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Original article

# Effect of pH on the bioprotection exerted by some strains of arbuscular mycorrhizal fungi

## Efecto del pH en la bioprotección ejercida por algunas cepas de hongos micorrízicos arbusculares

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**RESUMEN:** Arbuscular mycorrhizae have been widely described as favoring plant growth and making physical, biochemical and physiological changes in roots that lead to a better general condition of the plant and contribute to alleviate abiotic and biotic stress situations. As a result of their multiple benefits, their use in Cuban agriculture has been gradually increasing. INCA's generalist strain recommendation system is based, fundamentally, on the soil type and its associated fertility. Knowing how the bioprotection effect of different strains is integrated with pH can contribute to elucidate whether the effect is associated with a strain "*per se*" or depends on the effectiveness of the strains. For this purpose, an experiment was designed using a red-yellowish Argissolo soil, adjusting the $Ca^{2+}$ and $Mg^{2+}$ concentrations to a single level and three pH values (5.5; 6.5 and 7.2) with the aim of finding out whether differences in pH also influence the bioprotection exerted by these strains. *Rhizophagus irregularis*, *Glomus cubense* and *Rizophagus clarus* strains recommended for different pH ranges were used and *Fusarium oxysporum* f. sp. *phaseoli* was inoculated on 21-day-old bean plants as pathogen. Strains originated differentiated pH-dependent responses in the intensity of colonization, bioprotection exerted and active induction of peroxidases, indicating that the bioprotection effect was associated with the effectiveness of each strain at one or another pH.

**Key words:** mycorrhizae, *Fusarium oxysporum*, bean.

**RESUMEN:** Las micorrizas arbusculares han sido ampliamente descritas como favorecedoras del crecimiento vegetal y realizan cambios físicos, bioquímicos y fisiológicos en las raíces que conducen a un mejor estado general de la planta y contribuyen a aliviar las situaciones de estrés de carácter abiótico y biótico. Producto de sus múltiples beneficios se ha ido incrementando paulatinamente su uso en la agricultura cubana. El sistema de recomendación de cepas de carácter generalistas del INCA se basa, fundamentalmente, en el tipo de suelo y su fertilidad asociada. Conocer cómo se integra el efecto de bioprotección de diferentes cepas con el pH puede contribuir a dilucidar si el efecto se asocia a una cepa "*per se*" o depende de la efectividad de las mismas. Para ello se diseñó un experimento utilizando un suelo Argissolo rojo-amarillento, ajustando las concentraciones de $Ca^{2+}$ y $Mg^{2+}$ a un único nivel y tres valores de pH (5,5; 6,5 y 7,2) con el objetivo de conocer si las diferencias en el pH también influyen en la bioprotección ejercida por estas cepas. Se utilizaron las cepas *Rhizophagus irregularis, Glomus cubense* y *Rizophagus clarus* recomendadas para diferentes rangos de pH y se usó como patógeno *Fusarium oxysporum* f. sp. *phaseoli* que fue inoculado en plantas de frijol de 21 días de edad. Las cepas originaron respuestas diferenciadas dependientes del pH en la intensidad de la colonización, la bioprotección ejercida y la inducción activa de peroxidasas, indicando que el efecto de bioproteccion se asoció a la efectividad que presentaba cada cepa en uno u otro pH.

**Palabras clave:** *Fusarium oxysporum*, frijol, micorrizas.

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## INTRODUCTION

In addition to plant nutrition, arbuscular mycorrhizal symbiosis positively affects the ability of plants to overcome biotic and abiotic stresses, thereby commonly improving tolerance to unfavorable environmental conditions and resistance to pathogens (1). The establishment and maintenance of the association requires a high degree of coordination between both partners and a bidirectional (plant-fungus) control of the fair exchange of resources between symbionts (2). In fact, precise regulation of hormone levels has been proposed as a central mechanism in regulating the interaction (3).

