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ACTIVITYS PATTERN OF B-1,3-GLUCANASES AND QUITINASES IN THE AMF - SYSTEMIN INTERACTION IN TOMATO. II EARLY SYMBIOTIC PHASE

Patrón de la actividad de las β-1,3-glucanasas y quitinasas en la interacción HMA-sistemina en tomate. II fase temprana de la simbiosis

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ABSTRACT. Systemin (S) and jasmonic acid (JA) play a central role in the damage response in tomato (*Solanum lycopersicum*). JA is also known to regulate the arbuscular mycorrhizal (AM) symbiosis in this and other plant species. An experiment was made in which the possible participation of the systemin in early stages of the establishment of the mycorrhiza was evaluated. Exogenous systemin appliction to a very rapid and transient increase of root glucanase and quitinase activity patterns at 3, 6 and 12 days after emergence (dae). Arbuscular mycorrhizal fungi (AMF) species produced a gradual increment leading to transient peaks of activity, at 3 and 6 dae (for chitinase) and 9 dae (for β-1,3-glucanase). The pattern suggests the establishment of a pre-symbiotic dialogue plant - AMF which appeared to be partly modulated by systemin, judging by the synergic effect on β-1,3-glucanase activity observed in the systemin and AMF treatment. A similarly positive effect on β-1,3-glucanase activity was observed after systemin application at the earliest colonization stages. The above suggests that systemin could impact the tomato mycorrhizal process with the modulation of defense responses.

Key words: pectinolytic enzymes, mycorrhizae, defense mechanisms, sistemine

RESUMEN. La sistemina (S) y el acido jasmónico (AJ) juegan un papel central en la respuesta al daño en tomate (*Solanum lycopersicum*). El AJ es también conocido como un regulador de la simbiosis micorrízica arbuscular (MA) en esta y en otras especies de plantas. Se realizó un experimento en el cual se evaluó la posible participación de la sistemina en etapas tempranas del establecimiento de la micorrización. La aplicación exógena de la sistemina produjo un rápido y transciente incremento de los patrones de actividad de las glucanasas y quitinasas a los 3, 6 y 12 días post emergencia (dpe). Las especies de los hongos micorrízicos arbusculares (HMA) produjeron un incremento gradual con picos transcientes de actividad, a los 3 y 6 dpe (para quitinasa) y 9 dpe (para β-1,3-glucanasa). El patrón sugiere el establecimiento de un diálogo pre-simbiótico rápido planta-HMA que parece ser modulado en parte por la sistemina, avalado por el efecto sinérgico sobre la actividad de β-1,3-glucanasa en el tratamiento de la sistemina y HMA. Se observó un efecto positivo similar sobre la actividad de glucanasa después de la aplicación de la sistemina en las etapas más tempranas de la colonización. Esto sugiere que la sistemina podría tener un impacto sobre el proceso de micorrización del tomate con la modulación de las respuestas de defensa.

Palabras clave: enzimas pectinolíticas, mycorrhizae, respuesta de defensa, sistemina

INTRODUCTION

Under natural conditions, the roots of more than 80 % of the plants are associated with symbiotic soil fungi, where the Arbuscular Mycorrhizae (MA according its acronyms in Spanish) constitute one of the main groups (1). The radical colonization by AMF induces important changes in the expression

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of different genes related to defense responses in the host plant, which allow it to overcome biotic and abiotic stresses (2).

In this process, the induction of a systemic resistance response, similar to that produced by certain fungi and bacteria, occurs with the induction of diverse proteins related to pathogenicity (PRs), among which β-1,3-glucanases are reported, chitinase and enzymes related to oxidative processes (3,4).

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. These defense responses occur transiently, similar to what is observed in plant-pathogen interactions (5).

Other authors studying the amount of induced signal during the attack by herbivorous insects or by mechanical damage in tomato leaves (6), found the induction of a polypeptide hormone of 18 amino acids, systemin, involved in the expression of defense genes in Tomato plants and other Solanaceae. Once recognized by the target cells, the systemin produces the activation of a cascade of signals that involve the octadecanoic pathway producing the transient accumulation of jasmonic acid (AJ) in tomato leaves and the expression of genes related to the induced systemic response (7)

It has been reported that this hormone is required for the induction of the systemic response of the so-called systemic response proteins to damage (8), which includes several proteins associated with the signaling pathways and others similar to those induced by insects and herbivores (9).

