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Research Article

Barley seed coating with urease and phosphatase to improve N and P uptake

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ABSTRACT: In this work, barley seed coating with urease and phosphatase, free and immobilized in humic acids, was studied as a tool to enhance the use efficiency of N and P by plants. The seeds were encapsulated in gels of Ca-alginate by a method that allows gelation of the polymer, containing enzymes, from the surface of seeds outward. The coating process did not affect seed germination. A pot experiment with the coated seeds was carried out. The seed coating with free enzymes increased soil ammonium-N (NH_4^+-N) and inorganic P up to 39 and 54 %, respectively, and up to 62 and 64 %, with the immobilized enzymes, in comparison with control pots. Seed coating improved N (47-137 %) and P (29-61 %) uptake by plants. Moreover, a synergistic response in N and P uptake with both urease and phosphatase was observed. It can be concluded that the seed coating with urease and phosphatase exerted a positive effect enhancing the use of N and P by plants, which could lead to the development of natural fertilizers, minimizing the application of chemical fertilizers.

Keywords: soil enzymes, seed encapsulation, plant nutrients uptake, humic acids

Introduction

High rates of N and P fertilizers are commonly added to soils to increase crop yield. Unfortunately, many of these fertilizers are not efficiently used by plants, potentially causing long-term harm to the environment, such as greenhouse emissions and eutrophication (Lu and Tian, 2017). In fact, no more than 30 % and about 25 % of the N and P added, respectively, are taken up by plants in the first year of application (Panda, 2013).

It is well known that soil enzymes play an essential role releasing nutrients, such as N and P, as inorganic forms to be taken up by plants by mineralization of organic matter. Although soil N and P occurs in both inorganic and organic forms, up to 90 % of the N (Olk, 2008) and 20-80 % of P (Fransson and Jones, 2007) are in organic form. Among the enzymes involved in the hydrolysis of organic N compounds are amidohydrolases (L-asparaginase, L-glutaminase, amidase, urease and L-aspartase) (Ekenler and Tabatabai, 2004) and urease is one of the most important enzymes related to N transformation in the soil (Bai et al., 2012). The organic P compounds are mineralized by extracellular phosphatases, adaptive enzymes produced by plant roots and soil microorganisms in response to a need for P by plants (Schneider et al., 2001).

The development of biotechnological tools, such as seed coatings with stabilized enzymes to improve the utilization of N and P by plants, is an important challenge for sustainable agriculture. Enzymes can be stabilized by their association with humic colloids, which protect them against proteolytic activity and other processes leading to their inactivation in the soil (Burns et al., 2013). It has been reported that the coating of barley seeds with alkaline phosphatase, free and immobilized in humates, improved the use of soil P by plants (Pilar et al., 2009; Pilar-Izquierdo et al., 2012, 2013). Mvila et al. (2016) found that barley seed coating with urease and humic-urease-complexes significantly increased the plant N uptake.

In this context and taking into account that simultaneously application of N and P fertilizers gave a much stronger growth response of plants than either of them alone (Harpole et al., 2011; Fisk et al., 2014), we hypothesize that seed coating with both urease and phosphatase could exert a synergistic effect on N and P uptake by plants. Therefore, the aim of this research was to study the effect on plant growth and N and P uptake by plants of barley seed coatings by Ca-alginate with urease and phosphatase, free or stabilized in humic acids.

Materials and Methods

Enzyme immobilization

Urease (urea amidohydrolase, EC 3.5.1.5) was obtained from jack bean (*Canavalia ensiformis*). Alkaline phosphatase (EC 3.1.3.1) was obtained from *Escherichia coli* ATCC27257, a phosphatase hyperproductive strain supplied by the Spanish Official Culture Collection (Valencia, Spain), according to Pilar et al. (2003). Carboxymethylcellulose (CMC), *p*-nitrophenylphosphate (pNPP) and all other reagents were of analytical grade.

Alkaline phosphatase and urease were immobilized, respectively, by co-flocculation with humic acids (HA) and Ca^{2+}. The HA were obtained following the methodology proposed by Busto et al. (1997). The methods used to immobilize alkaline phosphatase and urease and the biochemical characteristics of the resultant enzymatic complexes were described by Pilar et al. (2003) and Mvila et al. (2016), respectively. Briefly, the humic-phosphatase complexes (HPC) were obtained by mixing in orbital shaking (150 rpm; 4 °C; 2 h) 1 mL of the alkaline phosphatase solution, 2 mL of HA and 4 mL of 0.1 M 2-amine, 2-methyl, 1-propanol (2A 2M 1P) buffer. The humic-urease complexes (HUC) were prepared by mix-

ing (150 rpm; 4 °C; 3 h) 2 mL of urease solution (0.25 mg mL^{-1}), 2 mL of HA (4.5 mg mL^{-1}) and 3 mL of phosphate–citrate–borate buffer (PCBB: 100 mM phosphate, 57 mM borate, 36 mM citrate) (pH 8). Then, the immobilization solutions were flocculated by the addition of 2 mL of 0.5 M $CaCl_2$ and shaken (150 rpm, 6 h, 20 °C). The suspensions were centrifuged (15,400 g; 15 min; 4 °C), and the resulting pellets were washed five times with 0.1 M calcium acetate buffer (pH 4.5). Then, the resultant enzymatic complexes, HPC and HUC, were resuspended in 0.3 mL of 2A 2M 1P or PCBB buffer, respectively, frozen and lyophilized.

