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INDUCTION OF DEFENSE MECHANISMS IN MYCORRHIZED TOMATO PLANTS AGAINST THE ATTACK OF *Oidiopsis taurica* (Lev.) Salm

Inducción de mecanismos de defensa en plantas de tomate (*Solanum lycopersicon* L.) micorrizadas frente al ataque de *Oidiopsis taurica* (Lev.) Salm

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ABSTRACT. In order to evaluate mycorrhized tomato plants against the attack of *Oidiopsis taurica* pathogen an experiment was conducted. Plants previously treated with arbuscular mycorrhizal fungus (AMF) *Glomus cubense* (40 spores g^{-1}) and *G. mosseae* (60 spores g^{-1}) were challenged with *O. taurica* (2 x 104 spores mL^{-1}), at 21 days of germination. Several enzymatic activities related to the induction of defense (PRX, PPO, β -1.3 glucanase, chitinase and phenylalanine ammonia lyase) were determined and mycorrhizal variables (visual density or fungal occupation (DV) and colonization rate) and percentage of damage caused by the pathogen were measured. Mycorrhizal species induced systemic responses in plants where higher levels of protection were observed in plants treated with *G. cubense.* These levels were not sufficient to prevent colonization of the pathogen and thus the damage caused by this, however, there was a differential response between both AMF studied. The low response induced by *G. mosseae* against the attack of the plant pathogenic fungus was remarkable, which leads to the conclusion that not all HMA have the same inductive response against a specific plant pathogen.

Key words: defense mechanisms, pathogenicity, tomato, AMF

RESUMEN. Se realizó un experimento con el objetivo de evaluar la respuesta del tomate micorrizado, frente al ataque del hongo fitopatógeno *Oidiopsis taurica* (Lev.) Salm. Para ello se enfrentaron plantas previamente tratadas con los hongos micorrízicos arbusculares (HMA) *Glomus cubense* (40 esporas g^{-1}) y *G. mosseae* (60 esporas g^{-1}) con *O. taurica* (2 x 104 esporas mL^{-1}), a los 21 días de germinadas. Se determinaron algunas actividades enzimáticas relacionadas con la inducción de defensa (PRX, PPO, β-1,3 glucanasa, quitinasa y fenilalanina amonio liasa), así como variables micorrízicas (densidad visual u ocupación fungica (D.V) y porcentaje de colonización) y porcentaje de daño producido por el patógeno. Las especies micorrízicas indujeron respuestas sistémicas en las plantas, donde los mayores niveles de protección se observaron en las plantas tratadas con *G. cubense*. Los niveles de inducción de respuesta no fueron suficientes para evitar la colonización del hongo fitopatógeno y por ende, el daño producido por este; sin embargo; existió una respuesta diferencial entre las dos especies de HMA estudiadas. Es interesante destacar la baja respuesta inducida por *G. mosseae* frente al ataque de este hongo fitopatógeno, lo cual permite concluir que no todos los HMA presentan la misma respuesta inductiva frente a un fitopatógeno específico.

Palabras clave: mecanismos de defensa, patogenicidad, tomate, HMA

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INTRODUCTION

The current trend to reduce the use of pesticides of any kind is known, for its negative impact on the soil and the man (1). The use of biological products in agriculture has reached great boom in recent years,

promoting plant yield and crop productivity through obtaining biofertilizer to replace polluting chemicals (2).

Considering this trend, arbuscular mycorrhizal fungi (AMF) have received special attention in the latest decades, firstly because they are naturally found in the ecosystems and can establish a mutual symbiosis with most economic plants and then because of their positive influence on host nutrition, its physiological condition and willingness to confront the attack of various pathogens (2, 3).

Among the fungal pathogens affecting vegetable crops is *Oidiopsis taurica* (Lev.) Salm., causing powdery mildew, whose incidence is increased by climatic changes occurred in warmer and humid winters. This pathogen is favored under 70 % relative humidity and temperatures between 10 and 35 °C reaching an optimum value at 25 °C, which are the average environmental conditions under which vegetables grow in our country[A].

Having in mind what was above stated, besides considering these AMF are essentially reported as bio-protectors against root pathogens (6), and the need to know their bio-protective capability against foliar pathogens, this work was conducted to evaluate the response of two AMF species in tomato (*Solanum lycopersicum* L.) plants against the attack of the phytopathogenic fungus that causes powdery mildew.

