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PATTERN OF B-1, 3-GLUCANASE AND CHITINASE ACTIVITY IN THE AMF - SYSTEMIN INTERACTION IN TOMATO. I. PRESYMBIOTIC PHASE

Patrón de la actividad de las β -1,3-glucanasas y quitinasas en la interacción HMA-sistemina en tomate. I. Fase presimbiótica

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ABSTRACT. The arbuscular mycorrhizal fungi and elicitor have been many used with diverse objectives in modern agriculture. Systemin is a polypeptide hormone with many possibilities like elicitor of answers of defense in Solanaceas. Thus its application with the AMF, would allow increasing the effects in the protection against diverse pathogens. An experiment was made in which the possible participation of the systemin in the establishment of the mycorrhiza and the induction of defense response in pre-symbiotic stages was evaluated. The exogenous application of systemin in the roots produce the local and systemic induction of β-1,3-glucanase and chitinase in early stages. A synergic effect between systemin and AMF (*F. mosseae*) on the activity of β-1,3-glucanases in root from the first later hour the application was observed. *F. mosseae* produce the induction to these enzymes in root, at 24 hour the inoculation.

Key Words: pectinolytic enzymes, *Mycorrhizae*, defense mechanisms

RESUMEN. Los hongos micorrícicos arbusculares y los elicitores han sido ampliamente utilizados en la agricultura moderna con diversos objetivos. La sistemina constituye una de las hormonas polipeptídicas con amplias posibilidades como elicitor de respuestas de defensa en Solanáceas. Su aplicación de forma conjunta con los hongos micorrícicos arbusculares, permitiría incrementar los efectos en la protección contra patógenos diversos. Para determinar la participación de la sistemina en el proceso de establecimiento de los HMA en los estadios tempranos de la simbiosis micorrícica arbuscular y su influencia en la inducción de respuestas de defensa en tomate se realizó un experimento en fase presimbiótica. La sistemina aplicada en las raíces produce la inducción rápida de las β-1,3-glucanasas y quitinasas en etapas tempranas, de forma local y sistémica. Se observó un efecto sinérgico entre la sistemina y del HMA (*F. mosseae*), sobre la actividad de las β-1,3-glucanasas en raíz, desde la primera hora posterior a la aplicación de ambos. *F. mosseae* produjo la inducción de estas enzimas en raíz, a las 24 hora posterior a su inoculación.

Palabras clave: enzimas pectinolíticas, *Mycorrhizae*, respuesta de defensa

INTRODUCTION

The arbuscular mycorrhizal fungi (AMF) belong to the *Glomeromycota* phylum, which is one of the most important soil microorganisms (1). During the process of establishment and development of the symbiosis between AMF and plants, induction of resistance response occurs with the induction of diverse proteins related to pathogenicity (PRs), among which the β-1,3- glucanases (GLN), chitinases (QUI), and enzymes related to oxidative processes (2,3).

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The induction of these mechanisms in the early stages of the mycorrhizal association establishment has received special attention due to its possible involvement in the regulation of the symbiosis, as well as in the protection of the plants against the attack of pathogens (4).

Systemin (S) was the first peptide hormone identified in plants, which induces defense responses in *Solanaceae* before insect affectation and mechanical damage (5). Recently, it has been linked to a decrease in the incidence of fungal necrotrophic pathogens (6), with the significant expression of genes that code for key components of the systemic defense signaling system (7). After recognition of the cell surface system by the receptor, a cascade of signals is activated that involves the transient accumulation of jasmonic acid (AJ) and as a consequence, the expression of genes related to defense responses (6,8). Among the proteins that are induced are the so-called Systemic Response Proteins to harm (9), which include several proteins associated with signaling pathways and others similar to those induced by insects and herbivores (10). Previous studies have shown the induction by the system of Proteins Related to Pathogenesis (PRs) (11,12).

Recent studies aimed at analyzing the possible role of systemin in the local and / or systemic modulation of the mycorrhizal association indicate that the application in mycorrhized tomato plants induces the accumulation of β-1,3-glucanases and chitinases (12).

In order to elucidate the possible interconnection of the signaling pathways induced by the system and the AMF, the pattern of activity of PRs proteins induced by this interaction was evaluated in the presymbiotic phase.