Seed coating

The barley seeds (*Hordeum vulgare* L., cvar Vanessa) were encapsulated in gels of Ca-alginate containing (i) free alkaline phosphatase (FP) or HPC, (ii) free urease (FU) or HUC and (iii) urease and phosphatase together (FU + FP) or (HUC + HPC). The barley seeds were encapsulated using the method described by Pilar et al. (2009) (modified from Patel et al. (2000)) and modified as follows: the encapsulation solution contained 2 % (w/v) calcium chloride, 6 % (w/v) CMC and different enzyme/CMC solution ratios (FP (8,912 U mL^{-1}): 0.4 mL mL^{-1}; HPC (58,866 U g^{-1}): 0.1 g mL^{-1}; FU (1,159 U mg^{-1}): 0.4 mg mL^{-1}; HUC (213 U g^{-1}): 0.1 g mL^{-1}; FU + FP: 0.4 mg mL^{-1} of urease and 0.4 mL mL^{-1} of alkaline phosphatase; HUC + HPC: 0.05 g mL^{-1} of each immobilized enzyme). The coating urease or phosphatase activity was defined by the ratio of the enzyme activity (urease or phosphatase) of the encapsulated seeds to the enzyme activity (urease or phosphatase) applied in the coating process. Determinations were replicated five times.

Enzyme coating stability and germination test

Seeds coated with FU, FP, HUC or HPC were stored for 32 days at 4 °C, and the residual urease or phosphatase activity was determined periodically. The germination rate of the seeds was evaluated, as described by Pilar-Izquierdo et al. (2012).

Assay of amidohydrolase activities

The activities of amidohydrolases (urease (EC 3.5.1.5), amidase (EC 3.5.1.4), aspartase (EC 4.3.1.1), glutaminase (EC 3.5.1.2) and asparaginase (EC 3.5.1.1)) were measured as described by Kandeler and Gerber (1988) with the following modifications. Briefly, the method is based on determining the NH_4^+ release when 0.5 mL of sample (HA) is incubated with 3.5 mL phosphate-citrate-borate buffer (PCBB: 100 mM phosphate, 57 mM borate, 36 mM citrate) at different pH value (7 for urease; 8.5 for amidase and aspartase; 10 for glutaminase and asparaginase) and 1 mL of substrate (urea, formamide, L-aspartic, L-glutamine and L-asparagine, for urease, amidase, aspartase, glutaminase and asparaginase, respectively) at 37 °C for 15 min. To assess the urease activity of soil and encapsulated seeds, samples of 1 g fresh soil and five seeds, respectively, were incubated with 4 mL PCBB. The NH_4^+ released was determined as described by Mvila et al. (2016). One enzymatic unit (U) was defined as the amount of enzyme that produced 1 μg of NH_4^+-N over 1 h under the assay conditions. The urease activity was expressed as U per 1 mL of enzyme (free or immobilized), 1 g of coated seed or 1 g of soil dry weight.

Assay of alkaline phosphatase activity

The alkaline phosphatase activity was determined using the artificial substrate p-nitrophenyl phosphate (pNPP) (Tabatabai and Bremner, 1969). The methodology used to evaluate the phosphatase activity of FP, HPC, encapsulated seeds and soil was described in detail by Pilar-Izquierdo et al. (2012). One enzymatic unit (U) was defined as the amount of enzyme that produced 1 μg of pNP during 1 h under the assay conditions. Phosphatase activity was expressed as U per 1 mL of enzyme solution, 1 g of immobilized enzyme, 1 g of dried weight soil or 1 g of coated seeds.

Pot experiment

A pot experiment was conducted in plastic pots (150 mL) containing 150 g of a non-amended soil to study the effect of the seed coating with the enzymes on the plant growth and N and P uptake. The soil used for the pot experiment was collected from the surface (0-10 cm depth) of a farm field located in Ribera del Arlanza (Burgos, Spain), air-dried at room temperature, sieved through a 2-mm mesh and stored at 4 °C until use. The soil was a sandy loam with 513 g kg^{-1} of sand, 436 g kg^{-1} of silt, and 76 g kg^{-1} of clay. The initial properties of the soil were: pH 7, total N 13.2 g kg^{-1}, total C 26.8 g kg^{-1}, organic C 20.7 g kg^{-1}, total P 0.41 g kg^{-1} and inorganic P 0.24 g kg^{-1}.