Recent studies aimed at analyzing the possible role of systemin in the local 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 (10). This paper shows changes in induction patterns of the induced systemic response (ISR) in mycorrhizal plants by unidentified mechanisms in the late stage.

In order to elucidate the possible role of systemin in the dialogue between the AMF and the plant, with the participation of common signaling mechanisms, the activity pattern of PRs proteins induced by this interaction was evaluated.

MATERIALS AND METHODS

Obtaining plant material and application of products

Tomato plants (*Solanum lycopersicum* L.) from the cultivar "Amalia" (11) were used. 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 made up of a mixture of typical leached Ferralitic Red soil and earthworm humus in a 1:1 (v:v), sterile relationship (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.

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

K^+	Ca^{2+}	Mg^{2+}	P	Organic matter	pH
(cmol kg^{-1})			(mg mL^{-1})	(%)	(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 (13)

100 seeds of the cultivar Amalia were used; they were sown directly on 1 kg pots. Inoculation with AMF was performed by coating the seeds (14). Later, at the time of the emergency, the tomato system was applied, for which a synthetic product was used (Laboratorios BQ SOS, from Mexico), it was added to the substrate in solution (1 mL per plant at 44,06). nmol). A control treatment was maintained that did not have AMF or systemin, in which distilled water was added at a rate of 2 mL per plant.

For the samplings a dynamic was followed from three to 24 days after the emergency (dpe), corresponding to 3, 6, 9, 15, 18, 21 and 24 dpe.

At the time of sampling the plants were washed and separated by organs, dividing the roots into two groups. A 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 to determine the establishment of mycorrhizal symbiosis

Colonization mycorrhizal

To quantify the mycorrhizal colonization, the roots were dried at 70 ˚C, digested with KOH, and then stained with trypan blue. The percentage of mycorrhizal colonization (% C) was evaluated according to the method of intercepts and the intensity of colonization (% I) (10).

Enzymatic assay

The extraction of total proteins and the enzymatic determinations were carried out according to the methodology described by Noval *et al*. (10). The total protein concentration was determined by the Bradford method (15), using the commercial kit (Bio-Rad Laboratories, USA). The determination of enzymatic activities *in vitro* 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 PR 521 microplate reader. For the chitinase activity (QUI); EC- 3.2.1.14), the colloidal chitin prepared from reactive grade chitin (Fluka) was used as the substrate and the absorbance was read at 585 nm in the spectrophotometer (10). All enzymatic activities were transformed to µKat (microkatals) and pKat (picokatals) per milligrams of proteins, as needed (16). 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 ten plants, in each sampling moment. The data were processed statistically according to the variance analysis of simple classification, comparing the means by means of 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 (17). The percentages of increments were calculated by comparing the treatments with the corresponding control.

RESULTS

Dynamics of the symbiosis establishment

From the first moments of evaluation (3 dae) the presence of fungal structures in the roots was detected, evaluated as percentage of colonization (% C) and intensity (% I) (Table II), with low levels in the first nine days (6,1 % C and 0,0824 % I). From 12 and 15 dae an increase occurred, with values that were maintained until the end of the experiment (46,56 % C and 1,8 % I), with statistical similarity among them (15 to 24 dae). During this phase, high levels of both indicators were reached for this crop, taking into account the results reported in previous studies, in which values of 13 colonization percentage and 0,61 percentage intensity were found as visual density in plants of mycorrhized tomato with *Funneliformis mosseae* (*Glomus mosseae sensu lato*) 32 days after germination (18).

Table II. Percentage of arbuscular mycorrhizal colonization (%C) and intensity or frequency of colonization (% I) of plants inoculated 3-24 days after emergence (dae)

Days	3	6	9	12	15	18	21	24
% colonization								
Fm	6,1 a	14,22 a	11,06	30,51	46,08 b	46,02 b	45,91 b	47,74 b
Fm + S	3,51 b	9,15 b	12,58	32,64	55,04 a	52,29 a	52,81a	56,06 a
Sx	0,209 ***	0,154 ***	0,266 n.s.	1,56 n.s.	0,385 ***	0,694 ***	1,211 ***	0,374 ***
% intensity								
Fm	0,08	0,19 a	0,30 a	1,33 b	2,34 b	1,07 b	2.00	1,78
Fm + S	0,07	0,12 b	0,17 b	2,21 a	4,18 a	3,52 a	3,50	2,26
Sx	0,008 n.s.	0,003 ***	0,007 ***	0,044 ***	0,047 ***	0,354***	0,32 n.s.	0,158 n.s.

