



## INTERACTIONS BETWEEN PHOSPHORUS AND HUMIC SUBSTANCES: INFLUENCE IN P BIOAVAILABILITY

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### Abstract

*A number of studies have reported the beneficial effects of the organic matter on the bioavailability of P in different soil types. These effects have been attributed to several factors, including the possible formation of phospho-metal-humic complexes, which could protect P from retrogradation in both acid and alkaline-calcareous soils. However, the experimental evidence supporting the existence of these complexes in solis is scarce and principally based on indirect arguments.*

*The aim of this article is to describe the different studies carried out in our laboratory in order to characterize the formation and stability of these complexes, as well as their effect protecting P from soil retrogradation and their potential availability for plants. These results are discussed considered along with other present in the literature.*

### 1. Introduction

Numerous studies have reported the close relationship between phosphorus (phosphate, P) bioavailability in soils and the concentration of soil organic matter (SOM) [1]. Other studies have shown the beneficial effect of organic amendments (OA) containing significant concentrations of humic substances on both plant available P and P plant uptake in different soil types [2,3]. Although a number of studies have related this beneficial effect to the action of those fractions of organic acids with lower molecular weight derived from plant root exudation and SOM decomposition [3], other studies showed that humic substances could also play a important role in this process [3].

Besides the possible action of the different fractions of SOM on soil P dynamics through the increase in soil microbial activity and the activity of soil enzymes [4], other more direct mechanisms could also be important. These include: (i) the competition between specific acidic fractions of SOM, principally those of lower molecular weight, and phosphate (P), which inhibits P soil absorption [5]; (ii) the modification of the precipitation pattern of calcium phosphates, which produces salt species with higher water solubility such as dicalcium phosphate dihydrate [6]; (iii) the mobilization of free P from precipitated phosphates (Fe-, Al- or Ca- phosphates) as a result of the formation of stable cation-humic complexes [7], and (iv) the mobilization of P through the possible formation of stable P-metal-humic complexes (8).

Many authors have proposed the formation of soluble or insoluble P-metal-humic complexes as one of the possible mechanisms of action of humic substances on soil P dynamics [8-12]. In many cases, the hypothesis has been based on indirect results mainly related to the correlation among the P distribution in solution, certain metals (Fe or Al) and humic substances after fractionation of the solution using size exclusion

chromatography) [11,13], the fixation of P in precipitated metal-humic complexes [12, 14, 15], or the variation in the  $^{31}\text{P}$  NMR spectra obtained in the presence of humic substances and metal-humic complexes [3,16]. However, specific studies aimed at investigating the formation and physicochemical characteristics (stoichiometry, stability and solubility) of these P-metal-humic complexes are very recent [17, 18, 19, 20, 21]. The aim of this review is to present the main physico-chemical and agronomical (soil interaction, plant root uptake) features of these complexes by integrating these different recently published works with new unpublished information.

## 2. Physico-chemical characterization of P<sup>-</sup>-metal-humic complexes

As have been pointed out above the main problem concerning the study of these types of complexes is to have an adequate methodology to separate the complexed P from the non complexed P.

Riggle and von Wandruszka (17) reported the use of a special, non-commercial, selective Co electrode to evaluate the concentration of free P in a solution containing different metal-humic complexes and several P concentrations. However, although this method is able to measure the concentration of free and complexed (Total P – Free P) it does not separate both fractions.

Guardado et al (18) explored the suitability of the use of a stabilized (bicarbonate) anion-exchange resin with the capacity to fix free P in order to both measure free and complexed P and isolate solutions containing this type of complexes. They compared the efficiency of the anion-exchange resin method with that of a more time-consuming ultrafiltration method in a system including iron (Fe)-humates and P. A summary of the results obtained is presented in Table 1.

Table 1.- Total complexed P (PTC) in reactions with Fe-HA at different pH, as measured using ultrafiltration (UF) or the interaction with an anion-exchange resin (AR)

pH	$C_T^*$ (mg/l)	$Fe_c^\alpha$ (mM)	$P_{TC}$ (AR)		$P_{TC}$ (UF)		$(P_{TC}/Fe_c)$ (AR)	$(P_{TC}/Fe_c)$ (UF)
			$\bar{x}$ (mM)	CV <sup>#</sup> (%)	$\bar{x}$ (mM)	CV (%)		
4	2279	4.036	0.738	5.6	0.818	4.0	0.183	0.203
6	2295	4.065	0.688	8.8	0.693	7.4	0.169	0.171
8	2220	3.932	0.540	0.9	0.459	1.1	0.137	0.117

\* Total Carbon;  $^\alpha$  Complexed Fe in Fe-HA; <sup>#</sup> Coefficient of variation (n=4)

The concordance between both methods was good thus showing the suitability of the anion (bicarbonate)-exchange resin method.