MATERIALS AND METHODS

The experiments were conducted at the areas of the National Centre of Animal Health (CENSA) using a completely randomized design with three replicates and two repetitions. The tomato variety "Amalia" (*Solanum lycopersicum* L.) was selected, which was obtained at the Department of Plant Genetics and Breeding from the National Institute of Agricultural Sciences (INCA) (4). The experiment was developed in 1-kg-sized containers, with a substrate made up by a mixture of Red Ferralitic soil and vermicompost on a 3:1 (v/v) ratio, sterilized by dry heat at 150 °C for one hour during three days. Some agrochemical characteristics of the substrate used are shown in Table I.

The AMF species Glomus cubense (Y. Rodr. & Dalpe) (INCAM-4) (5) and *Glomus mosseae* (Nicolson & Schenck) (INCAM-2) (6) coming from the strain collection of the National Institute of Agricultural Sciences (INCA) were used, whose inocula had a titer of 40 and 60 spores g^{-1} fresh soil respectively, which were inoculated by seed coating, besides a dynamic mycorrhization set up for both strains from the third day up to the 26th day.

Sampling was performed to determine fungal colonization in non-inoculated plants with the pathogen; thus, a root sample of at least 10 plants randomly taken per treatment was formed. They were dried at 70 °C and stained by the method described by Phillips and Hayman (7), evaluating colonization percentage by the intercept method (Grid line intersects) (8). The intensity of colonization or visual density (% VD) was calculated (9).

The experiment was repeated twice in 2003 and 2004, within January-March period, which is the most favorable time for this crop. Plants were kept under glasshouse conditions until they were inoculated with the pathogen and placed inside mesh houses, where sunlight incidence was attenuated in the areas of CENSA, San José de Las Lajas, Mayabeque, Cuba.

The *O. taurica* inoculum was collected from infected tomato plants of Amalia variety kept under growing conditions at 20 ± 2°C, 70% relative humidity and natural photoperiod. Fresh spores were gathered by washing infected leaves with distilled water to prepare an inoculum concentration adjusted to 2 x 104 spores mL^{-1}, which was sprayed upon the leaves of 21-day-old plants, at the rate of 5 mL. Three replicates were used, each of them having three plants kept under closed humidified chambers (covered with nylon) to enhance pathogen establishment.

The following treatments were settled:

- Control plants (neither AMF nor pathogens)
- Mycorrhized plants with *G. mosseae*
- Mycorrhized plants with *G. cubense*
- Inoculated plants with *O. taurica*
- Mycorrhized plants with *G. mosseae* and inoculated with *O. taurica*
- Mycorrhized plants with *G. cubense* and inoculated with *O. taurica*

Five days after inoculation, symptoms and signs of the disease caused by the pathogen were evaluated and sampled to determine the enzymatic activity, where planting material was removed by organs. Data were transformed according to the scale reported by Schaefer *et al.* (10) and analyzed using Kruskal Wallis' nonparametric multiple comparison test supplemented by Mann-Whitney's comparison test and the corresponding one to Bonferroni's correction (11).

[A]Gómez G. Comunicación personal (INISAV). 2008

Table I. Agrochemical characteristics of the substrate

Substrate	K^+	Ca^{2+} (cmol kg^{-1})	Mg^{2+}	P (ppm)	Organic matter (%)	pH (H_2O)
Typical lixiviated Red Ferralitic Soil (8,9): worm humus (3:1)	0,6	18,9	6,0	160,0	6,9	7,3

Proteins were extracted through the method described by Solórzano[B] and the concentration (19) was quantified, reading the absorbance at 595 nm in a spectrophotometer (Ultrospec Plus Spectophotometer, Pharmacia LKB); therefore, a bovine serum albumin pattern curve was manufactured from a stock solution of 1 mg mL^{-1}.

The enzymatic activities determined are shown in Table II and expressed in units of enzymatic activity according to the following formula:

Specific activity (SAU) = Enzymatic activity/protein concentration

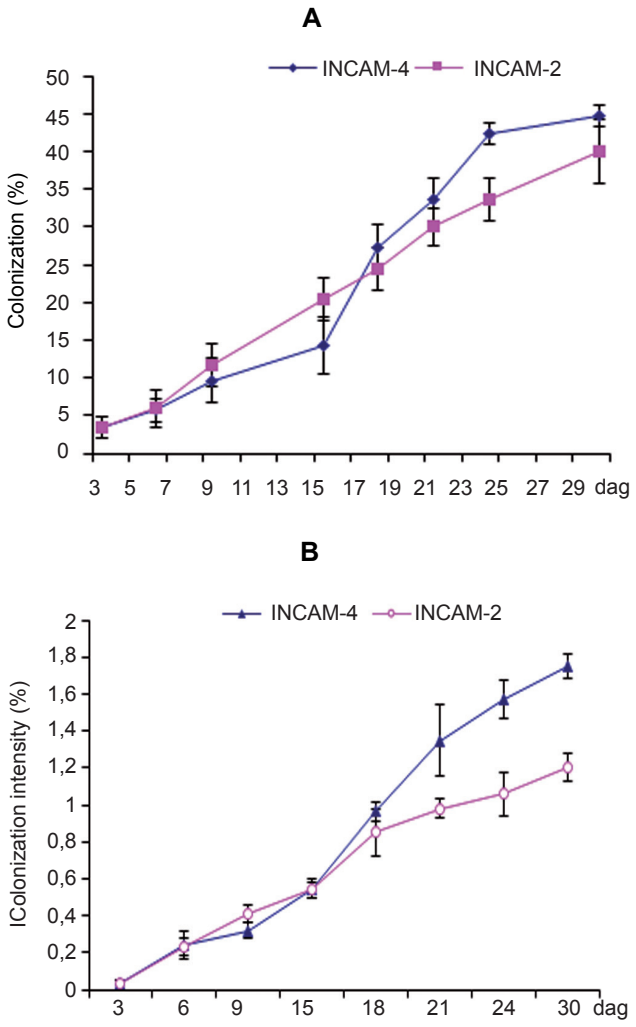
The confidence interval of means at 95 % probability was calculated according to the number of repetitions and data reproducibility. A Multivariate Analysis of Main Components was developed to determine the relationships of pathogens with enzymatic activities reflected in plants and severity of symptoms. The Microsoft® Statgraphics Plus, Version 5.1 was used. The analysis of mean comparison and determination of confidence intervals were performed by the Statistic program version 6.1.

RESULTS AND DISCUSSION

Figure 1 shows the percentages of fungal colonization and occupation (% DV) for each of the treatments under study.

Colonization dynamics of AMF strains is shown in Figure 1A, where increased levels of fungal colonization differed significantly between both strains at the 21st day. Even though colonization levels tended to be higher from the 15th to 24th days for *G. cubense*, there were no statistically significant differences in the ability of strains to colonize tomato plants of the variety under study.

Fungal occupation dynamics is shown in Figure 1B. For both strains, visual density percentages are within the levels reported for this crop (1,3-1,8 %) (12).



The bars represent confidence intervals of the mean of treatments for p≤0.05 (n=3). T1 refers to control plants (M-) whereas T2 and T3 to plants inoculated with *G. cubense* and *G. mosseae* respectively

Figure 1. Colonization dynamics (A) and intensity (B) detected for the treatments under study

However, the strain *G. cubense* presented higher levels of VD from the 18th day on, which correspond to a greater amount of exchanging structures between the tomato plants from Amalia cv. and this AMF strain, such behavior could be related to the conditions of substrate used since this is the strain recommended for these fertility conditions (13).

[B]Solórzano, E. Proteínas de defensa y estudio enzimática en la interacción tomate-*Alternaria solani*. [Tesis de Doctorado]. Universidad Agraria de la Habana. 2002. 100 pp.

Table II. Enzymatic determinations performed at the experimental study

Enzyme	Quantification method	Substrate	λ (nm)	Units (SAU)
β-1,3-glucanases (PR2)	Dangrois *et al.*	Laminarine	450	µKat mg^{-1} protein
Chitinases (PR3)	Boller *et al.*	Coloidal chitine	585	pKat mg^{-1} protein
Peroxidases (PRX)	Fric	Guayacol & H_2O_2	470	nKat mg^{-1} protein
Phenyl alanine ammonia-lyase (PAL)	Nagarotna *et al.*	Fenil alanine	275	nKat mg^{-1} protein
Poliphenol oxidase (PPO)	Alexander *et al.* Cited by Noval B.M (12)	Pyrogallol	420	nKat mg^{-1} protein

Through specific genetic markers, the intensity of colonization and the presence of arbuscules were evaluated in *Medicago truncatula* (Gaertn) mycorrhized with *G. intraradices* (Biermann & Lindermar) (14). These researchers found that at the onset of AMF-plant interaction, arbuscules increased slowly, followed by a stage in which colonization levels and arbuscule content was higher between the 21st and 49th days with the presence of all their developing and senescence stages.