Arbuscular mycorrhizal fungi (AMF) are not only edaphic microorganisms, but the extraradical mycelium, which is the most abundant structure and is responsible for nutrient and water uptake, among other effects, is found in the soil. Even within the symbiosis it is unusual because the bulk of the structures are found in the soil and not within the host; therefore, then edaphic conditions in mycorrhizal functioning is important (4). Although there is no strict partner specificity in arbuscular mycorrhizal (AM) symbiosis, the outcome of the interactions that are established depends on the interacting partners and environmental conditions (5). In this sense, the National Institute of Agricultural Sciences (INCA) has proposed a system for recommending efficient strains in terms of nutrition and ecoservices, based, fundamentally, on the type of soil and its associated fertility (6).

This system aims to maximize the effects of these fungi, with a consequent decrease in fertilizer application and maximum yield, derived from the ecoservices of this symbiosis. It includes pH as a determinant of the maximization of the effects, which is associated with $Ca^{2+}$ and $Mg^{2+}$ levels, so that these could also be associated with the effective response of the strains.

On the other hand, plant pests must be managed to maintain food quality and abundance. To this end, different approaches have been used to prevent and mitigate the effect of pests (7). In the framework of current agriculture, AMF use has become vitally important, not only because of their contribution to plant development and nutrition, but also because of effects as anti-stress agents that these microorganisms have, both on biotic and abiotic stresses (8). In this sense, AMF use as anti-stress agents against diseases has gained increasing interest. Different mechanisms have been proposed by which these fungi are able to induce protection in their plant hosts, including improvements in nutrition, changes in radical exudates, induction of active defense mechanisms, and even translocation of signals using the hyphal network to induce defensive mechanisms in neighboring plants, so that this response can prevent the development and establishment of the pathogen (9).

Different authors have emphasized the fact that the establishment and symbiotic efficiency of these microorganisms are influenced by the edaphic environment, specifically by soil fertility and pH (6,10).

This work aimed to elucidate to what extent soil pH can influence the symbiotic efficiency and, therefore, the bioprotection effect exerted by three AMF strains.

## MATERIALS AND METHODS

*Plant material:* Beans (*Phaseolus vulgaris* L.) of the Preto Estrela variety were used, provided by the seed group of EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária, in Portuguese) Agrobiology.

*Experimental conditions:* Starting from a red-yellowish Argissolo soil (11), which corresponds to an Acrisol Rhodic Santi according to World Reference Base with pH-$H_2O$ of 4.94 and exchangeable $Ca^{2+}$ and $Mg^{2+}$ contents of 0.17 and 0.05 $cmolcd^{-1}$, it was proceeded to prepare three pH conditions (5.5; 6.5 and 7.2) with $CaCO_3$ and adjusted to similar $Ca^{2+}$ and $Mg^{2+}$ concentrations at the three pHs of 1.63 and 0.26 $cmolcd^{-1}$ using $CaSO_4$ and $MgSO_4$. With the procedure described, the possible masking caused by varying amounts of $Ca^{2+}$ and $Mg^{2+}$ in the different treatments, which prevents establishing the effect of pH, was eliminated.

*Arbuscular mycorrhizal fungi (AMF):* The AMF strains *Rhizophagus irregularis* (Blaszk, Wubet, Renker & Buscot) Walker & Shüßler (INCAM-11, DAOM-711363) and *Glomus cubense* (Y. Rodr. & Dalpé) (INCAM-4, DAOM-241198), from the collection of the Mycorrhizae Laboratory from INCA, San José de las Lajas, Mayabeque, which were preserved in an osmotically protected solution (12). *Rizophagus clarus* (Nicol. & Schenck) Walker & Shüßler strain (A5, CNPAB) from the Biological Resource Center Johanna Döbereiner (CRB-JD) EMBRAPA Agrobiology, Seropédica, Rio de Janeiro, Brazil, preserved in substrate at 4 ºC whose titer was 75 spores $g^{-1}$ of substrate and which was applied in the planting niche at 1 g rate, was also used. This strain is isolated from the soils of Brazil and, therefore, is adapted to the acidic conditions of soils in this area.

Inoculation with AMF was performed at planting and plant emergence for Rhizophagus *irregularis* and *Glomus cubense* using a liquid inoculum at a rate of 60 spores $mL^{-1}$ and in the planting niche for *Rhizophagus clarum*, at a rate of 1 g of solid inoculum with a spore content of 75 spores per g of soil.