This methodology was used to obtain the stability constants and the maximum binding capacity (MBA) of the P-Fe humate interaction. Thus, a pH-Dependent stability of P-Fe-HA complexes in solution was estimated using an apparent stability constant (K) calculated by the Scatchard method (22) (Figure 1):

$$(PTC/MBA)/ PTF = K - (PTC/MBA) K \text{ or } \theta/ PTF = K - \theta K$$

PTF being the total free P in the equilibrium; PTC the total complexed P; and  $\theta$  the Sites bound / Maximum metal binding capacity (MBA) (PTC / MBA) ratio, where K was obtained from the plot  $\theta/ PTF$  vs  $\theta$ .

MBA was estimated by extrapolating the straight-line segment corresponding to binding at high P ion saturation in a PTC vs. PTC/ PTF plot. Only results corresponding to P: Fe-HA reaction ratios without precipitation of HA were considered in the calculation of K.

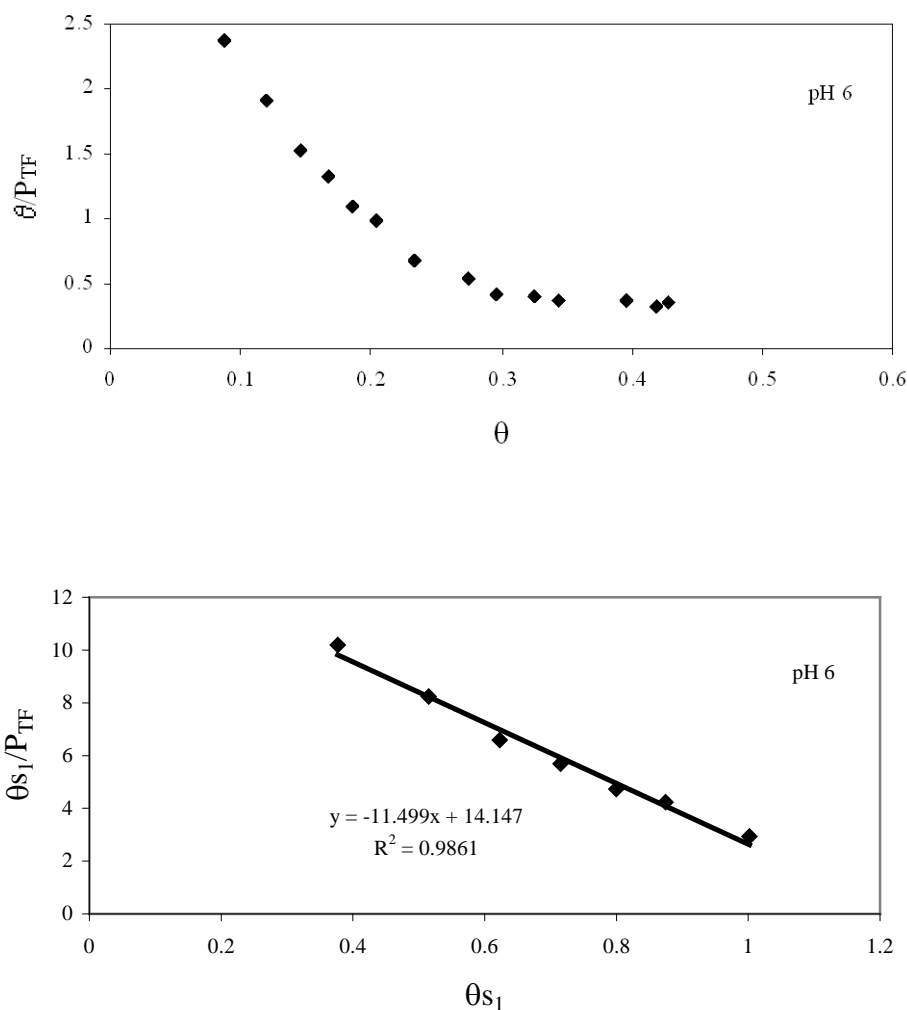


Figure 1.- Application of the Scatchard to the interaction of Fe-humate (Fe-HA) and P at pH 6

From this study, it was concluded that only a relatively small proportion of HA complexed Fe was involved in P fixation (from 10 to 20%, assuming 1:1 Fe:P stoichiometry). Two main causes could explain these results: (a) the formation of stable HA-Fe-HA aggregates through the formation of Fe bridges, which reduce the concentration of complexed Fe with free coordination bonds available for P fixation; and (b) the existence of a restricted group of Fe complexing sites in HA with the electronic configuration necessary to form stable P-Fe-HA bonds.