Fm: *Funneliformis mosseae*. Fm/S: *Funneliformis mosseae*+systemin
Means with common letters do not differ significantly according to the Tukey procedure (HSD) (p≤0.05)

It was observed that systemin acted as enhancer of mycorrhization, from 12 dae, when evaluating both colonization percentage and intensity, with percentages of 6,98-19,44 and 26,96-228,97, respectively, when compared to the levels reached by the plants where *F. mosseae* was inoculated without the elicitor (*Fm*) application during 12-24 dae.

This period coincides with the evaluation time. In the mycorrhized plants (*Fm*) there is an important increase of both indicators of the establishment and functioning of the symbiosis, with the consequent increase of the metabolic processes related to it. After the recognition of the system a series of signaling processes are activated, which lead to the accumulation of JA (7) and can play a crucial role in the processes of establishing and maintaining the MA symbiosis in a functional way (19-21).).

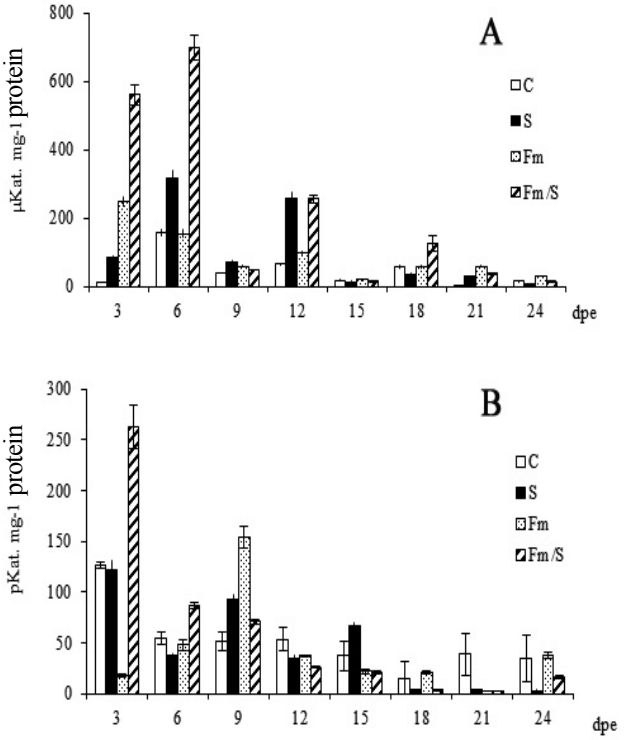
The mycorrhizal roots exhibit an increase in the levels of this hormone accompanied by the expression of genes that are induced by this acid and of genes that code for their own biosynthesis; however, contrasting results have been reported with its application, where it has been observed, both decrease in the rate of mycorrhization, and improvement of the AMF-plant interaction (22).

Jasmonates belong to a different kind of lipid metabolism, among which are the oxylipines. Recent studies have revealed the regulation of these compounds during the arbuscular mycorrhizal symbiosis, where the modification of the expression pattern of genes related to the oxilipine pathways is produced (23). These reports could explain the results observed with the combination of the AMF and the elicitor, if it takes into account that the systemin is an inducer of defense responses linked to the accumulation of jasmonic acid and secondary signals such as oxylipines (6).

The effect observed on the mycorrhization with the application of the elicitor, is of great practical use, since it could increase the benefits that these fungi offer to the crops.

Activity pattern of enzymes related to defense: glucanase and chitinase

The activity patterns of the GLN enzymes in the roots of tomato plants only mycorrhized (*Fm*), showed a tendency to reach the highest levels in the first stages of the establishment of mycorrhizal colonization, at 3 and 6 dae (Figure 1A), with values of 248,64 and 154,52 µKat mg^{-1} protein, respectively. In relation to the QUI activity, in mycorrhizal plants an increase occurred later, reaching a maximum of activity at nine dae with 154,05 pKat mg^{-1} protein (Figure 1B), a response that coincided with the development of the first phases of the process of establishing the symbiosis, given by the presence of the fungus inside the root at low levels (6,1-11,06 % C and 0,08-0,30 % I). Subsequently, the activity gradually decreased as the mycorrhization process progressed, until reaching similar or lower values to the control.



(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 3-24 days post-emergence (dae) induced by the application of AMF and systemin

It was found that the exogenous application of systemin, independently, produced increases in the activity of GLN in the early stages, reaching the highest levels at 3 and 6 dae (83,47 and 317,64 µKat mg^{-1} protein , respectively), with a second moment of increase at 12 dae (256,67 µKat mg^{-1} protein), which showed significant differences among them, in relation to the confidence intervals observed.

