604

45. Nakamoto K (1986) Infrared and Raman spectra of inorganic and coordination compounds: Wiley.

46. Baran EJ (2001) Review: Spectroscopic studies of oxovanadium coordination compounds. Journal of Coordination Chemistry 54: 215–238.

47. Dhakal RP, Ghimire KN, Inoue K, Yano M, Makino K (2005) Acidic polysaccharide gels for selective adsorption of lead(II) ion. Separation and Purification Technology 42: 219–225.
48. Yaul AR, Pethe GB, Aswar AS (2010) Synthesis, spectral, catalytic, and thermal studies of vanadium complexes with quadridentate Schiff bases. Russian Journal of Coordination Chemistry 36: 254–258.

49. Islam MK, Tsuboya C, Kusaka H, Aizawa SI, Ueki T, et al. (2007) Reduction of vanadium(V) to vanadium(IV) by NADPH, and vanadium(IV) to vanadium(III) by cysteine methyl ester in the presence of biologically relevant ligands. Biochimica Et Biophysica Acta-General Subjects 1770: 1212–1218.

50. Kanamori K, Sakurai M, Kinoshita T, Uyama T, Ueki T, et al. (1999) Direct reduction from vanadium(V) to vanadium(IV) by NADPH in the presence of EDTA. A consideration of the reduction and accumulation of vanadium in the ascidian blood cells. Journal of Inorganic Biochemistry 77: 157–161.

51. Andrezalova L, Gbelcova H, Durackova Z (2013) DNA damage induction and antiproliferative activity of vanadium(V) oxido monoperoxido complex containing two bidentate heteroligands. Journal of Trace Elements in Medicine and Biology 27: 21–26. doi: 10.1016/j.jtemb.2012.04.007 PMID: 22575540

52. Olness A, Gesch R, Forcella F, Archer D, Rinke J (2005) Importance of vanadium and nutrient ionic ratios on the development of hydroponically grown cuphea. Industrial Crops and Products 21: 165–171.