With the aim of investigating these two hypotheses, we studied the size distribution changes associated with HA metal complexation and P fixation in metal-humic complexes [19]. To this end, two different methods, which were expected to show different molecular aggregation behaviors, were employed to obtain P--Metal-HA complexes. The main difference between these methods was the order of addition of the different compounds involved in the reaction.

Likewise, the relationship between the physicochemical characteristics of M-HA binding sites and their involvement in P fixation, were investigated through the analysis of the MBA and the stability of P-M-HA complexes involving two trivalent (Fe and Al) and five divalent metal bridges (Zn, Cu, Mn, Ca and Mg). Metal selection was based on two main criteria: (a) their ability to form humic complexes with different physicochemical properties; and (b) their potential role in P soil dynamics in both acid (Fe, Al, Zn, Cu, and Mn) and calcareous soils (Ca and Mg).

In Figure 2 we present the organic carbon profiles obtained by ultrafiltration for the humic system (HA), the iron-humate (Fe-HA) and the P-Fe-HA complexes obtained by both methods (M1 and M2), at different pH.

As can be observed, in all cases the formation of the P-Fe-HA complex involves a significant reduction of the aggregation associated to Fe HA complexation, thus indicating that P complexation implies the breaking of poly-dentate Fe-HA bonds. However, there were not significant differences between synthesis methods, thus suggesting that the cause of the low concentration of HA complexed Fe involved in P fixation might be linked to the electronic requirements associated with the P binding site.

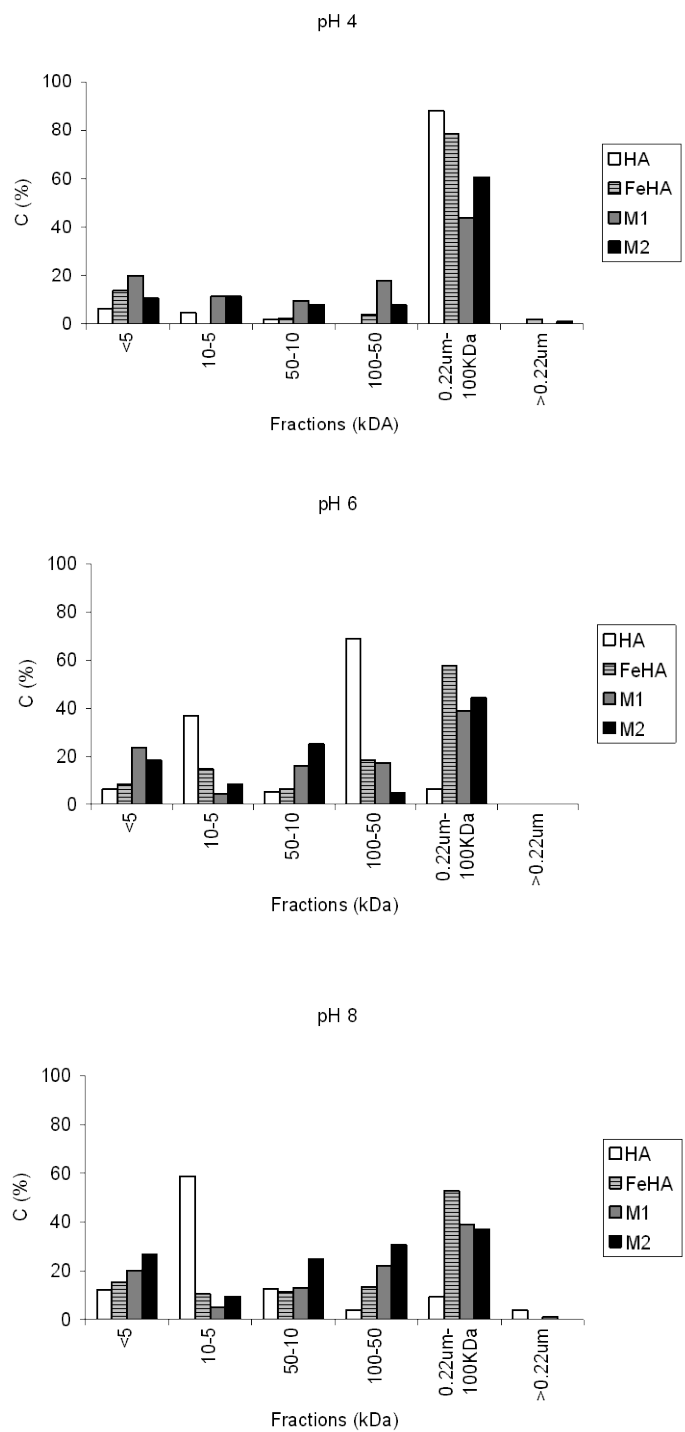


Figure 2.- Organic carbon profiles as a function of the sizes for HA, Fe-HA, and P-Fe-HA for method 1 (M1) and 2 (M2), at different pH

Regarding the P complexation ability (MBA) of the different metal-HA complexes, a summary of the results obtained is presented in Tables 2 and 3.