MATERIALS AND METHODS

Obtaining plant material and application of products

Tomato plants (*Solanum lycopersicum* L.) from the cultivar 'Amalia' were used (13). The seeds were superficially disinfected with 10 % commercial sodium hypochlorite for 10 min, followed by three washes with sterile distilled water (H_2O d), after which they were sown. The substrate was composed of a mixture of typical leached red ferralitic soil and vermicompost humus in a 1: 1 (v: v), sterile ratio (Table I). The plants were grown in a growth chamber, under controlled conditions, with a photoperiod of 8 h of darkness at 16 °C and 16 h of light at 28 °C.

The *Funneliformis mosseae* (T.H. Nicolson & Gerd) AMF species (14) (Fm) was selected from the Mycorrhizal Laboratory strain of the National Institute of Agricultural Sciences (INCA according its acronyms in Spanish).

Table I. Physical-chemical characteristics of the substrate formed by the mixture of Red ferralitic leached typical soil and earthworm humus in 1: 1 ratio (v: v)

K^+	Ca^{2+}	Mg^{2+}	P	Materia	pH
(cmol kg^{-1})			(mg mL^{-1})	Orgánica (%)	(H_2O)
0,31	25,5	9,0	160,0	14,7	7,1

Methods of analysis: pH $_{(H2O)}$, organic matter (Wakley-Black), P (Oniani, H_2SO_4 IN), K and other changeable cations, Maslova (NH_4Ac pH 7), the CCB by sum of the bases (15)

Trays of 120 wells of 10 mL capacity each were used where a disinfected seed was planted in each one. 21 days after the emergence, the mycorrhizal fungus was inoculated and the system was applied to the substrate, independently and in combination, according to the design of the treatments. The AMF was prepared in a liquid suspension with a concentration of 20 spores mL^{-1}, of which 1 mL per plant was added to the substrate, in the mycorrhized treatments and the tomato synthetic system (Laboratories BQ SOS, from México) was added to the substrate (1 mL per plant at 44,06 nmol) (12), in the corresponding treatments. In the control treatment, distilled water (2 mL per plant) was added. For the samplings a dynamic was followed during the 48 hours after the inoculation (hai): 1, 2, 4, 8, 12, 18, 24 and 48 hai.

At the time of sampling the plants were washed and separated by organs, dividing the roots into two groups. One portion was used for the determination of enzymatic activities, which was frozen in liquid nitrogen and stored at -70 °C, while the other was dried in an oven at 70 °C for the determination of the establishment of the mycorrhizal symbiosis (colonization).

Mycorrhizal colonization

To quantify mycorrhizal colonization, roots dried at 70 °C were digested with KOH, and then stained with Trypan blue. The percentage of mycorrhizal colonization (% C) and the intensity of colonization according to the intercepts method (% I) (12) were evaluated.

Enzymatic test

Protein extraction and enzymatic determinations were carried out according to the methodology described by Noval *et al*. (12). The protein concentration was determined by the Bradford method (16), using the commercial kit (Bio-Rad Laboratories, USA).

The determination of *in vitro* enzymatic activities in the roots and leaves of tomato plants was carried out. For quantification of β-1,3-glucanase activity (GLN; EC-3.2.1.39) laminarin was used as the substrate and the absorbance was read at 450 nm in a SUMA microplate reader, PR 521. For the chitinase activity (QUI; EC- 3.2.1.14) the colloidal chitin prepared from reactive grade chitin (Fluka) was used as a substrate and the absorbance was read at 585 nm in the spectrophotometer (12). All enzymatic activities were transformed to microkatals (μKat) and nanokatals (Kat) per milligrams of proteins, as needed (17). The absorbance readings in the different methods used were performed in a spectrophotometer, Ultrospec Plus Spectrophotometer, Pharmacia LKB. All substrates were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).

Experimental design and statistical analysis

The experiment was developed following a completely randomized design with three repetitions; each conformed by 10 plants, in each sampling moment. The data were processed statistically according to the analysis of variance of simple classification, comparing the means using the confidence intervals with a degree of significance of 95 %. The experiment was repeated three times in time, of which a representative one was selected. All comparisons were made according to the statistical package STATGRAPHIC Plus Version 5.1 (18). The percentages of increments were calculated by comparing the treatments with the corresponding control.