Uncoated (control) and coated seeds with the different enzymatic treatments were previously germinated on moistened filter paper in a plant growth chamber at 26 °C for 96 h. For each seed treatment, five seedlings were planted in a plastic pot at a depth of 10 mm. The pots were placed in a growth chamber and the plants were grown under a temperature regime of 20/15 °C (light/dark) and 16 h photoperiod per day. The pots were watered daily with deionized water (15 mL). Each experiment was replicated five times. Plants were harvested at 7, 14, 21, 28 and 35 days after planting (DAP), (5 plants, 1 per each replicate pot at each time). After harvesting, length, dry weight, N and P contents were determined in the shoots. Soil samples were taken to evaluate NH_4^+-N, inorganic P, and urease and alkaline phosphatase activity in the bulk soil.

Plant and soil analysis

To determine the shoot dry weight the samples were oven-dried at 105 °C for 48 h. The contents of P and N of shoots were determined according to Hayes et al. (2000) and Hevia and Cioccia (1988), respectively. To determine the N and P uptake by plants, dry weight was multiplied by N and P contents of the shoot, respective-

ly. The soil NH_4^+-N was extracted with KCl (Keeney and Nelson, 1982) and determined according to Keeney and Bremner (1966) and modified by Beck (1983). Inorganic P of soil was extracted with 0.5 M H_2SO_4 (Saunders and Williams, 1955) and the P in the solution was measured according to Murphy and Riley (1962).

Statistical methods

The variance and regression analyses were applied to data using Statgraphics Centurion XVI.I for Windows. Data in Tables and Figures are given as mean values and the standard errors of the mean (SEM). All determinations were replicated five times. To compare significant differences in the means, the Duncan test was used at $p < 0.05$, unless otherwise stated.

Results

Seed coating and germination test

The enzyme activity (urease or phosphatase) retained in the coating layer of encapsulated seeds with free enzymes (FP, FU, FU + FP) was higher (between 33 and 46 %) than that of seeds encapsulated with the immobilized forms (HUC, HPC, HUC + HPC) (varied from 24 to 33 %) (Table 1). The seed coating with free enzymes (FU, FP, FU + FP) resulted in higher values of enzyme activity for urease than for phosphatase. Moreover, in each case, similar values of enzyme activity retained in the coating layer were observed regardless of the seed coating performed, applying the enzymes individually (FU or FP) or in combination (FU + FP). Thus, the coating urease activity of seeds encapsulated with FU and FU + FP was of 46 and 44 %, respectively, and the coating phosphatase activities of seeds encapsulated with FP and FU + FP were of 33 and 35 %, respectively. Seed encapsulation with HPC and HUC + HPC resulted in similar values of coating phosphatase activity (25 %). However, when seeds were coated with the immobilized enzymes applied together (HUC + HPC), the coating urease activity was significantly increased in comparison with the seed encapsulation with only HUC (33 and 24 %, respectively).

Table 1 – Coating urease and phosphatase activities of barley seeds encapsulated with free urease (FU), immobilized urease (HUC), free urease and phosphatase (FU+FP), free phosphatase (FP), immobilized phosphatase (HCP) and immobilized urease and phosphatase (HUC+HPC). Values are presented as mean (n = 5) ± SEM.

Enzyme applied to the seed coating	Coating urease activity	Coating phosphatase activity
	%	
FU	46 ± 2.6	–
HUC	24 ± 0.3	–
FP	–	33 ± 4.9
HPC	–	25 ± 0.2
FU+FP	44 ± 0.5	35 ± 1.6
HUC+HPC	33 ± 0.5	25 ± 2.4

The HA did not show phosphatase activity. However, the humic support showed amidohydrolase activities of 11.7, 3.4, 2.4, 9.7 and 11.1 U mL^{-1} for urease, amidase, asparaginase, aspartase and glutaminase, respectively. After immobilization with the urease solution the activities of the enzymes, except for urease (activity: 152 U mL^{-1}), did not change.

The stability of the enzymes added to the seed coating layer after 32 d of storage is shown in Figure 1. The immobilized enzymes (HUC and HPC) showed higher stability than the free enzymes (FU and FP). Moreover, the stability of HUC and HPC in the coating layer resulted in similar values of retained activity, whereas significant differences in these values were found between FP and FU. Thus, after 32 d of storage, the seeds treated with HPC and HUC retained 72 and 76 % of their initial activity, respectively, while seeds coated with FP and FU retained 42 and 22 %, respectively.

The coating process did not affect the viability of seeds. The germination percentage of the encapsulated seeds ranged from 87 to 96 % in comparison to uncoated seeds (Table 2). Although these values were

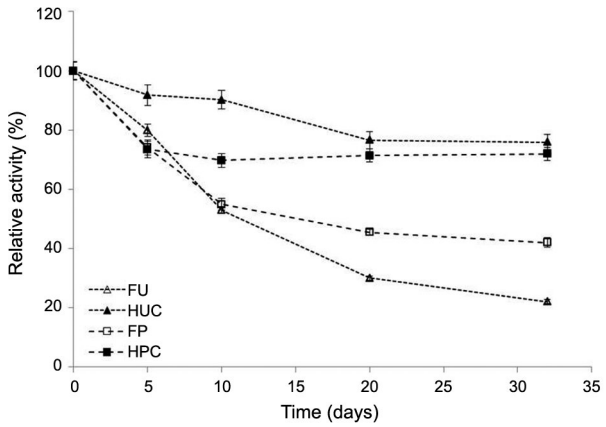

Figure 1 – Relative enzyme activity (urease or phosphatase) in the seed coating layer of barley seeds coated with free urease (FU) or phosphatase (FP), and immobilized urease (HUC) or phosphatase (HPC). Values are presented as mean (n = 5) and SEM (error bars).