Table 2.- Maximum binding ability for phosphate corresponding to the binding sites of higher stability (MBAH) in P-M-HA complexes obtained using the method 1 (M1) and method 2 (M2)

	M1			M2			
	*MBA <sub>H</sub> /[HA]			*MBA <sub>H</sub> /[HA]			
pH	4	6	8	pH	4	6	8
<b>Fe</b>	0,052	0,046	0,028	<b>Fe</b>	0,060	0,078	0,083
<b>Al</b>	0,044	0,059	0,031	<b>Al</b>	0,053	0,091	0,124
<b>Ca</b>	0,014	0,018	0,023	<b>Ca</b>	0,024	0,021	0,040
<b>Mg</b>	0,016	0,025	0,015	<b>Mg</b>	0,017	0,027	0,034
<b>Cu</b>	0,025	0,033	0,020	<b>Cu</b>	0,031	0,050	0,063
<b>Mn</b>	0,023	0,012	0,033	<b>Mn</b>	0,022	0,042	0,088
<b>Zn</b>	0,026	0,040	0,028	<b>Zn</b>	0,039	0,050	0,043

\* MBA values are referred to the HA quantity and are expressed as mmol PO<sub>4</sub><sup>3-</sup> g<sup>-1</sup> of HA.

Table 3.- Apparent stability constant values for the binding sites of higher stability (logK<sub>H</sub>) for P-M-HA complexes obtained using the method 1 (M1) and the method 2 (M2)

	M1			M2			
	LogK <sub>H</sub>			LogK <sub>H</sub>			
pH	4	6	8	pH	4	6	8
<b>Fe</b>	3.27	3.98	4.09	<b>Fe</b>	4.13	3.55	4.07
<b>Al</b>	4.03	4.06	3.92	<b>Al</b>	3.33	4.05	4.10
<b>Ca</b>	3.66	3.66	3.48	<b>Ca</b>	3.47	3.65	3.07
<b>Mg</b>	3.51	3.61	3.56	<b>Mg</b>	3.17	3.46	3.43
<b>Cu</b>	3.79	3.65	3.55	<b>Cu</b>	3.63	3.54	3.36
<b>Mn</b>	3.02	4.05	3.94	<b>Mn</b>	3.05	3.05	3.11
<b>Zn</b>	3.78	3.36	3.57	<b>Zn</b>	3.53	3.69	3.69

The results concerning MBA confirmed previous results indicating the involvement of a small fraction of HA complexed metal in P fixation.

As for stability constants, in general trivalent cations (Fe and Al) presented the highest stability.

It is also noteworthy that the order of magnitude of the apparent stability constants for P-M-HA complexes was very close to that corresponding to M-HA complexes obtained by fluorescence spectroscopy following the Ryan and Weber method (data not shown). This result was consistent with the results obtained by Riggle and von Wandruszka [17], although they found higher stability values, probably because of a different P concentration ranges used in the experiments.

Given that M-HA complexes play an important role in soil complexed metal dynamics [9,14-16], this work suggests that P-M-HA complexes might also affect soil P dynamics.

In order to study those structural and/or electronic conditions that could govern the P:metal humic interaction, we investigated P binding in specific metal humic complexes using two complementary experimental techniques : Infrared spectroscopy (FTIR), and Fluorescence [20]. We also complete the study carrying out a theoretical study using molecular modeling methods (principally molecular mechanics and semi-empirical quantum chemical methods). In order to compare the influence of divalent and trivalent metals we

have considered humic-metal-phosphate complexes involving Al(III), Fe(III) and Zn(II) [20].

The analysis of P metal-HA reaction using both fluorescence and FTIR confirmed the interaction of P with metal-HA binding sites. As an example, the fluorescence pattern of the P-Fe-HA interaction at pH 6 is presented in Figure 3.