RESULTS AND DISCUSSION

Dynamics of the symbiosis establishment

To monitor the establishment of the arbuscular mycorrhizal symbiosis, the percentage (% C) and intensity (% I) of the colonization in the roots of the plants under study was evaluated (Table II). In the first 18 hai, the presence of fungal structures in the roots was not detected (data not shown), which were observed after 24 hai, at which time the inoculated treatments showed statistical similarity in both indicators. At 48 hai the treatments showed statistical differences, both in the percentage of colonization and in the intensity.

In the variant where the combined application of the AMF and the elicitor was carried out, levels of mycorrhization were lower than those observed in the plants where only *F. mosseae* was inoculated. This response may be due to the occurrence of two events in the roots of the plants where both products were applied, the induction of rapid responses by the system (9,11) and the stress produced by the processes of penetration and dissemination of the fungus mycelium in the roots (19-21), processes that have been documented in the literature, but independently.

Table II. Percentage of arbuscular mycorrhizal colonization (% C) and colonization intensity (% I) of plants inoculated 24 and 48 days after emergence (dae)

Tratamientos inoculados	% Colonización				% Intensidad			
	24		48		24		48	
	hpi							
Fm	2	a	3,75	a	0,03	a	0,17	a
Fm/S	1,5	a	2,25	b	0,02	a	0,03	b
Esx	0,35		0,25		0,01		0,03	

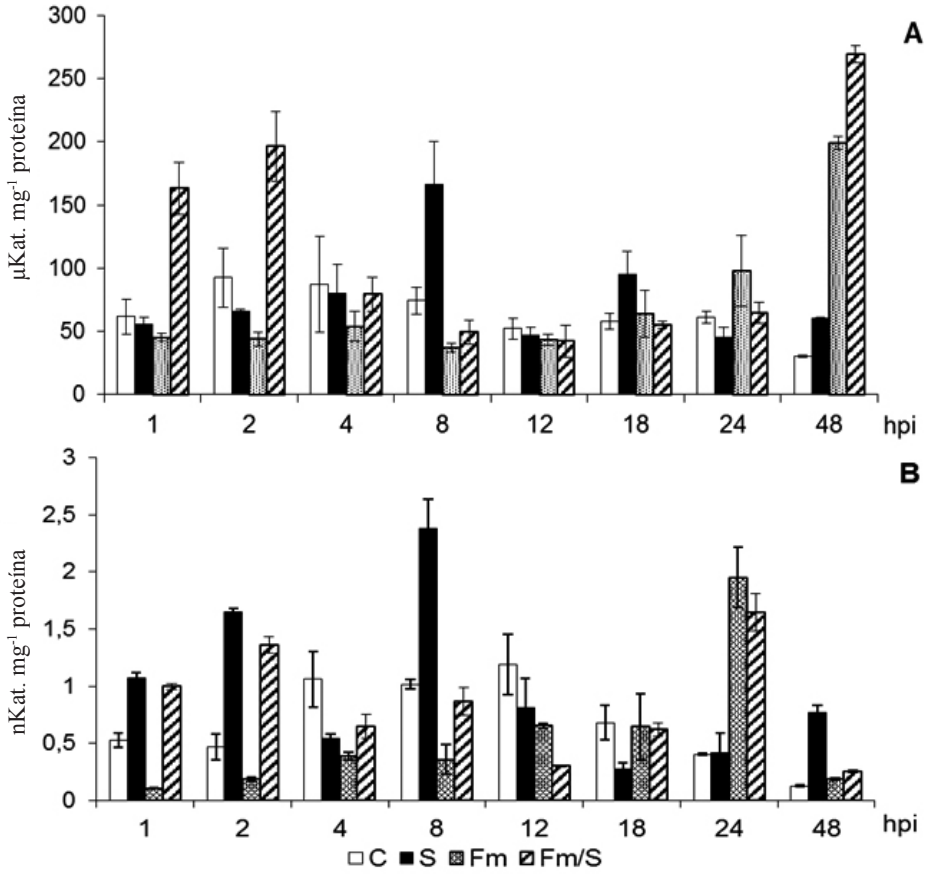
Means with common letter are not significantly different according to Procedure Tukey (HSD) (p≤0,05) (22) Fm: *Funneliformis mosseae*; Fm / S: *Funneliformis mosseae* + systemin

In both indicators, low levels were observed (1,5-3,75 % C and 0,023-0,16 % I), which indicates that it was in the early stages of the establishment of the *F. mosseae* mycelium in the roots. In spite of this, the evaluation of the colonization in early stages allowed to detect the initial moments of the establishment of the symbiosis, which occurred between 24 and 48 hours.