Table 2 – Germination test of seeds coated with urease free or immobilized (FU or HUC), phosphatase free or immobilized (FP or HPC), free urease and phosphatase (FU+FP), and immobilized urease and phosphatase (HUC+HPC). Values are presented as mean (n = 5) ± SEM.

Enzyme	Germination
	%†
FU	87ns
HUC	92
FP	96
HPC	94
FU+FP	90
HUC+HPC	89

†In relation to untreated seeds. Control germination = 90 %; ns = not significant.

slightly lower than in the control, the differences were not statistically significant. Moreover, the germination of the coated seeds was delayed 24 h in comparison to untreated seeds.

Effect of seed coating on soil urease and phosphatase activity

The urease and phosphatase activities in the bulk soil of pots planted with the coated seeds are shown in Figure 2. In general, the urease activity was higher in pots planted with seeds coated than in control pots at all the assessed times (Figure 2A). On average during the study period, the seed coating significantly increased the urease activity in the bulk soil (p = 0.0141) (Figure 2B). Thus, the pots planted with coated seeds with the immobilized and free enzymes showed values of urease activity in the bulk soil between 164-187 U g^{-1} dried soil and 160-175 U g^{-1} dried soil, respectively. Contrastingly, in the control pots, the urease activity was 155 U g^{-1} dried soil, which represent increases between 6-21 and 3-13 % in relation to control pots.

In general, seed coating with the immobilized enzymes (HUC, HPC and HUC+HPC) significantly increased (p = 0.000) the phosphatase activity in the bulk soil, whereas no effect was observed when seeds were coated with free enzymes (FU, FP and FU+FP) (Figures 2C and 2D). Thus, on average during the study period, values of phosphatase activity in pots planted with the immobilized enzymes between 468 and 486 U g^{-1} dried soil and 408 U g^{-1} dried soil in control pots, were observed, which represents increases between 15 and 19 % in relation to the control (Figure 2D). Moreover, a decrease in the soil phosphatase activity with time was observed in all pots from 14 DAP (Figure 2C).

Effect of seed coating on soil NH_4^+-N and inorganic P

The effects of seed coating with urease and/or phosphatase on both soil NH_4^+-N and inorganic soil P are shown in Figure 3. Overall, the soil NH_4^+-N was significantly higher (p = 0.000) in pots planted with treated seeds than in control pots (Figures 3A and 3B). Thus, on average during the study period, the pots planted with coated seeds with the free and immobilized enzymes showed values of soil NH_4^+-N in the bulk soil between 4.2-5.0 mg kg^{-1} of dried soil and 4.6-5.9 mg kg^{-1} of dried soil, respectively. On the other hand, values of 3.6 mg kg^{-1} of dried soil were found in the control pots. Moreover, the highest increases in soil NH_4^+-N were found when seeds were treated with the enzymes added to-