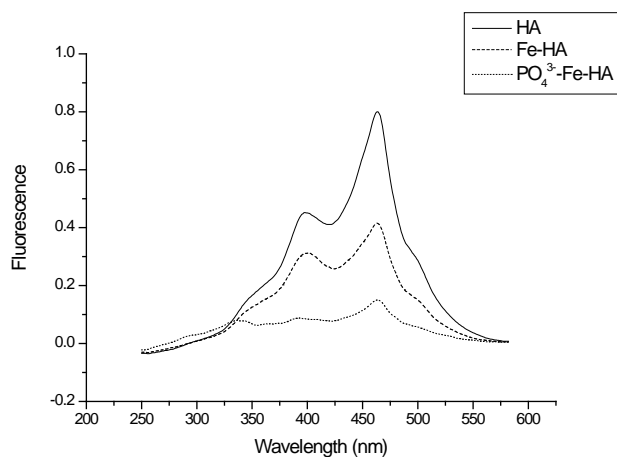


Figure 3.- Synchronous Fluorescence Spectra ( $\Delta\lambda = 18$  nm) for HA, Fe-HA complexes and P-Fe-HA complexes at pH 6

As can be observed, P complexation is associated with a very important quenching effect of the fluorescence with respect to HA and Fe-HA, thus confirming the interaction of P with HA through HA complexed Fe.

This interaction was also confirmed by  $^{31}\text{P}$  NMR in liquid state for P-Ca-HA complexes (Figure 4).

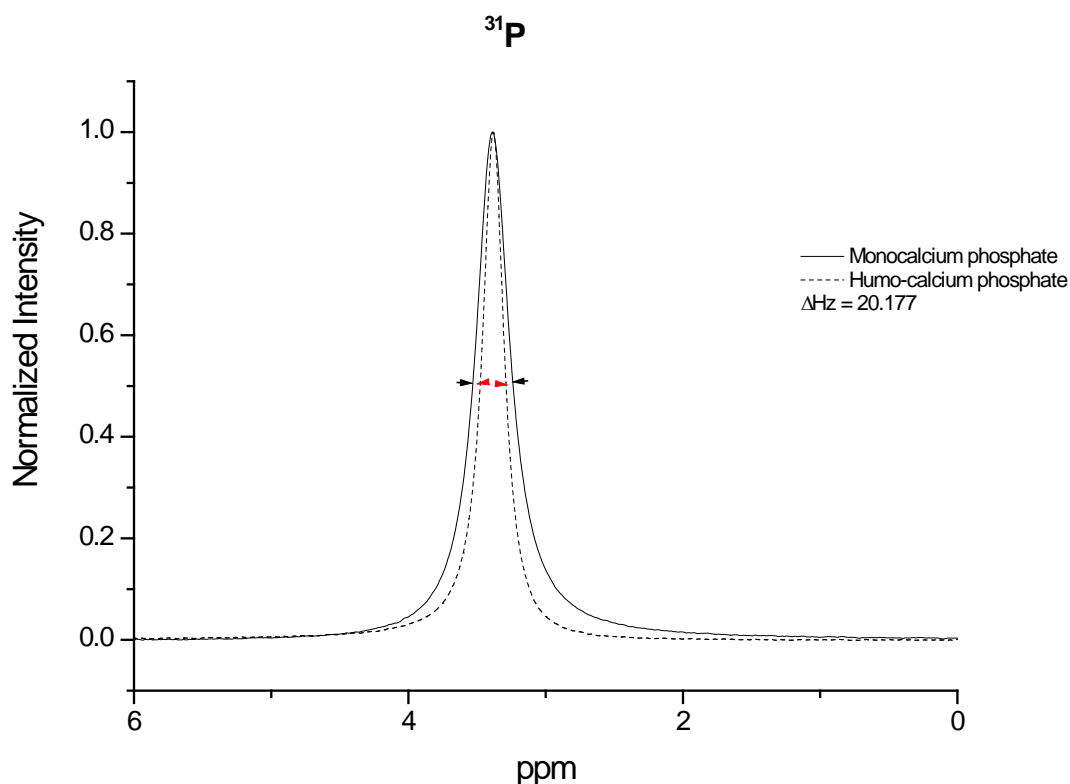


Figure 4.-  $^{31}\text{P}$  NMR for monocalcium phosphate and P-Ca-HA complex

As was expected the incorporation of Ca to the coordination sphere of P and the influence of HA on the coordination sites occupied by Ca implied a reduction in the width of the band.

It was very interesting the results obtained from the modeling study of the P-metal-HA interaction. Thus, a theoretical study of the chemical interaction between the different elements of the molecular system (HA, metals, and phosphate) was carried out using both Molecular Mechanics and Semi-empirical Quantum Mechanics methods. The operational and theoretical approaches were made assuming that inter-molecular chemical interactions generally have two main, successive, steps:

- (i) Step 1. First, a spatial convergence of the interacting molecules that is governed by electrostatic forces related to the electric charge distribution (atomic charges) on each molecule (the inter-molecular convergence interaction is of an electrostatic nature) is assumed. This first step, which is named electrostatic interaction, was studied through the energy minimization of the molecular system using molecular mechanics. Both MM+ and Amber force fields were used, and the geometry and the atomic charges of the individual molecules were previously obtained using the semi-empirical quantum mechanics method PM3 [20].
- (ii) Step 2. Second, a chemical interaction between specific regions of the molecules that have achieved the bond distance as a consequence of the electrostatic interaction (Step 1) is assumed. This step, which involves the creation of new chemical bonds, is named binding interaction. To study the binding interaction, we carried out the energy minimization of the chemical system obtained from the electrostatic interaction, using the semi-empirical quantum chemical method PM3 [20].