The pots were 1 kg pots with one plant per pot, to which Hoagland's solution adjusted to a phosphorus concentration equivalent to a quarter of the initial solution (1/4 P equals 0.204 g $L^{-1}$) was added weekly, to maintain a supply of nutrients that would allow the correct development of the crop, as well as to achieve effective mycorrhization.

Plants were grown under semi-controlled conditions of temperature (24 ºC ±2 ºC), relative humidity (80-85 %) and natural photoperiod (14 hours light-10 hours' dark). The experiments were carried out under greenhouse conditions at EMBRAPA Agrobiologia, Seropédica, Rio de Janeiro, Brazil.

The treatments established are shown in Table 1.

*Pathogen*: The pathogen used was *Fusarium oxysporum* f. sp. *phaseoli* maintained in PDA and provided by the

phytopathogen laboratory of EMBRAPA Brasilia, Brazil. The phytopathogen inoculum was obtained from colonies grown for 15 days in Petri dishes of 90x14 mm, containing the sporulation medium for beans (Agar 15 g; dextrose 20 g; bean leaves 200 g and complete with distilled water at 1000 mL pH 5.9) (11) and a mixed inoculum was made with races 149 and 151, which are those with the highest incidence in Brazilian conditions. To the plates, 20 mL of sterile distilled water was added and the mycelium was collected with a Drigalski spatula. The concentration was adjusted to 105 spores $mL^{-1}$ by counting in a Neubauer chamber. Inoculation was carried out on 21-day-old plants, applying the pathogen by spraying in the root zone at a rate of 5 mL $plant^{-1}$.

A split-plot experiment was developed in which each plot corresponded to a pH with eight treatments for each plot, consisting of a single inoculation of each AMF strain and an uninoculated control combined with inoculation or not of the pathogen, according to Table 1. Eight pots per treatment with three plants each were used. Plants inoculated with *Fusarium oxysporum* were kept separate from non-inoculated plants.

After inoculation with the pathogen, 21 days after the plants emerged, they were kept in a humid chamber closed with nylon for 48 hours. In all treatments, symptoms and signs of the disease were evaluated daily, counting as day zero the moment the plants were inoculated and up to day five, which corresponded to plants 25 days after germination. At each moment, the percentage of leaf necrosis per leaf was evaluated, calculating the average of the measurements on all the leaves of each plant. The severity of the damage was evaluated by measuring the degree of wilting on each plant, according to the scale of Pozo and collaborators (13). During each evaluation, destructive sampling of plants from a pot was performed to determine the frequency and intensity of mycorrhizal colonization, as well as total protein extraction to evaluate peroxidase enzyme activity.

*Determination of fungal occupancy:* A pool of roots from three plants/treatment was sampled, dried at 70 °C and stained with 5 % ink in 2 % acetic acid (14). Colonization frequency was assessed by the intercept method (15) and colonization intensity (D. V) by the method described (16).

*Enzyme extraction and Peroxidase determination:* Root and leaf samples were macerated in liquid nitrogen independently and homogenized in a 1:2 ratio (g $mL^{-1}$), with extraction buffer (sodium acetate, 0.1 M, pH 5.2; containing 5 g polyvinylpyrrolidone and 0.05g β- mercaptoethanol, in 100 mL of extraction solution). The homogenate was shaken in sieve for 45 min, in ice bath, then filtered through gauze and centrifuged at 14 000 x G, at 4 °C for 25 min, in refrigerated centrifuge. The supernatant was stored at -80 °C until use for total protein concentration and peroxidase enzyme activity determinations. Protein concentration was determined by the method described by Bradford (17).

*Peroxidase activity (PRX) (Enzyme classification (E.C) 1.11.1.7):* It was performed according to the continuous method described (18). The oxidation rate of guaiacol was determined in spectrophotometer (Ultrospec Plus Spectrophotometer, Pharmacia LKB), registering the absorbance values at 470 nm. The variation of absorbance was taken for two minutes at 10-second intervals. Enzyme activity was expressed as µmoles of product formed min-1 $mL^{-1}$ of enzyme.

Subsequently, the specific activity was calculated from the ratio of enzyme activity and protein concentration of each sample.