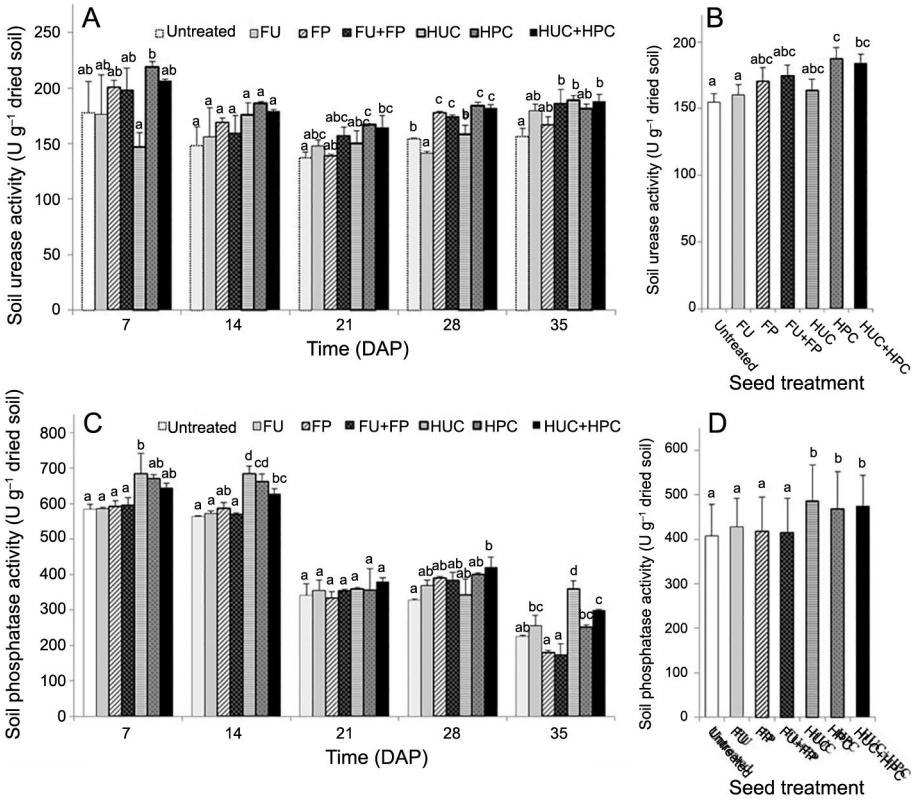

Figure 2 – Urease (A and B) and phosphatase (C and D) activities in the bulk soil of pots planted with seeds untreated and coated with free urease (FU) or phosphatase (FP), free urease and phosphatase (FU+FP), urease (HUC) or phosphatase (HPC) immobilized, and urease and phosphatase immobilized (HUC+HPC). DAP: days after planting. Urease activity at t = 0: 132 U g^{-1} dried soil. Values are presented as mean (n = 5) and SEM (error bars). Different letters mean significant difference according to the Duncan test at $p < 0.05$.

gether in form free (FU + FP; 39 %) and immobilized (HUC + HPC; 62 %). On the other hand, an increase in the soil NH_4^+-N with time, to a maximum at 28 DAP, followed by a sharp decrease, was observed in all pots (Figure 3A).

In general, the seed coating with the enzymes significantly increased (p = 0.000) the soil inorganic P (Figure 3C and 3D). Thus, the pots planted with coated seeds showed mean values between 0.20 and 0.25 g kg^{-1} dried soil versus 0.15 g kg^{-1} dried soil for control pots (Figure 3D), which meant increases between 30 and 64 % over control (Figure 3D). The highest increases were observed when seeds were coated with the enzymes added together, in the free (FU + FP; 54 %) and immobilized (HUC + HPC; 64 %) forms. Furthermore, it was observed that, in all pots, the soil inorganic P increased during the first days and then decreased, remaining in stable levels from 21 DAP until the end of the experiment (Figure 3C).

Effect of seed coating on plant growth and N and P uptake

The effect of seed coating on plant growth is shown in Figure 4. The data analysis at different DAP showed that the shoot length of plant growth from coated seeds was only significant at 7 (p = 0.000) and 14 (p = 0.0058) DAP, with all plants reaching a similar shoot length at the end of the pot experiment (35 DAP) (Figure 4A). Thus, at 7 DAP, higher values of shoot length (between 38-64 %) were found when compared to the control pots (128-153 mm over 93 mm of control plants). However, in general, the seed coating significantly increased (p = 0.000) the shoot length (Figure 4B).

The seed treatment significantly increased (p < 0.01) the shoot dry weight of the plants (Figures 4C and 4D). Thus, on average during the study period, there were increases in the shoot dry weight of plants grown from encapsulated seeds between 14 and 22 % (56 and 66 mg per plant over 49 mg per plant of control plants) in comparison with the control pots. Moreover, no significant differences between treatments were found (Figure 4D).

The seed coating significantly increased (p = 0.000) the N and P uptake by plants (Figure 5). In general, the seed coating exerted a higher effect in the N (Figures 5A and 5B) than P (Figures 5C and 5D) uptake. Thus, on average during the study period, the N uptake increased from 47 to 137 % (1.28 to 2.06 mg per plant) over control pots (0.87 mg per plant) (Figure 5B), whereas the increases in P uptake ranged from 29 to 61 %