The model selected to represent binding sites in HA was a salicylic acid moiety. This selection was based on the following main reasons:

The study was carried out on the systems involving Al(III) and Zn(II) because these two cations are well parameterized in PM3 semi-empirical method. The metals were modeled as  $Zn^{2+}$  and  $Al^{3+}$  in their pentahydrated and hexahydrated forms respectively because they are their main molecular forms in the pH interval considered in the study.

Two main ionized forms for phosphate are present in the pH range of the study:  $H_2PO_4^-$  and  $HPO_4^{2-}$ . We present here the results obtained using  $HPO_4^{2-}$  to model the phosphate anion. The results using  $H_2PO_4^-$  were qualitatively similar to those corresponding to  $HPO_4^{2-}$ .

The study was developed following two steps: firstly, we considered the formation of the metal-HA complex; secondly, we considered the interaction of phosphate with the metal-HA complex.

The relative stability of the interactions was assumed to be directly related to the value of the binding energy (E) calculated for the molecular interaction. The binding energy corresponding to the molecular interaction ( $\Delta E_r$ ) is calculated by the subtraction of the sum of the binding energy of the individual molecules involved in the interaction from the binding energy corresponding to the new molecule formed as a result of the interaction [20].

The calculated interactions for the Al-SA(-1) (A) and Al-SA(-1)-P are presented in Figure 5.



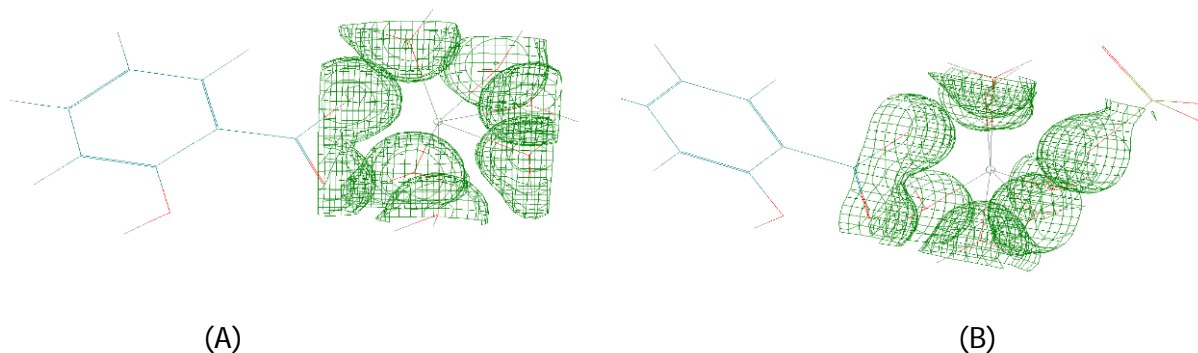


Figure 5.- Binding interaction between the salicylic binding model (-1 charge) and Al. (The grid shaped globes represent the electronic density distribution for a contour value of  $0.05 \text{ e a}0^{-3}$ ) (A); and Binding interaction between Al-SA(-1) complex and phosphate. (The grid shaped globes represent the electronic density distribution for a contour value of  $0.05 \text{ e a}0^{-3}$ ) (B)

The results obtained for both the Al-SA- P and the Zn-SA- P interactions indicate that the stability, and even the formation, of phosphate-metal-SA (and possibly phosphate-metal-HA) complexes is conditioned by the stability of the metal-SA(HA) complex: the more the stability of metal complexes the less the stability (and the possible formation) of phosphate complexes. This result could provide a possible explanation for the fact that only a relatively low fraction of complexed metal intervenes in phosphate fixation in metal-HA. Only the metal fraction involved in HA complexes with low stability will be able to form stable phosphate complexes. The theoretical study indicates that this situation might be related to the metal fraction complexed with only one carboxylate. Thus, the results obtained suggest that in the case of Zn-HA- P complexes, only the fraction of Zn complexed by a carboxylic group participates in phosphate binding. This would be also the case for the more stable fraction of Al-HA- P. This carboxylate may be integrated in a more complex binding site (like the salicylic structure) or, more probably, may correspond to isolated groups. This result also provides a complementary explanation for the relatively low fraction of complexed metal that is involved in the formation of stable P-metal-HA complexes, since several studies indicate that the majority of carboxylic groups are probably grouped with other carboxylates (phthalate type structure) or with phenols (salicylate type structure). So that the relative concentration of isolated carboxylic groups in HA is expected to be rather low.