Colonization levels were appropriate in this paper according to crop age under similar conditions (14), indicating that the presence of arbuscules, as exchanging structures inside the root, remained at high levels of representation and the fungal colonization intensity measured as VD was high for both AMF species.

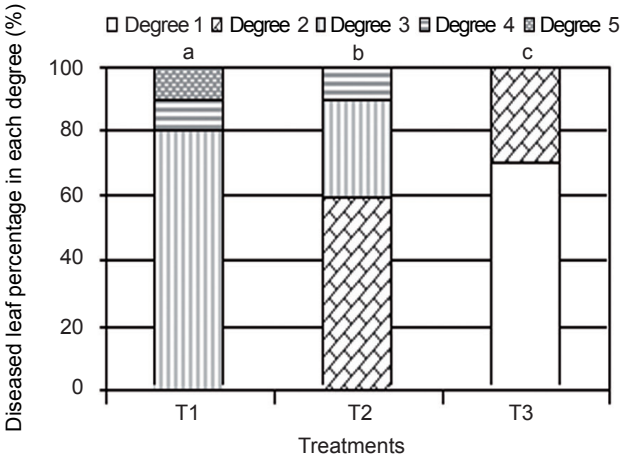
This result allowed to prove that every time both AMF species are represented by higher values in the mycorrhizosphere, according to VD levels observed for crop age, corresponding to what has been previously reported (13).

The molecular dialogue established between the plant and AMF determines the colonization levels they reach and mycorrhizal efficiency observed as colonization intensity (13), in the case of tomato crop, percentages of fungal colonization and occupation measured as visual density are between 40 and 45 % and 4 to 4,3 % respectively for plants that have completed their life cycle (14). In the case of those plants used in the experiment, colonization dynamics was performed to demonstrate that both strains were represented at appropriate levels for crop age, besides using a sterilized substrate to reduce or prevent colonization by native soil AMF, so that control plants did not develop colonization.

Leaf necrosis rates observed in tomato plants under different treatments are presented in Figure 2. It is noted that in plants inoculated with *G. cubense* and *G. mosseae*, necrosis percentages were lower than the control. Plants inoculated with G. cubense highlighted by decreasing two degrees the intensity of damages caused by the pathogen.

PRX activation for plant-AMF-*O. taurica* interaction is represented in Figure 3. Enzyme activity in the root confirms the fact this is locally induced by AMF (15) and was superior to mycorrhized plants compared to control plants.

With regard to the root of plants facing *O. taurica*, the enzyme activity decreased in plants inoculated with AMF compared to noninoculated ones and facing the pathogen, also no substantial changes were observed at the level of activity of this leaf enzyme, between the control plants and those mycorrhized with *G. mosseae*. There were differences between these plants and the ones mycorrhized with *G. cubense*.



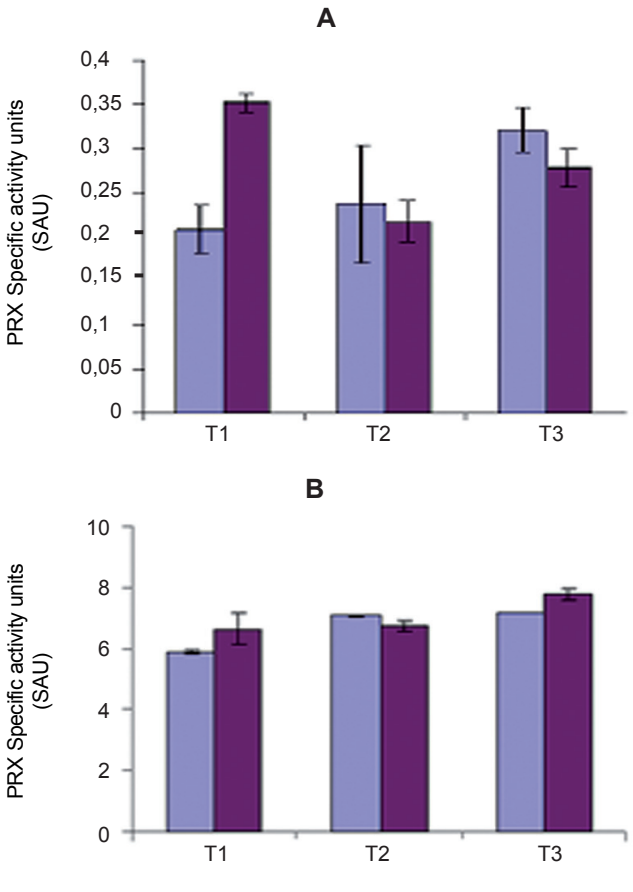
T1: control plants, T2 and T3: mycorrhized plants with G. mosseae and G. cubense, respectively
Non common letters indicate significant differences according to Bonferroni´s correction for p≤ 0,05 (n=6)