*Statistical analysis of data:* Parametric and non-parametric techniques were used according to the variable analyzed. The severity of the disease produced by the pathogen is presented according to the following formula % of healthy plants=100 % wilt detected, to visualize a severity dynamic.

## RESULTS AND DISCUSSION

One of the premises established for mycorrhizal fungi to exert their effect is that they must be established and with sufficient fungal structures that allow them to exert their action (7). In this sense, Figure 1 represents colonization intensity levels that include not only the presence or absence of the fungus in the root as in the case of frequency or colonization, but also the amount of fungal structures that are detected as a better indication of the functioning of the symbiosis (15,19).

The strain provided by the EMBRAPA Agrobiology group presented the highest mycorrhizal intensity at the most acidic pH, while at pH 6.5 the highest occupancy was found with the inoculation of *G. cubense*, followed by the inoculation of *R. irregulare* and with a lower performance of the *R. clarus* strain. At pH 7.2 the behaviors of *R. irregulare* and *G. cubense* were very similar and superior to those obtained when inoculating *R. clarus*. Results that have been achieved in Cuba on the management of mycorrhizal

**Table 1.** Treatments established at each pH value

| Treatment | Denomination | Description |
|---|---|---|
| **Control** | **Control** | **Plants without inoculation** |
| *Rhizophagus clarus* | *R. clarus* | Inoculated plants with *R. clarus* |
| *Glomus cubense* | *G. cubense* | Inoculated plants with *G. cubense* |
| *R. irregularis* | *R. irregularis* | Inoculated plants with *R. irregularis* |
| Control+*Fussrium oxysporum* | Control + P | Inoculated plants with *F. oxysporum* |
| *R. clarus+F. oxysporum* | *R. clarus* + P | Inoculated plants with *R. clarus* and with *F. oxysporum* |
| *G. cubense+F. oxysporum* | *G. cubense* + P | Inoculated plants with *G. cubense* and with *F. oxysporum* |
| *R. irregularis+F. oxysporum* | *R. irregularis*+ P | Inoculated plants with *R. irregularis* and with *F. oxysporum* |

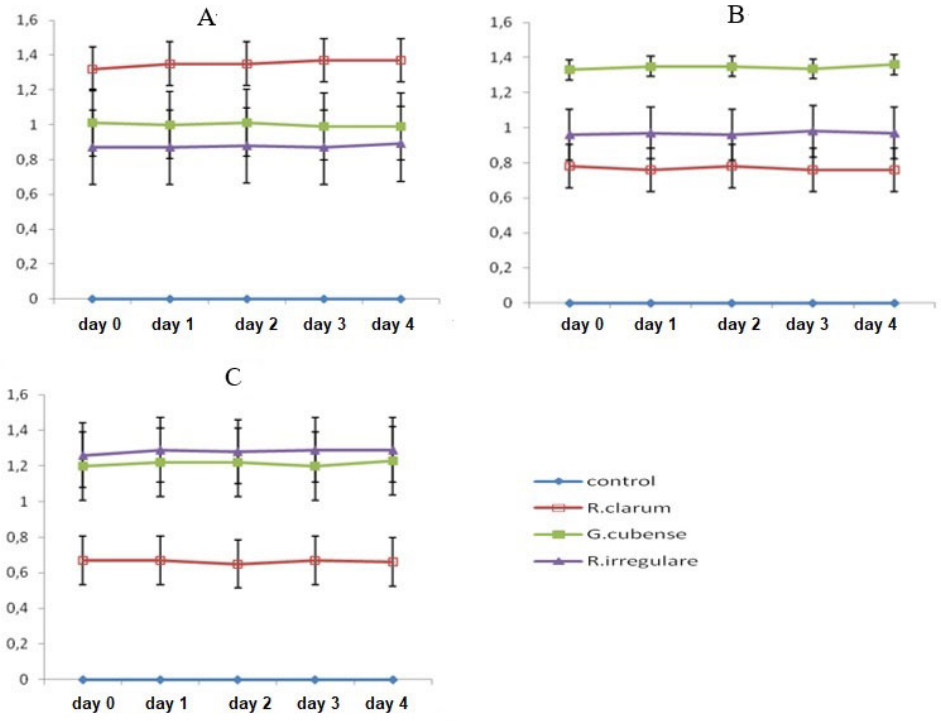
inoculants include the use of efficient strains of generalist character, whose efficiency condition is dependent on the pH of the soil or substrate in which the inoculated crop will be developed (6). *G. cubense*/INCAM-4 strain is recommended for use in soils whose pH-$H_2O$ fluctuates between 5.8 and 7.2; while *R. irregulare*/INCAM-11 strain is recommended for pH between 7 and 8, with an overlapping or transition zone of efficiency of both strains between pH 7 and 7.2. Results of this work corroborate the criteria already described by different authors (6) and allow the inclusion of this type of soil of Brazilian origin, which as it is observed, changes the efficient strain as a function of pH, with which the types of soils in which they are fulfilled are increased and are not circumscribed to Cuban soils. In the case of *R. clarus* strain, it is a strain isolated from Brazilian soils, recommended to inoculate diverse crops in the most acid soils of this country and, therefore, which will present the highest effectiveness in the acid pH.