Activity pattern of enzymes related to defense: glucanases and chitinases

The activity patterns of the GLN enzymes in the roots of the tomato plants inoculated with *F. mosseae* independently (Fm) showed activity levels similar or lower than those obtained in the control treatment in the first 18 hai (basal level) with significant differences between them (Figure 1A). After 24 hours, there was a gradual increase that reached a significant point at 48 hai (98,05 and 199,08 μKat mg^{-1} protein), with increases of 60,44 % and 558,85 %, respectively.

Regarding the QUI activity, it was observed that in the independently mycorrhized plants (Fm) the activity of this enzyme decreased until 12 hours, in relation to the levels reached by the control, with which they showed significant differences (Figure 1B). At 24 hours there was a transient increase (1,95 nKat mg^{-1} protein), which differed statistically with the other variants studied.



A) β-1,3-glucanase (GLN) and (B) chitinase (QUI). The GLN and QUI activity was analyzed in root samples (n=10). Control (C) (white bars), systemin (S) (black bars), *Funneliformis mosseae* (*Fm*) (left slanted bars) and *F. mosseae* plus systemin (Fm/S) (right slanted bars). The data show the determination of enzymatic activity by triplicate ± SE in the radical extracts corresponding to representative experiments replicated three times with similar results. The vertical bars indicate the confidence intervals for each mean (p≤0,05)

Figure 1. Enzymatic activity in roots of tomato plants evaluated at 1-48 hours after inoculation (hai) induced by the application of AMF and/or systemin

It has been found that expression regulation of chitinases occurs in mycorrhizal plants, and a speculative model of the suppression mechanisms of the constitutive expression of this enzyme (control treatment), induced by the MA in early stages has been proposed (23). In the same it is observed that in late stages the accumulation of specific chitinases induced exclusively by mycorrhization occurs. This mechanism could explain the process of regulating the spread of AMF at the root.

The exogenous application of the system, independently induced the GLN activity at eight, 18 and 48 hours, being significant the levels reached at eight hours (166,33 µKat mg^{-1} protein), corresponding to 123,85 % of increments in relation to the QUI activity, there was an early induction from the first moments of evaluation, 1 and 2 hai (1,07 and 1,64 38 nKat mg^{-1} protein), corresponding to 103,97 and 251,75 % of increments, respectively.

There was a second increase at eight hours (2,38 nKat mg^{-1} protein) which showed the highest level reached in the experiment, with 133,96 % increases and significant differences with all the variants studied. The induction of the GLN and QUI produced by the systemin *per se*, in the first hours after its application in the roots, suggests that in the roots of the tomato plants there must be recipients of the systemin, where the SR-160 protein or a similar one participates, which acts as a primary signal, in a similar way to how it has been shown in leave (24).