Summarizing, the results obtained indicate that the P complexation in metal-HA complexes occurs and that it is principally mediated by the interaction of P with acid groups of HA through metal bridges. Likewise, this study also indicates that only a small fraction of metal-HA binding sites are involved in P fixation. This fact seems to be related to the electronic features of binding sites. Thus the stability and capacity of P fixation in metal-HA binding sites is inversely proportional to the stability of metal-HA complexes: the more the stability of metal complexes the less the stability (and the possible formation) of phosphate complexes.

### 3. Agronomical consequences of P-metal-humic complex formation

#### 3.1. Influence of P complexation in metal-humic complexes on P soil retrogradation

In order to study the influence of metal-HA P complexation on P soil absorption and precipitation, we investigated the concentration of P remaining in soil solution when the P

added to the soil is complexed by different metal-HA complexes. We used several soils. The results obtained for a soil with high P fixing capacity are presented in Figure 6.

As can be noted, the three P-metal (Fe, Al and Ca)-humic complexes significantly decreased the concentration of soil fixed P. The efficiency was very high for P-Fe-HA followed by P-Al-HA and P-Ca-HA. This result was qualitatively in line with the stability of these complexes presented in Table 3. The results also show the low capacity of free HA to protect P; and probably this capacity is mediated by the complexation of Fe or Al in the soil since this soil has high contents of interchangeable Fe and Al. In fact, in other soils with higher pH and lower Fe and Al concentrations the effect of free HA on P soil fixation was not significant (data not shown).

Thus, these results confirm that the formation of this type of complex in soil is probably involved in the P protection effect of soluble organic matter.