It should be noted that previous studies comparing the effectiveness of INCAM strains with several species of forage grasses and in different soils showed a change in the effectiveness of the strains by soil type and explained by changes in pH (20). Although the high correlations in natural conditions between pH and Ca and Mg contents in soils masked the referred effect.

AMF are adapted to a wide spectrum of edaphic conditions that are related to their occurrence, development, and effectiveness (21) and influence the functioning of the mycorrhizal symbiosis subject to the interaction of several edaphic factors (4), with special emphasis on pH (22). The pH is considered one of the most important chemical properties of the soil (23), due to the effect it has on the physical, chemical and biological characteristics of the soil, as well as on crop yields. This variable can determine, from the biological point of view, the type of organism that develops on a soil, due to its great influence on the availability of nutrients. In this regard, it is stated that fungi and the group of actinomycetes bacteria constitute the two large groups of soil microorganisms, and the predominance of one group or the other depends on local conditions, especially pH and moisture content (24). Among these microorganisms are arbuscular mycorrhizal fungi, which live in mutualistic symbiosis with about 95 % of the species of the plant kingdom (6).

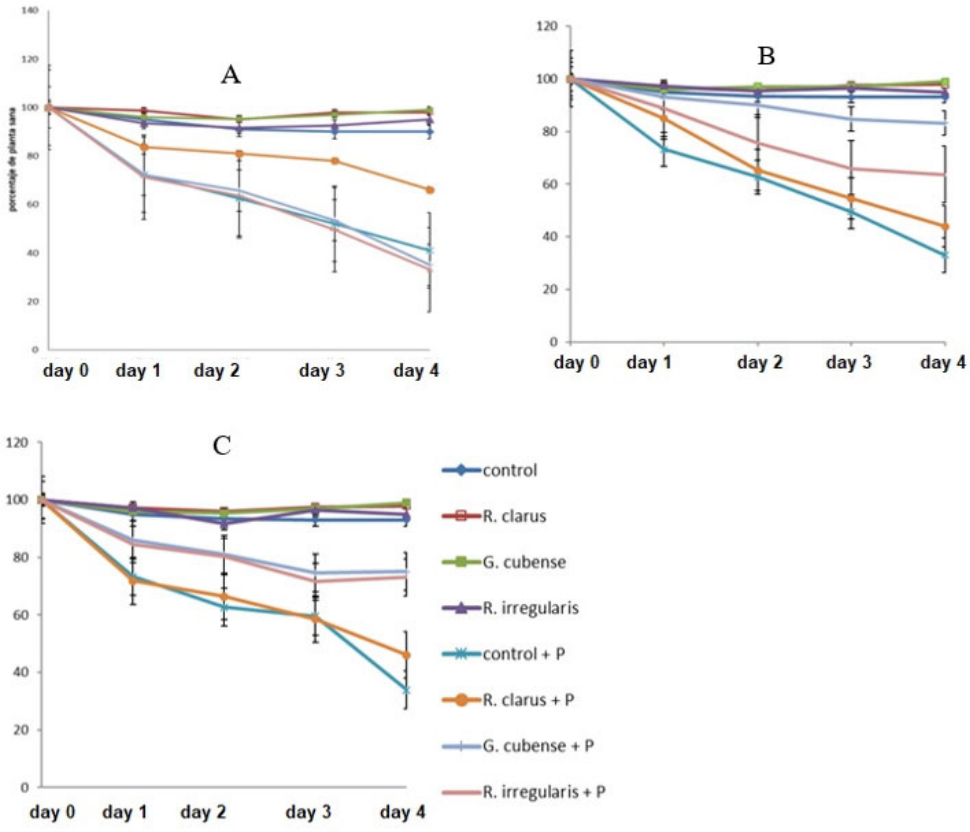
It is considered that the acidity condition of the soil, expressed through its pH, is linked to the functioning of AMF (25). The effects of pH on nutrient availability, the regulation of ion exchange processes, and the diversity of microorganisms associated with the mycorrhizosphere are currently explicable (26,27). In addition, other more direct effects on germination and sporulation have been reported (25). However, it is considered that the mechanism that explains and conditions the changes in function and effectiveness of these strains with soil pH is still not sufficiently clear.