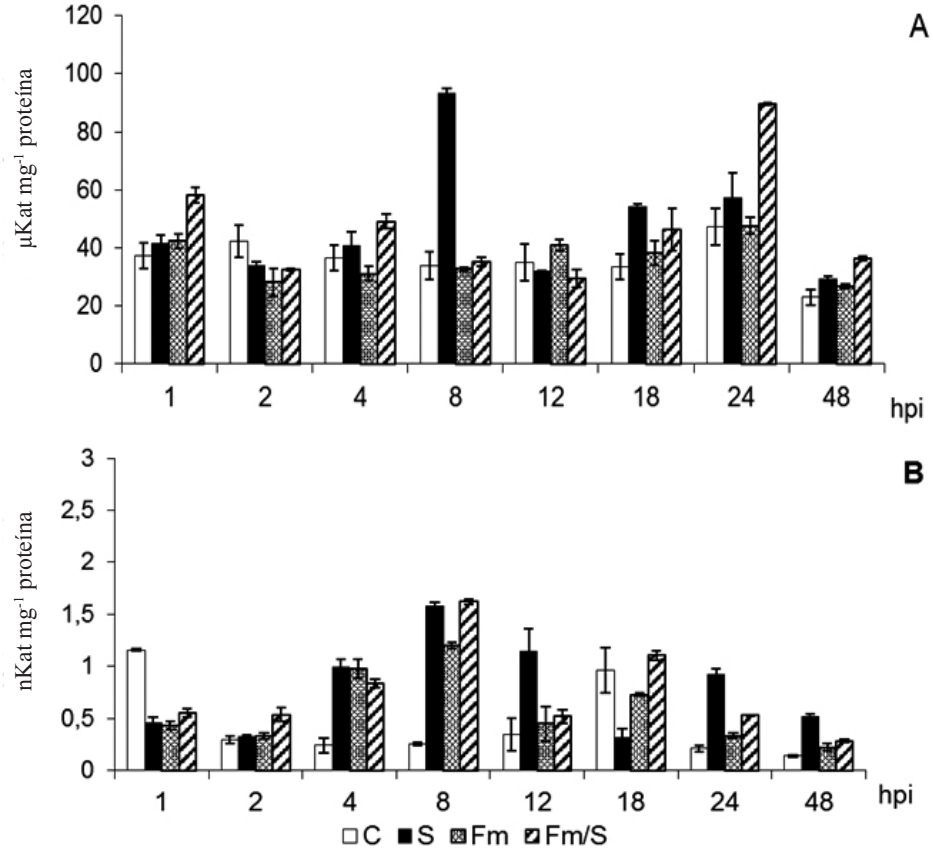
A synergistic effect was observed with the joint application of Fm and systemin, with important levels of GLN activity at 1 and 2 hai (163,16 and 196,53 µKat mg^{-1} protein with 223,31 and 258,37 % increase in relation to the treatments where they were applied independently) and at 48 hai with 269,22 µKat mg^{-1} protein and a significant increase of 790,99 % in relation to the control.

In relation to the QUI activity, an inhibitory effect was observed in the plants inoculated with Fm which was overcome during the early stages (1-8 hai) when combined with the application of the systemin (Fm/S). No synergistic effect between Fm and systemin was observed on the activity of this enzyme.

When analyzing the quantification of GLN and QUI activity in leaves, it was observed that lower levels were obtained than those obtained in the roots (Figure 2). In this organ was also found the presence of a basal level of GLN given by the control treatment, with average values of 38,26 µKat mg^{-1} protein (Figure 2A), which was not modified by the inoculation of the AMF when applied independently (Fm). Likewise, the presence of basal levels of the QUI enzyme (control) was found, which were overcome by the treatments, in most of the times in which the evaluation was performed (Figure 2B). The inoculation with Fm, independently induced increases in the activity of this enzyme at four and eight hai (0,98 and 1,2 nKat mg^{-1} protein) with increases of 304,47 and 374,79 %, respectively; however, in the rest of the moments evaluated, this variant did not surpass the control.

The application of the systemin exogenously produced a significant induction of GLN activity at eight hai, with a second peak, more discrete, at 18 hai (93,37 and 54,35 µKat mg^{-1} protein), which showed increases of 175,87 and 62,30 %, respectively. In relation to the QUI induction was observed at four hai, which was maintained over time (8 and 12 hai), with values of 0,99, 1,58 and 1,14 nKat mg^{-1} protein and increases of 310,09; 522,77 and 234,71 %, respectively. Subsequently, during the 24 and 48 hai, a second induction of lesser magnitude occurred than previously observed (0,91 and 0,51 nKat mg^{-1} protein), at which time the basal levels were lower.

In a similar way to that obtained in leaf root, a synergic effect was observed between AMF and elicitor (Fm/S) on GLN activity in early stages (1 hai), reaching levels of 58,22 µKat mg^{-1} protein, which was more significant at 24 hai, with 89,63 µKat mg^{-1} protein and 89,46 % increase; however, in relation to the QUI, no synergic effect was observed in any of the moments evaluated.