The effect on the activity of the QUIs was later, observing levels that exceeded the control at 9 and 15 dae (92,13 and 67,05 pKat mg^{-1} protein, respectively).

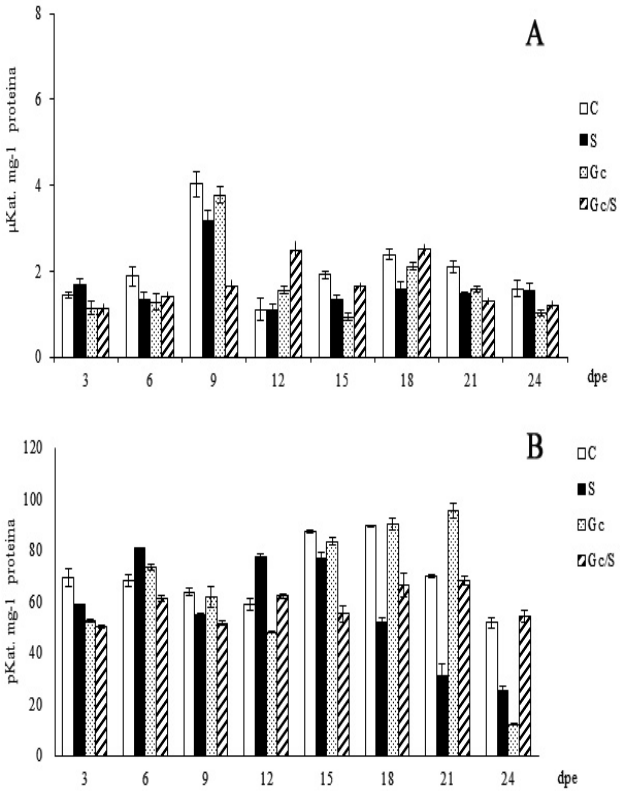
In analyzing the results in treatment of a synergistic effect between combinations *F. mosseae* and systemin (*Fm*/S) it was found. This was marked on the activity of the GLU with a rapid and transient induction at 3 and 6 dae (560,49 and 699,07 µKat mg^{-1} protein), which showed statistically significant differences with the treatments where both were applied independently and increases of 125,42 % and 352,42 %, respectively.

In relation to the QUI activity in root (Figure 1B), a rapid and transient induction was also observed at 3 dae with a synergistic effect of AMF and systemin (*Fm*/S) (263,1 pKat mg^{-1} protein), which corresponded to an 87,07 % increase. Only at 9 dae there was an activity induction of this enzyme in all the evaluated treatments, in relation to the control (36,75-199,06 % increase); time in which the plants inoculated with *Fm* showed the highest values (154,05 pKat mg^{-1} protein). In late stages both *Fm* and systemin produced the repression of the activity.

When analyzing the quantification of GLN activity in leaves, it was observed that the treatments showed statistically significant differences between them; although in a general way, in all of them very low levels were reached, in relation to those obtained in root, which oscillated between 1,64 and 2,06 µKat mg^{-1} protein (Figure 2A). Only at 9 dae, an increase in activity was observed in the control plants, which was followed in decreasing order by *F. mosseae* and by systemin (4,02, 3,77 and 3.16 µKat mg^{-1} protein, respectively).

At 12 dae, a synergistic effect (2,47 µKat mg^{-1} proteins) was found with a 90 % increase in relation to the applied bioproducts. Independently, it exceeded all the treatments under study. This effect was maintained at 15 and 18 dae, which despite showing statistical differences in relation to the treatments where F. mosseae and the inducer were applied separately did not surpass the basal levels (control).

The activity patterns of QUI enzymes in the leaves of tomato plants independently mycorrhized (*Fm*) (Figure 2B), showed similar levels with values close to or below the control, with a mean of 71,49 pkat mg^{-1} protein, except for the 18 dae, when it reached a maximum of 103,08 pKat mg^{-1} protein. In the plants that were mycorrhized with *F. mosseae*, without the application of the elicitor (*Fm*), there was an increase in the activity of this enzyme at 21 dae (95,27 pKat mg^{-1} protein). In general, in the leaves, activity levels lower than those obtained in the roots were observed for both enzymes.