Figure 2. Leaf necrosis in tomato plants inoculated with the treatments under study and facing *Oidipsis taurica*

It has been suggested that the oxidative burst which occurs due to the cytotoxicity of active oxygen species such as superoxide anion and hydrogen peroxide, as well as production speed are the first line of defense against pathogen invasion. This causes the direct killing of the pathogen or retains its entrance, because of cell wall hardening by oxidative cross-reaction with the structural proteins of the wall. The toxicity of active oxygen species can also contribute to host cell death (16). Although AMF species differences for enzyme activation were evident, it was found that even the lowest levels were satisfactory to stop pathogen advance, expressed by the severity reduction recorded for both AMF species. However, it was also very evident that *G. cubense* species behaved more efficiently, reducing the severity in two levels relative to control plants.

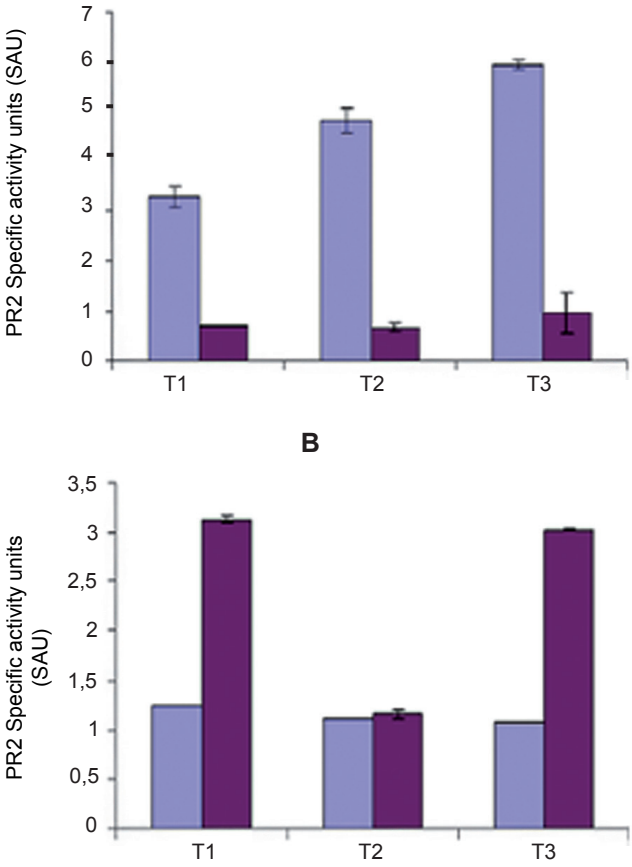
It has been noted there is some compatibility between *G. cubense* and tomato (12), so that by activating this mechanism to the levels observed in this study, it suggests there was an attempt to induce a programmed cell death mechanism at a higher percentage to try to circumscribe the pathogen to the inoculating area. By increasing this process, related to oxidative metabolism in the treatment where plants were challenged with the pathogen, compared to others, it was once again proved that symbiosis establishment not only benefits plant agronomic traits, but also its protection against pathogens.

Figure 4 presents PR2 induction in roots and leaves from AMF inoculated plants facing pathogen attack.



The first bar represents plants without the pathogen
T1: control plants; T2 and T3: mycorrhized plants with *G. mosseae* and *G. cubense* respectively
Error bars represent the confidence interval of the mean for p≤0,05 (n=6)

Figure 3. PRX activity detected in roots (A) and leaves (B) from tomato plants facing the attack of *Oidiopsis taurica*



PR2 specific activity units (SAU)
The first bar represents plants without the pathogen
T1: control plants; T2 and T3: mycorrhized plants with *G. mosseae* and *G. cubense* respectively
Error bars represent mean confidence interval for p≤ 0,05 (n=6)

Figure 4. PRX activity detected in roots (A) and leaves (B) from tomato plants facing the attack of *Oidiopsis taurica*

The local induction of β 1,3 glucanase in mycorrhized plant roots was higher than in the control plants (Figure 4A), meanwhile there was no systemic response induction in AMF inoculated plants, since PR2 activity levels in both were not appreciably modified compared to control plants (Figure 4B).