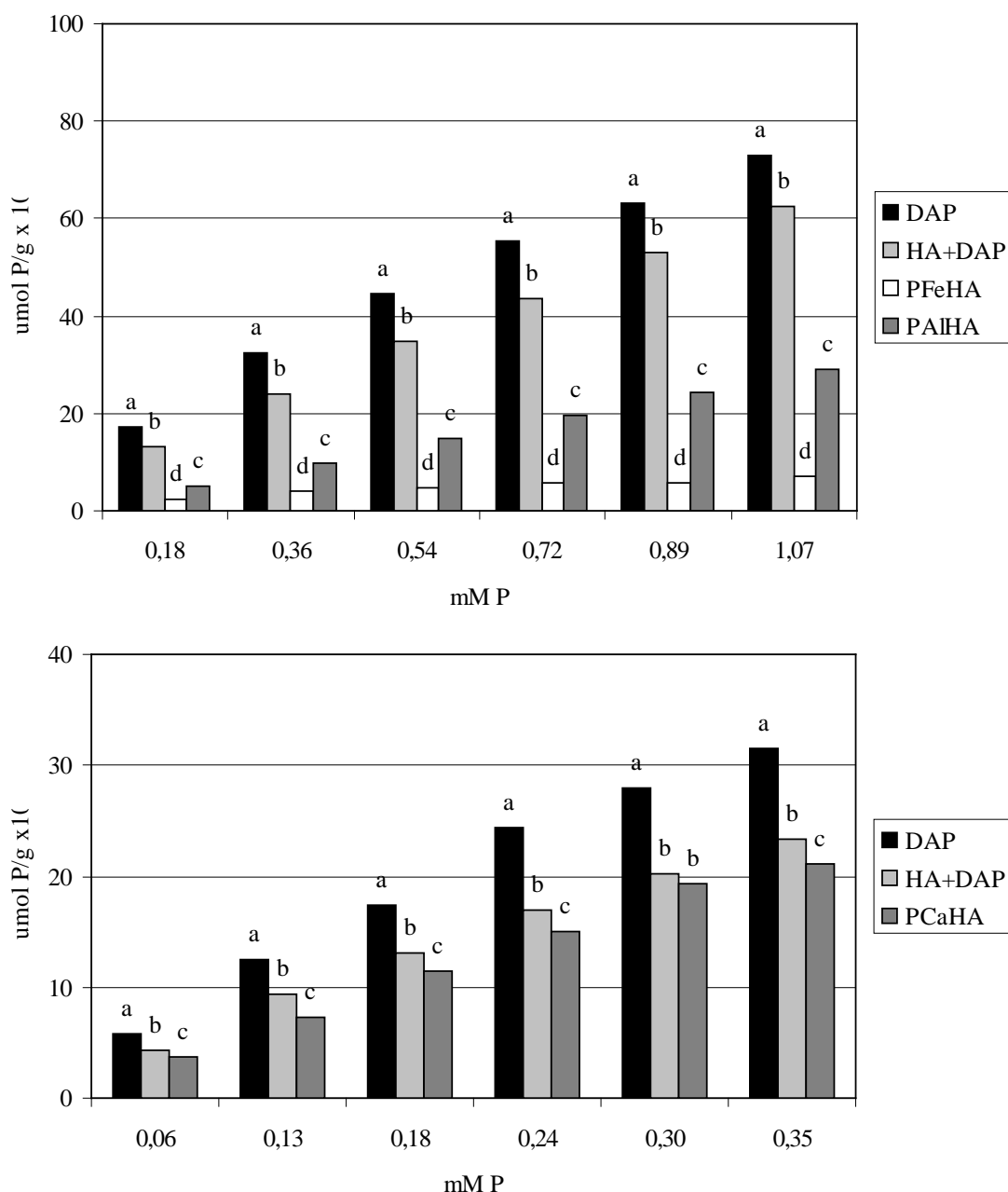


Figure 6.- Protection study for Guardo soil (pH 4.5)

These results are also reflected in the fraction of potentially plant available P recovered after the interaction between a soil with high P capacity fixation and this type of complex (P-Ca-HA complex).

In Figure 7 we present the bicarbonate- and HCl- extracted P fractions for a monocalcium phosphate and a P-Ca-humic complex in an acid soil with high P fixation capacity.

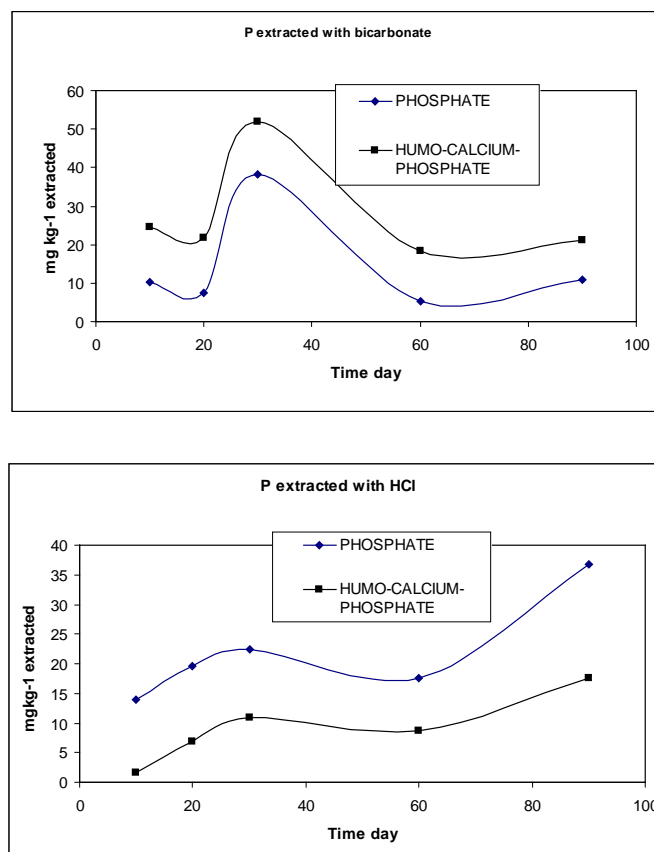


Figure 7.- Bicarbonate- and HCl- extracted P fractions for a monocalcium phosphate and a P-Ca-humic complex in an acid soil with high P fixation capacity

The results show that the application of P-Ca-Humic complexes was associated with higher concentrations of potentially available P (bicarbonate extracted fraction), and lower concentrations of retrograded P (HCl extracted fraction), thus showing the protecting effect suggested by the above mentioned results of P soil absorption isotherms.

Now, the question that arises is if plants are able to assimilate the P fixed in metal-humic complexes.

### 3.2. Plant ability to take up P complexed in metal-Humic complexes

In order to study the plant capacity to assimilate the P fraction complexed by metal-humic complexes, we have compared the growth of wheat plants receiving soluble phosphate and P complexed by Fe-humic complexes.

As can be seen in Figure 8, there were not significant differences between the developments of the plants growing with these different P forms, although different uptake mechanisms could be involved.



Figure 8.- Growth of wheat plants cultivated with phosphate and phosphate complexed by Fe-humates

In conclusion, these studies show that P complexation by metal-humates is associated with a significant P protection from soil retrogradation that is reflected in both soil P absorption isotherms and the fraction of potentially plant available P. Likewise, these results also show that a model plant (wheat) is able to assimilate the P complexed in Fe-humic complex that was the complex with the highest stability.

All these results stress the important role that these types of complexes could play in both the protection of P from soil retrogradation and the potential P –bioavailability in soils.

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