Figures 2 and 3 present levels of protection exerted by the different strains at the pHs used in the experiment and the specific activity of peroxidases detected.




A: pH=5.5, B: pH=6.5 and C: pH=7.2. The ordinate axis represents the intensity of colonization measured as described in Materials and Methods and the abscissae the time in days. The bars correspond to the confidence intervals for p≤0.05 %

**Figure 1.** Intensity of colonization detected for each of the strains tested at the different pH-$H_2O$ established during the experiment




A: pH=5.5, B: pH=6.5 and C: pH=7.2. Bean plants (*P. vulgaris* L.) of the Preto Estrela variety, 21 days old, the AMF strains *Rhizophagus clarus* (R. clarus), *R. irregularis* (R.irregulare) and *Glomus cubense* (G. cubense) and *Fusarium oxysporum* f. sp. *Phaseoli* as pathogen (P), grown on bean sporulation medium, were used. The ordinate axis shows the percentage of healthy plants by the formula presented in Materials and Methods and on the abscissa axis the time in days. Bars correspond to the confidence intervals for p≤0.05 %

**Figure 2.** Percentage of healthy plants found at different pHs during the confrontation between mycorrhizal plants inoculated or not with the pathogen

Plants that were not inoculated with the pathogen showed no damage to the leaves and no significant differences between treatments. In the treatments inoculated with strains of F. oxysporum, although damage to plants was found at all pH levels, regardless of the inoculation of one or another AMF strain, although in the latter the damage was much less, with the bioprotection effect achieved with each strain varying with pH.

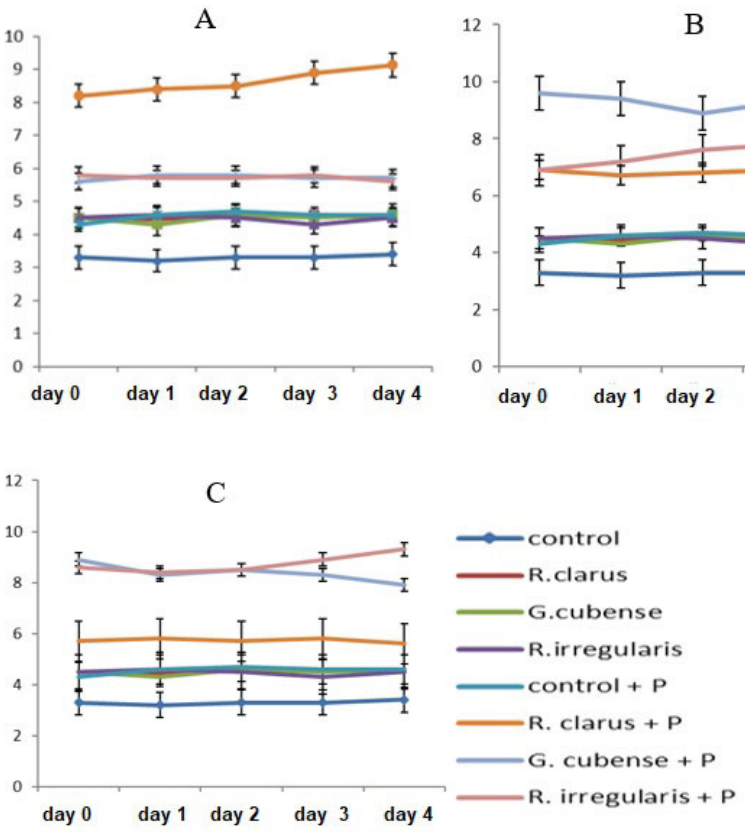
The magnitude of the bioprotection effect produced by each AMF strain at a given pH was associated with the degree of fungal intensity reached by that strain at the same pH (Figure 1), such that at each pH the strain or strains with the highest colonization intensity presented, at the same time, the greatest bioprotection effect. Therefore, results indicated that the bioprotection effect was associated with the degree of effectiveness or mycorrhizal functioning presented by each strain, which was pH-dependent.