(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)

Figura 2. Enzymatic activity in leaves of tomato plants evaluated 3-24 days after emergence (dae) induced by the application of AMF and systemin

GLN are abundant in plants and have a crucial participation in different physiological processes. They play a dominant role in cell division, in the exchange of compounds in plasmodesm and in the reduction of abiotic stresses (4). In relation to the function of enzymes in the MA symbiosis, the presence of the substrate of these hydrolases, the β-1,3-glucans, has been found in the wall of the members of the Glomaceae and Acaulosporaceae family, which suggests the possibility that they may be involved in the partial degradation of the cell wall of the AMF and of the plants, facilitating the process of penetration in the radical cell (18).

It was observed that the application of the system produced a transient increase in the activity of this enzyme in root at 6 dae. It has been identified that this polypeptide hormone produces the accumulation of proteinase inhibitors and at least 19 more proteins;

those that include components of signal transduction pathways and other proteins whose function in the defense of plants have not been fully identified. However, recently peptidomic studies revealed their possible linkage with the expression of PRs proteins. In a quantitative analysis of 14 peptides related to damage in tomato, a new peptide was obtained (CAPE1). It showed a response similar to that observed by systemin, with the induction of expression of genes related to activity against pathogens and herbivores such as protease inhibitors (PI-1 and PI-2) and related protein genes with the pathogenesis PR-1b, β-1,3-glucanase (PR-2) and chitinases, among others. Similar to the system, the peptide CAPE1 activates the biosynthesis of AJ (24).

As it is known, the QUI participate in different physiological processes, with diverse functions, which could have produced the punctual increase of isoforms of the enzyme not related to mycorrhization, as observed in the control treatment. These enzymes are abundant in the roots where they can act by releasing molecular signals that act as endogenous elicitors of morphogenesis. It has been observed that they could participate in the process of root development, by inducing the differentiation of tomato calli in *in vitro* culture and in embryogenesis in carrots (25).

Regarding the systemin, it is unknown what could be the cause of the repression of the activity of the QUI in root to the 12 dae, applied independently, when compared with the control plants. On the other hand, a negative influence of the components of the signaling pathway of this elicitor with the induction of QUI has not been found either.

During the initial stages of the establishment of MAs, QUIs that are not related to symbiosis predominate, those that produce attenuated defense responses; while in the most advanced stages the accumulation of specific chitinases of mycorrhization occurs (26). In a mycorrhization study of tomato plants of the variety “Amalia” inoculated with *R. clarum*, an increase in the activity of this enzyme was associated with mycorrhization at 32 days. Apparently in the present experiment this moment of induction occurred at 9 dae in the root and at 21 dae in leaves, which confirms the occurrence of a rapid signaling process that led to the induction of systemic defense responses (18).

The possible participation of AJ in the signaling required for the establishment of induced systemic response (ISR) in mycorrhizal plants, as part of the resistance induced by mycorrhizae, recognized by MIR (27) has been suggested. . This produces a priming effect, dependent on the AJ (28), this hormone is crucial in the amplification of the response to mechanical damage in tomato (8,7,29). Several authors report the induction of ISR in mycorrhized plants (30); however, the contribution of the AJ to the MIR is not totally clear, as well as the control of this long-distance response (27).

The use of biofertilizers and elicitors in agricultural practice has taken a big boom in the last decade, which has been used for different purposes. A synergistic effect of AMF and systemin was observed on the induction of defense-related proteins in early stages, locally, suggesting the possibility of the convergence of the mechanisms of action by which both induce SRI.

The fact that the systemin stimulates the establishment processes of the mycorrhizal symbiosis, evaluated as percentage of colonization and intensity of the same, supports the relevant role of the AJ in the processes of establishment and maintenance of the MA symbiosis, which has been suggested by different authors. The results show the existence of a receptor for this elicitor in the roots, similarly it has been identified in leaves.

CONCLUSIONS

- The systemin potentiated the establishment of the mycorrhizal symbiosis with *F. mosseae*, as well as its action on the induction of GLN and QUI, by producing a rapid and transient increase in root, in the early stages of the mycorrhization establishment. It is known that this elicitor produces rapid activation of defense responses in which different elements that lead to the accumulation of AJ and ethylene, which are inducers of PRs protein expression, participate. However, these hormones have not been linked to repression processes of the activity of these proteins, observed in later times.
- The results suggest an important role of systemin in its interaction with the arbuscular mycorrhizal symbiosis, by modulating the defense responses during the process of establishing mycorrhization, with a long-term effect, in such a way that the symbiosis is enhanced by an unknown mechanism that could involve the regulation of PRs proteins.

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