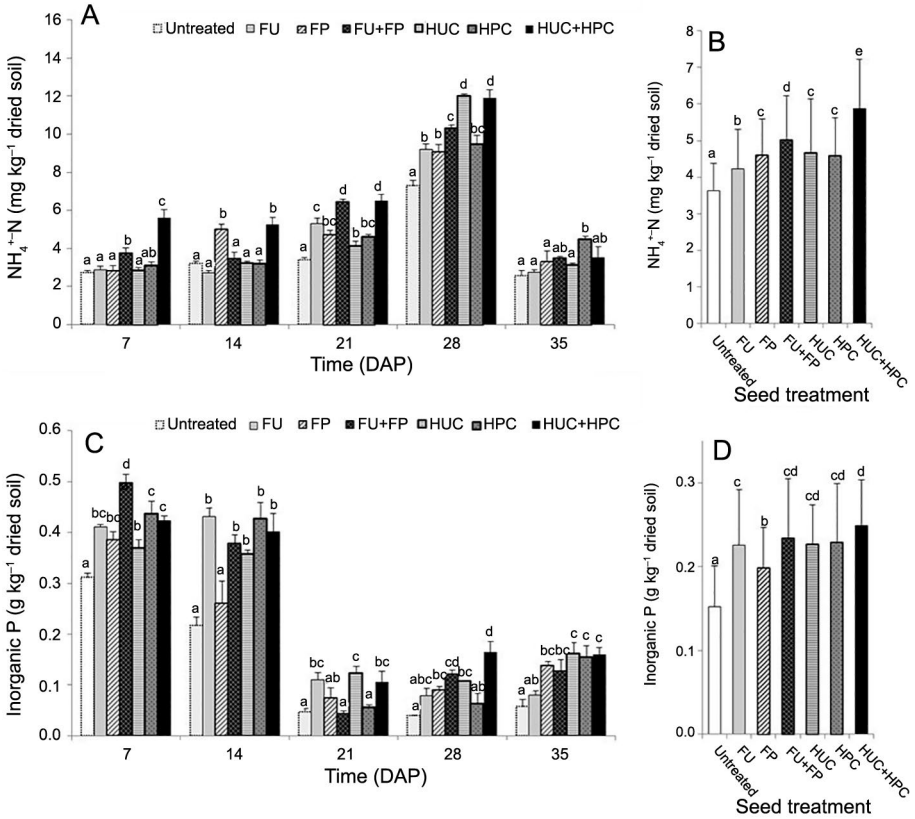

Figure 3 – Ammonium-N (NH_4^+-N) (A and B) and inorganic P (C and D) in the soil of pots planted with seeds untreated and coated with free urease (FU) or phosphatase (FP), free urease and phosphatase (FU+FP), immobilized urease (HUC) or phosphatase (HPC), and immobilized urease and phosphatase (HUC+HPC). DAP = days after planting. NH_4^+-N at t = 0: 2.5 mg kg^{-1} dried soil. Inorganic P at t = 0: 0.24 g kg^{-1} dried soil. Values are presented as the mean (n = 5) and SEM (error bars). Different letters mean significant difference according to the Duncan test at p < 0.05.

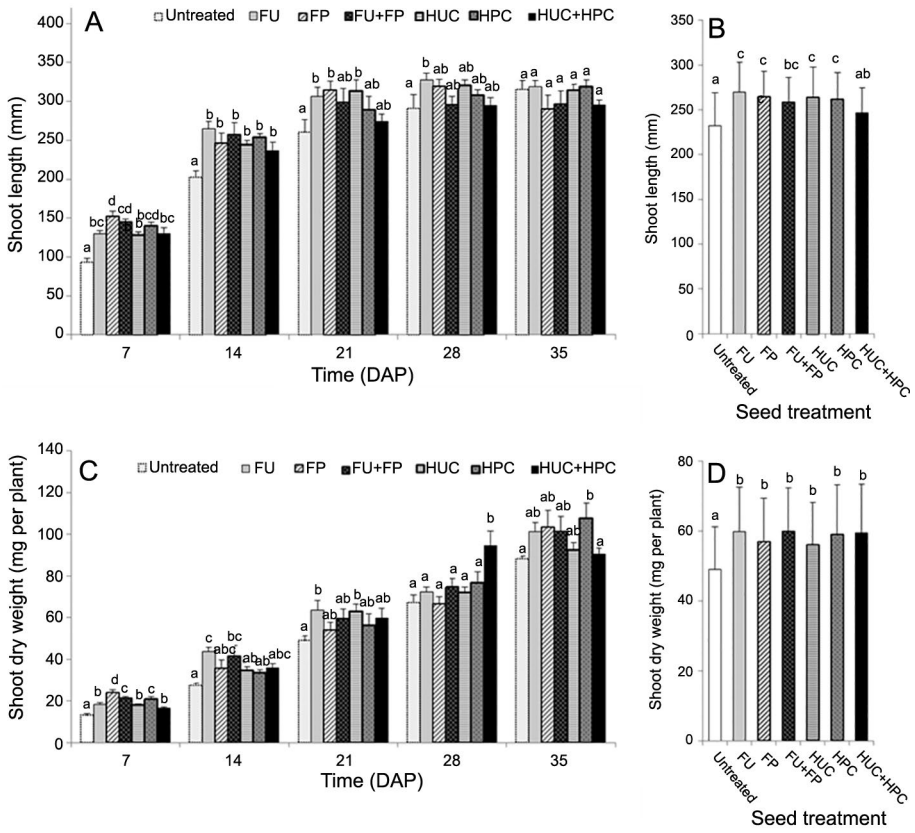

Figure 4 – Length (A and B) and dry weight (C and D) of plants grown from seeds untreated and coated with free urease (FU) or phosphatase (FP), free urease and phosphatase (FU+FP), immobilized urease (HUC) or phosphatase (HPC), and immobilized urease and phosphatase (HUC+HPC). DAP = days after planting. Values are presented as mean (n = 5) and SEM (error bars). Different letters mean significant difference according to the Duncan test at $p < 0.05$.

(0.40 to 0.50 mg P per plant) in comparison with control pots (0.31 mg P per plant) (Figure 5D). Moreover, the uptake of both N and P increased with time until 28 DAP and then decreased at the end of the study period, with a sharper decline for P (Figures 5A and 5C).

Regarding N uptake, the highest increases were found when seeds were coated with the enzymes applied together, showing increases of 200 and 265 % in plants grown from seeds coated with FU + FP and HUC + HPC, respectively, at the end of the pot experiment. Likewise, on average during the study period, seed encapsulation with FU + FP and HUC + HPC yielded the highest values of N uptake, namely, 1.71 and 2.06 mg per plant, respectively, over 0.87 mg per plant of control pots (increases of 96 and 137 %, respectively, over control).