β-1,3-glucanase (GLN) and (B) chitinase (QUI). The GLN and QUI activity was analyzed in root samples (n = 10). Control (C) (white bars); systemin (S) (black bars), Funneliformis mosseae (Fm) (left slanted bars) and F. mosseae + systemin (Fm/S) (right slanted bars). The data show the determination of enzymatic activity by triplicate ± SE in the radical extracts corresponding to representative experiments replicated three times with similar results. The vertical bars indicate the confidence intervals for each mean (p≤0,05)

Figure 2. Enzymatic activity in tomato leaves evaluated at 1-48 hours after inoculation (hai) induced by the application of AMF and/or systemin

In the mycorrhizal plants, the GLU induction was produced only after 24 hai with maximum activity at 48 hai, while the QUI only showed levels higher than the control at 24 hai. In this phase, recognition occurs between the symbionts, with the activation of a series of signal cascades, resulting in the production of hormones related to defense (25-27). It has been suggested that the initial stages of radical colonization by AMF are accompanied by the transient induction of plant defenses followed by suppression in late stages of the interaction (4,28,29).

Although the 24 and 48 hai were the final evaluation moments of the experiment, these correspond to the process early phases of establishing the MA symbiosis as demonstrated by the levels of colonization and intensity achieved in them (Table II).

Previous studies have shown that the exogenous application of the system in the roots induces defense responses at the local and systemic level (12) and the accumulation of Systemic Response Proteins to Damage (SWRPs) (9). In the present work the character of the induction systemin of rapid responses is corroborated, which was more marked on the QUI activity with increments from the first hours of evaluation (1 and 2 hai), showing at 8 hai the highest level of all the treatments under study. For GLU, the response was later showing increases only at 8 hai, with a second moment of induction at 18 and 48 hai but of lower intensity.

Most studies conducted with systemin focus on the detection of genes that are expressed by mechanical damage or by the attack of herbivorous insects (7,9) and monitor protease inhibitors, as well as other proteins induced by the jasmonic acid (30). In studies of the accumulation of mRNA of different proteins induced by the application of the systemin in tomato, it has been found that the detection of protease inhibitors occurs two hours after treatment with the elicitor, however, a lipoxygenase and the species Active oxygen levels were expressed in the first 30 minutes post-induction (31).

The synergism observed in very early phases with the application of *Fm* and systemin on GLU activity, shows the possible component joint action of the signal translation path of the elicitor and of the activated responses during the mycorrhizal symbiosis in the presymbiotic phase, corresponding to signaling processes and beginning of the formation of the appressoria.

It is known that after the reception of the system a series of reactions are activated where the calcium intake occurs, the activation of the activity of the MAPK, the production of active oxygen species, the increase of the activity of the enzyme ATPaseH^+, with the consequent hyperpolarization of the membrane, the accumulation of hormones such as jasmonic acid and the accumulation of secondary signals such as oxilipins (32).

Different authors have reported on elements that constitute secondary signals or defense response inducers of during the mycorrhization process. In this way it has been observed that in mycorrhized plants the expression of kinases is produced early, some of them dependent on cadmodulin and Ca (CCaMKs) (26,33), and on elements of the signaling process as receptors of the symbiosis (SYMRK), which are required as the primary signal of the mycorrhization process (34).

Several of these components are common to signal transduction pathways induced by both AMF and systemin, which are important elements of the signaling pathway that lead to the accumulation of jasmonic acid (6,26,27,35) and in this way to the induction of defense responses, among which are the PRs proteins.

With regard to the activity of the QUI, it seems that the response observed in the treatment where *Fm* and the system were applied together is not the product of a synergistic effect. In the moments evaluated between 1 and 8 hai, the same pattern of behavior was observed to the one found with the application of the elicitor independently, while the response observed at 24 hai, apparently, it is due to the effect of the AMF already shows similar levels obtained by this, when applied as independent (*Fm*), at which time the system reached low levels.

CONCLUSION

The results suggest an important role of systemin in its interaction with the arbuscular mycorrhizal symbiosis, by modulating early and transient defense responses during the process of establishing mycorrhization, enhancing the AMF action, which is reflected in the induction of PR proteins. The system produces rapid induction of β-1,3-glucanases and chitinases in the first hours after their application to the roots. A synergistic effect of this polypeptide hormone was obtained with the AMF (*F. mosseae*) on the induction of β-1,3-glucanase and chitinase, from early stages to root (1 hai), while in leaf it was later. *F. mosseae* only produced the induction of these enzymes in the root, in late stages.

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