It is noteworthy the high relationship between the bioprotection effect presented by INCAM4 and INCAM11 strains and the recommendation of efficient AMF strains as a function of pH as a criterion for their inoculation (6). The results also indicated the benefit of using these strains as the basis for inoculants, since they not only achieved high mycorrhizal performance and the benefits commonly associated with it (20), but also a greater bioprotection effect.

The bioprotection effect related to mycorrhizal functioning has been associated with the induction of plant defensive mechanisms and within these mechanisms peroxidases have been one of the linked PR-proteins (7,28). Figure 3 shows that in any of the pH treatments that did not receive the pathogen, basal levels of PRX activity did not differ from each other, whether inoculated with AMF strains or the control.

However, in the presence of the pathogen, in all cases, enzyme induction was higher. In the case of mycorrhizal plants, significant differences were found between the peroxidase inductions caused by AMF strains and this effect was pH-dependent. At each pH, the strain that produced the highest values of peroxidase induction was the one that caused the greatest bioprotection effect and, at the same time, achieved the highest fungal intensity.

The mechanisms induced by AMF to attenuate oxidative damage and protect plant cell function encompass a number of tolerance mechanisms activated by these fungi such as improvements in nutrition and water uptake,




A: pH= 5.5, B: pH=6.5 and C: pH=7.2. The dots represent the mean of the sampled treatments. The ordinate axis represents the specific activity calculated according to Materials and Methods and the abscissae represent the time in days. The bars correspond to the confidence intervals for p≤0.05 %

**Figure 3.** Peroxidase activity dynamics in each of the treatments analyzed at the different pHs at which the experiment was established

modulation and expression of genes that are related to signaling and thus stress response (7). The functioning of these fungi, however, is mediated by edaphic conditions among which pH stands out as an important element since this chemical property of the soil determines in many cases the efficiency of the endophyte, the percentage of spore germination, and the development of arbuscular mycorrhizae (29,30).

Soil acidity limits plant productivity, inhibits root elongation and reduces phosphorus (P) solubility. It is for this reason that some AMF species may be affected by this acidity condition, such as most *Glomus* species (30). The relationship established between soil pH ranges and the effect of mycorrhizal colonization is truly complex, depending not only on the fungal species, but also on the type of soil, the form in which the nutrients are found (fundamentally P and N and other elements such as Cu, Zn, Mo, B, etc.) and to a lesser extent on the plant species on which it develops (27).

Strains that compose the EcoMic® biofertilizer produced in Cuba, two of which were part of this study, can be classified according to Opik (30), as generalists with the crops and specific with the pH of the soil (6). In this sense, the efficiency of strains is maximized in the conditions in which they perform their best function, not only in the uptake of nutrients and water (20,31,32), but also in terms of the protection mechanisms they induce in plants. They are demonstrated here in the induction of peroxidases, which is reversed in a protection of biological membranes (7,28). This was always better in the strain that worked at the pH for which it is recommended, thus making it clear that, in our case, bioprotection is a further attribute of the effectiveness of the symbiosis.

## CONCLUSION

The inoculation of efficient AMF strains produces a significant bioprotection effect that is directly associated with the induction of peroxidases and both are a consequence of the degree of mycorrhizal functioning reached by different strains. The latter depends on the pH of the soil in which the mycorrhizal plants develop. At least with the strains studied, the bioprotection effect is not associated with a strain "per se" and is another attribute of the effectiveness of mycorrhizal functioning.

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