When facing the pathogen, no differences between treatments were observed in roots, whereas in the leaf system, there was a marked activity decrease in *G. mosseae* inoculated plants and no differences between treatments 1 and 3.

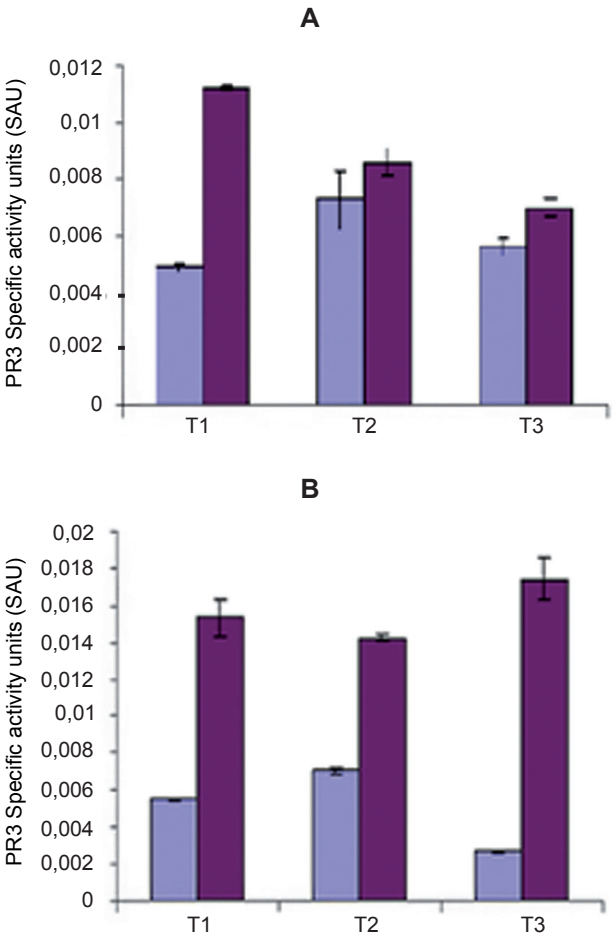
Figure 5 shows chitinase induction in leaves and roots from AMF inoculated plants against pathogen attack. Root activity showed a higher local response in mycorrhized plants, which was superior for the interaction with *G. mosseae* (Figure 5A).

When facing *O. taurica*, a significant activity decrease was appreciated for both AMF strains compared to control plants.

Figure 5B shows PR3 activity induction in leaf system, where a differential response was observed between mycorrhized strains regarding the systemic induction of chitinase activity, which was higher in those mycorrhized with *G. mosseae* than in the control plants and lower in the ones mycorrhized with *G. cubense*.

In general, there were no significant differences between the control and mycorrhized plants facing pathogen attack at the enzymatic induction levels, even though there was a tendency to decrease the activity in *G. mosseae* mycorrhized plants and chitinase activity was high in every treatment, due to the presence of the pathogen.

Chitinases and glucanases are among the most studied PR proteins, which are involved in the responses of active plant defense against fungal pathogens. These enzymes catalyze the hydrolysis of polysaccharides representing the main cell wall components of several fungi.



PR3 specific activity units (SAU)
The first bar refers to plant activity without the pathogen
T1: control plants; T2 and T3: mycorrhized plants with *G. mosseae* and *G. cubense* respectively
Error bars represent mean confidence interval for p≤ 0,05 (n=6)

Figure 5. PR3 activity detected in roots (A) and leaves (B) from tomato plants against pathogen attack

Moreover, chitin, a chitinase substrate, is not present in higher plants. Chitinases by itself or combined with glucanases can degrade cell walls isolated from some fungi and restrict their *in vitro* growth efficiently (12).

Mycorrhization has been shown to induce defensive responses in plants subsequently suppressed to the extent that AMF are recognized and such compatibility between the plant and the symbiont is mediated by sym genes (17), and as mycorrhizal efficiency mainly depends on soil fertility and AMF strain (13, 18), it is observed that both AMF strains do not induce systemic responses, because they did not differ significantly in the leaf enzymatic activity values from non mycorrhized plants (T1).