In the case of P, an average during the study period, the highest uptake values were found when seeds were coated with urease immobilized and free, alone or together with phosphatase in both cases. Values of P uptake of 0.50, 0.48, 0.47 and 0.45 mg per plant were found when seeds were coated with HUC, HUC + HPC, FU and FU + FP, resulting in increases in the P uptake of 61, 53, 49 and 44 % in comparison to control plants (0.31 mg per plant).

Discussion

The values of the urease and phosphatase activities held on the seed coating layer may be related with the encapsulation process (Table 1). Encapsulation implies the formation of a Ca-alginate capsule inside where the enzymes added are enclosed. Diffusional restrictions caused by the presence of this capsule could lead to the entrapped enzyme activity not to be fully expressed (Tanriseven and Doğan, 2001). These diffusional restrictions may vary depending on the characteristic of the coating layer, which could be affected by the type and concentration of the reagents used during encapsulation, and affect to a greater or lesser extent due to the concentration and properties of the enzyme applied.

Seed encapsulation with the enzymes in the immobilized form resulted in a higher enzyme coating stability (Figure 1), which can be explained by the association of the studied enzymes to the humic support. Many researchers (Mvila et al., 2016; Dong et al., 2008; Li et al., 2013) have reported increased stability of proteins bound to humic substances.

The delay of 24 h in the germination of the encapsulated seeds observed in this work may be related to

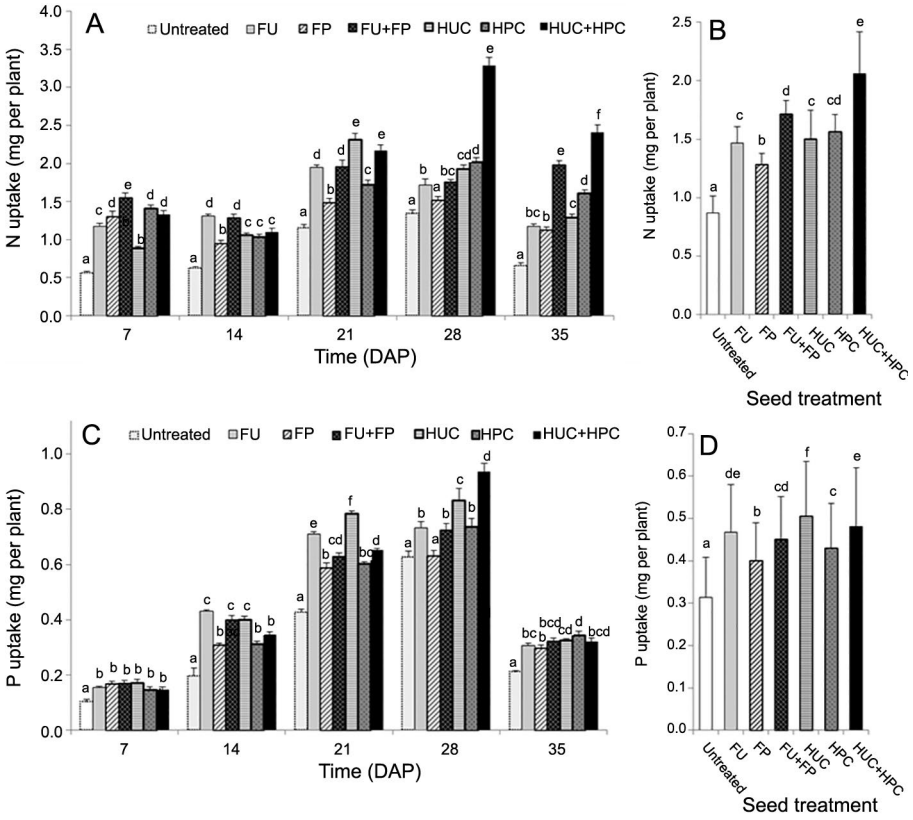

Figure 5 – Nitrogen (A and B) and P (C and D) uptake of plants grown from seeds untreated and coated with free urease (FU) or phosphatase (FP), free urease and phosphatase (FU+FP), immobilized urease (HUC) or phosphatase (HPC), and immobilized urease and phosphatase (HUC+HPC). DAP = days after planting. Values are presented as mean (n = 5) and SEM (error bars). Different letters mean significant difference according to the Duncan test at $p < 0.05$.

the alginate capsule itself, which may result in a physical barrier that restricts water uptake and gas exchange, an important aspect to insure optimal germination of seeds (Murphy, 2016).

The determination of enzyme activities in the rhizosphere is often hampered by the lack of methodologies to obtain sufficient amount of soil samples for laboratorial analyses (Gianfreda, 2015). In the present research, to estimate the effect of the seed coating with enzymes in the soil, the urease and phosphatase activities in the bulk soil were assessed. It should be noted that increases in urease and/or phosphatase activities in the bulk soil occurred regardless of the kind of enzyme added to the coating (urease and/or phosphatase) (Figures 2B and 2D). These increases are partly due to an effect of the alginate capsule. In this sense, Wingender et al. (1999) suggested that the interactions between enzyme and polysaccharides, such as alginate, affected their catalytic activity. In any case, these results did not reflect what happens in the soil rhizosphere, where an increase in the enzyme activities tested, because of seed coating, may be expected. Pilar-Izquierdo et al. (2013), applying an agar technique to visualize the phosphatase activity in the rhizosphere, reported that encapsulation of barley seeds with FP and HPC increased the rhizosphere phosphatase activity, although increases in the phosphatase activity in the bulk soil were not observed.