This result is consistent with previous reports (1, 3, 18) that confirm PR proteins have not been detected in mycorrhized plant leaves. This is because these enzymes not only act in the defensive response as it occurs in pathogenic interactions, but also they allow AMF intraradical growth, thereby reducing enzyme fragment size and do not constitute systemic response elicitors (3). However, these situations are changed when plants are subjected to biotic stress, showing differences in enzymatic induction that in some cases differ from control plants and allowed to decrease the severity recorded.

Figure 6 shows the induction of phenyl alanine ammonia lyase (PAL) in plant roots and leaves. The local induction of PAL activity showed no differences between mycorrhized plants, which were higher than the control. When facing *O. taurica*, a decreased activity was observed in AMF inoculated plants with respect to the control. Furthermore, it was shown that when plants were facing the pathogen, the activity was lower than in plants without the pathogen under the same conditions. This phenomenon may be due to the fact that such confrontation took place in leaf tissue and that enzymatic induction metabolism represents energy expenditure for the plant (19).

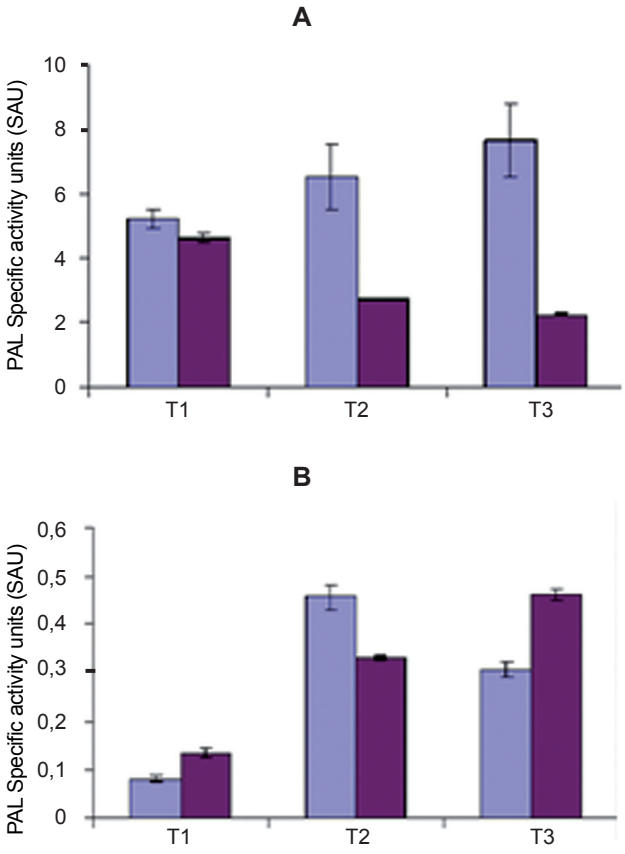
It should be easier for the plant to induce this system wherever the pathogen is acting, thus, considering cell economy, such induction was lower in the root zone, although the activity is observed, which in this case may allow the plant to have sentinel enzyme levels.

Figure 6B shows the induction of leaf system activity. Such induction of systemic response in mycorrhized plants was significant in those inoculated with *G. mosseae*. When plants were inoculated with *O. taurica*, activation was evident for both strains, it being superior in *G. cubense* mycorrhized plants.

Phenylalanine ammonia lyase is involved in the phenylpropanoids taking part at the deposition of phenolic compounds in cell walls and its subsequent reinforcement (20); therefore, increases of its activity, as it is seen in plants from Panel B treatments, make us suppose this is being reinforced against pathogen advance, so that a damage reduction should be expected.

Figure 7 shows the result from main component analysis for PRX, PR2, PR3 and PAL activity combined with the effect produced by the pathogen in tomato plants var. Amalia. It was found that the first two components explained 82,32 % of data variability. The first component is contributed by the variables of pathogen severity, β 1,3 glucanase in leaves and roots, PRX in roots and chitinase in roots and leaves, whereas the second component by PRX and PAL in leaves.

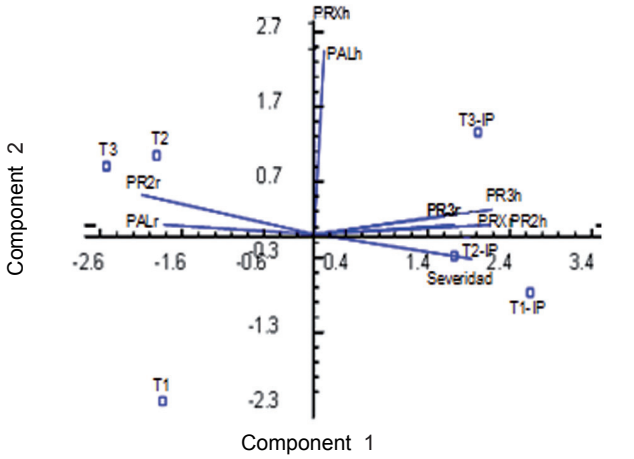
As it is noted, no relationship was found between the damage caused by the pathogen and PRX and PAL activity in leaves. This relationship resulted positive with chitinase in roots and leaves, with PRX in roots and with β 1,3 glucanase in leaves, but negative with β 1,3 glucanase and PAL in roots.



PAL specific activity units (SAU)
The first bar refers to plant activity without the pathogen
T1: control plants; T2 and T3: mycorrhized plants with *G. mosseae* and *G. cubense* respectively
Error bars represent mean confidence interval for p≤ 0,05 (n=6)

Figure 6. PAL activity detected in roots (A) and leaves (B) from tomato plants against pathogen attack

	Component	Component
Severity	**0,372049**	-0,0888877
PR2 r	**-0,407121**	0,147174
PR2 h	**0,332437**	0,0765588
PRX r	**0,416619**	0,0330246
PRX h	-0.000397718	**0,702557**
PR3 r	**0,329851**	0,0365701
PR3 h	**0,41855**	0,0930152
PAL r	**-0,35665**	0,0363865
PAL h	0,023688	**0,677191**



PRX refers to Peroxidase activity, PR2 to β 1,3 glucanase, PR3 to chitinase activity and PAL to phenyl alanine ammonium lyase
T1: non mycorrhized plants T2: *G. mosseae* and T3: *G. cubense*
r: enzyme activity values in roots and h: in leaves
IP: refers to plants facing pathogens

Figure 7. Main component analysis for *O. taurica*

When comparing the plants facing the pathogen with non-inoculated ones, it was observed that the presence of pathogen decreases glucanase expression and PAL in roots but increases chitinase activities in roots and leaves, PRX in roots and β 1,3 glucanase in leaves (Figure 7).

AMF are inducers of defense mechanisms, therefore, they may have some influence on plant biotic stress. However, the fundamental proposition to obtain AMF induced resistance is that mycorrhizal association should be well established (21), a premise that is fulfilled in this study.

AMF beneficial effect on biosecurity against root pathogens is widely documented in literature, among which are species of *Aphanomyces*, *Cylindrocladium*, *Fusarium*, Macrophomina, *Pythium*, *Rhizoctonia*, *Sclerotium*, *Verticillium* and *Thielaviopsis* (1, 17). It is reported that mycorrhization protects tomato plants from the attack of *Erwinia carotovora* Smith bacterium, and decreases microorganism population in the rhizosphere. Other groups of pathogens in which AMF influence was studied due to its damage in agriculture are gall-forming nematodes of *Meloidogyne incognita* species (1). The reduction of disease index caused by *P. parasitica* Dastur (*Phytophthora nicotianae* Breda de Hann) has been linked to protein induction from a group of PRs by *Glomus mosseae*.

However, there are few studies related to biosecurity of mycorrhized plants against leaf pathogens. This study shows that inoculation with both AMF strains reduced disease severity caused by this pathogen, which is a new report of their use on biosecurity.

With regard to mycorrhized plants, AJ levels increase in relation to non mycorrhized plants (22) and such increases have been found to activate the genes involved with plant defense enzymes, such as the ones from phenylpropanoids and PRs production (22). Concerning the mildew, it was observed that damage decrease was associated to increased induction of β 1,3 glucanase in leaves and chitinases in leaves and roots, which are mechanisms induced through jasmonate. Furthermore, it should be noted

that even though mycorrhized plants showed their defensive system activations, in some cases, they had higher activations when facing pathogens, leading to the conclusion that there was a defensive response conditioning that allowed to reduce pathogen severity (3, 23, 24, 25).

CONCLUSIONS

In general, it was observed that:

- AMF *G. mosseae* and *G. cubense* were settled in the mycorrhizosphere at appropriate levels for crop age
- Both species were effective in reducing *O. taurica* severity, *G. cubense* being more effective for its severity decreases of up to two levels with respect to control plants
- PR differential induction was observed in roots and leaves that was effective in reducing pathogen severity
- Response conditioning of mycorrhized plants determined pathogen severity reductions

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