The increase in the soil NH_4^+-N observed in all pots (Figures 3A and 3B) suggests that the mineralization of soil organic N has taken place. This increase could be explained by a combination of factors. First, the experiment under laboratory conditions could lead to N mineralization, which would explain the increase in the soil NH_4^+-N observed in the control pots. Abbasi et al. (2011) have reported similar results. The authors observed increases in the NH_4^+-N concentration of soils incubated under controlled conditions. On the other hand, since, in general, all the pots planted with coated seeds showed higher values of soil NH_4^+-N than control pots, N mineralization seems to be related to the coating applied to the seeds (Figure 3B). Moreover, the higher increases observed when urease and phosphatase were added together in the coating could be attributed to synergistic interactions between these enzymes.

Likewise, the decrease in the soil NH_4^+-N concentration found in all pots at the end of the pot experiment (35 DAP) may be partly due to the transformation of NH_4^+-N to NO_3^--N via nitrification, a process responsible

for most NH_4^+ loss in agricultural fields (Robertson and Groffman, 2015). Thus, increases in the NO_3^--N in soils incubated under laboratory conditions were found (Abbasi et al., 2011; Abbasi et al., 2001). Moreover, the decrease in the soil NH_4^+-N could also contribute to the increase observed in the N uptake by plants. Similarly, the changes in NH_4^+-N concentration found in the pots with time may be explained by the soil microbial activity (Robertson and Groffman, 2015).

The increases in the inorganic P in the bulk soil seem to indicate that P mineralization has occurred (Figures 3C and 3D). The higher increase in the soil inorganic P observed when seeds were coated with the enzymes added together (FU + FP and HUC + HPC) seems to confirm the effect of the enzymes added to the seed coating layer acting synergistically. On the other hand, the decrease in the soil inorganic P observed in all pots with time could be attributed to the plant uptake and the immobilization of inorganic P forms into organic P forms by microbial biomass.

The analysis of the bulk soil may not adequately reflect the effect of seed coating with the enzymes increasing the availability of N and P by plants. Thus, a more reliable indicator of soil nutrient bioavailability to plants is the analysis of the nutrients in plant tissues (Elliott et al., 1997). The increase in the uptake of N and P by plants grown from coated seeds seems to indicate the beneficial effect of seed coating with enzymes increasing the availability of these nutrients. Moreover, the enhancement in the use of N and P by the plants was reflected in the highest shoot dry weight observed in plant grown from seeds coated with enzymes, although there was no positive effect to the shoot length at the end of the study period. These increases in the N and P uptake are in line with the higher contents of NH_4^+-N and inorganic P found in pots planted with coated seeds compared to control pots. The higher increases in the N uptake observed when seeds were coated with urease and phosphatase together added seem to support the hypothesis of synergistic interactions between these enzymes, as indicated above, increasing the availability of N in the rhizosphere. Moreover, the higher values in N uptake of plants grown from seeds coated with the enzymes immobilized together could be related to the greater stability of enzymes associated with humic support. On the other hand, cycles of N and P are coupled in such a way that the availability of one of these nutrients could influence the availability of the other. Thus, Block et al. (2013) found that increased N availability enhanced bioavailable P. This fact could also explain the similar increases in P uptake observed when seeds were coated with urease added alone or together with phosphatase. The urea hydrolysis in the soil could result in an increase in soil pH and NH_4^+-N, which could lead to the dissolution of organic matter enhancing the P availability accordingly (Ouyang et al., 1999).

The different response found between the N and P uptake could be related to the characteristic of the soil and the plant species used in the experiment. The decreases in the N and P uptake of shoot at the end of the study period may be attributed to a dilution effect as shoot biomass increases. Many researchers for other crops (Elliott et al., 1997; Ziadi et al., 2008) have reported similar results.

In summary, seed coating with urease and/or phosphatase exerted a positive effect by increasing the N and P uptake by plants. In general, this effect was higher when seeds were coated with immobilized enzymes, which can be attributed to their higher stability in the seed coating layer. Moreover, a synergistic response in the N and P uptake because of the seed coating was observed. However, the short-term experiments carried out in laboratory did not reflect what happens in the field; therefore, experiments in natural conditions should be carried out.

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Authors' Contributions

Conceptualization: Ortega, N.; Pilar-Izquierdo, M.C.; Busto, M.D.; Perez-Mateos, M. Data acquisition: Mvila, B.G. Data analysis: Ortega, N.; Pilar-Izquierdo, M.C.; Busto, M.D.; Design and Methodology: Ortega, N.; Pilar-Izquierdo, M.C.; Busto, M.D. Writing and editing: Ortega, N; Pilar-Izquierdo, M.C.

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