

Glyoxalase I (GLX-I) analysis in native maize from Oaxaca, Mexico, infected with *Aspergillus flavus* *in vitro*

Análisis de glioxalasa I (GLX-I) en maíces nativos de Oaxaca, México, infectados con *Aspergillus flavus* *in vitro*

Análise de Glyoxalase I (GLX-I) em milho nativo de Oaxaca, México, infectado com *Aspergillus flavus* *in vitro*

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Abstract

The glyoxalase system plays an important role in various physiological processes in plants when they are subjected to different types of stress, whether physical, chemical or biological. *Aspergillus flavus* is an aflatoxin-producing fungus that contaminates dry grains, leading to a gradual deterioration of the grains and a significant reduction in their nutritional value. The objective of the present study was to evaluate the activity of the enzyme glyoxalase I (GLX-I) in maize coleoptiles from Oaxaca in response to infection caused by *Aspergillus flavus*. Nine maize samples from four different races were analyzed. The samples were inoculated with a suspension of *Aspergillus flavus* spores of known concentration and total protein extraction and quantification were performed on the coleoptiles, and GLX-I activity was determined by quantifying the amount of S-lactoylglutathione produced per minute. In addition, analysis of gene expression by reverse transcriptase polymerase chain reaction (RT-PCR) was performed. The inoculated maize coleoptiles showed symptoms of infection, color changes and wilting. The concentration of total proteins decreased significantly in the extracts of four samples in the presence of the fungus. In the GLX-I analysis, two samples had the highest enzymatic activity in the infected coleoptile extract with respect to the healthy one, in addition to presenting greater expression of the gene in the RT-PCR assay, this due to the response to *Aspergillus flavus* infection.

Resumen

El sistema glioxalasa juega un papel importante en diversos procesos fisiológicos en plantas cuando son sometidas a diferentes tipos de estrés, ya sean físicos, químicos o biológicos. *Aspergillus flavus* es un hongo productor de aflatoxinas que contamina granos secos, lo que conlleva a un deterioro paulatino de los granos y la reducción significativa de su valor nutricional. El objetivo del presente estudio fue evaluar la actividad de la enzima glioxalasa I (GLX-I) en coleoptilos de maíces de Oaxaca como respuesta a la infección producida por *Aspergillus flavus*. Se analizaron nueve muestras de maíz de cuatro razas diferentes. Las muestras se inocularon con una suspensión de esporas de *Aspergillus flavus* de concentración conocida y a los coleoptilos se les realizó la extracción y cuantificación de proteínas totales y se les determinó la actividad de GLX-I cuantificando la cantidad de S-lactoilglutathión producida por minuto. Además, se realizó el análisis de la expresión del gen por reacción en cadena de la polimerasa transcriptasa reversa (RT-PCR). Los coleoptilos de maíz inoculados presentaron síntomas de infección, cambios de coloración y marchitamiento. La concentración de proteínas totales disminuyó significativamente en los extractos de cuatro muestras ante la presencia del hongo. En el análisis de GLX-I, dos muestras tuvieron la mayor actividad enzimática en el extracto de coleoptilo infectado con respecto al sano, además de presentar mayor expresión del gen en el ensayo de RT-PCR, esto debido como respuesta a la infección por *Aspergillus flavus*.

Palabras clave: GLX-I, estrés, proteínas, *Aspergillus*.

Resumo

O sistema glioxalase desempenha um papel importante em vários processos fisiológicos em plantas quando estas são submetidas a diferentes tipos de estresse, seja físico, químico ou biológico. *Aspergillus flavus* é um fungo produtor de aflatoxinas que contamina grãos secos, levando a uma deterioração gradual dos grãos e uma redução significativa em seu valor nutricional. O objetivo do presente estudo foi avaliar a atividade da enzima glioxalase I (GLX-I) em coleóptilos de milho de Oaxaca em resposta à infecção causada por *Aspergillus flavus*. Nove amostras de milho de quatro raças diferentes foram analisadas. As amostras foram inoculadas com uma suspensão de esporos de *Aspergillus flavus* de concentração conhecida e a extração e quantificação da proteína total foram realizadas nos coleóptilos, e a atividade do GLX-I foi determinada pela quantificação da quantidade de S-lactoilglutathione produzida por minuto. Além disso, foi realizada a análise da expressão gênica por reação em cadeia da polimerase com transcriptase reversa (RT-PCR). Os coleóptilos de milho inoculados apresentaram sintomas de infecção, alterações de cor e murchamento. A concentração de proteínas totais diminuiu significativamente nos extratos de quatro amostras na presença do fungo. Na análise de GLX-I, duas amostras apresentaram a maior atividade enzimática no extrato de coleóptilo infectado em relação ao saudável, além de apresentarem maior expressão do gene no ensaio de RT-PCR, isso devido à resposta à infecção por *Aspergillus flavus*.

Palavras-chave: GLX-I, estresse, proteínas, *Aspergillus*.

Introduction

Maize is the main agricultural product in Mexico (Rosado and Villasante, 2021), 18,151,034 million hectares are planted annually, with an average harvested area of 17,229,616 ha (Agrifood and Fisheries Information Service [SIAP], 2021), and its diversity is found in traditional agricultural systems. The crops plant native varieties and through their knowledge, the preferences and practices they have developed, they will be able to maintain the diversity in this crop (Fernández *et al.*, 2013; Cabrera *et al.*, 2015). The so-called “creole or native breeds” are the result of the traditional manipulation of the peasants and the environmental variability present in the numerous ecological niches in which they are cultivated, which contribute to the conservation and generation of the genetic diversity of the crop, coming to form new types, varieties or races (Arteaga *et al.*, 2016; Orozco-Ramírez *et al.*, 2016). Of the 59 breeds identified in the country (Fernández *et al.*, 2013), 35 are reported in Oaxaca, which represents 59 % of the total existing diversity in Mexico (Aragón-Cuevas *et al.*, 2006; Kato, 2009). Maize production in Mexico is characterized by being cultivated in the rainy season, which during its growth and storage is exposed to contamination by fungi, including *Aspergillus flavus*, generating economic losses of production and production problems public health due to the consumption of food contaminated by aflatoxins (Martínez *et al.*, 2013).

On the other hand, stress is an unavoidable limiting factor for agriculture and is becoming a major problem in the modern world, reducing up to 50 % of crop yields globally (Bandyopadhyay *et al.*, 2016). Plants grown under natural conditions are constantly subjected to a wide variety of abiotic stresses such as salinity, drought and toxic metals, which cause the production of cytotoxic compounds such as methylglyoxal (MG) and the response of macromolecules to eliminate it (Hasanuzzaman *et al.*, 2017a; Ben *et al.*, 2018). Methylglyoxal is a natural metabolite and a highly reactive cytotoxic compound, produced when organisms are subjected to some type of stress, mainly abiotic (Ghosh, 2017; Borysiuk *et al.*, 2018); it can damage and modify proteins, lipids, carbohydrates and DNA, resulting in cell death, therefore it is important for cells to detoxify to survive (Rohman *et al.*, 2016). MG is removed by the glyoxalase system mainly by two enzymes, GLX-I and GLX-II. GLX-I catalyzes the transformation of MG into S-D-lactoylglutathione through the use of a reduced glutathione (GSH) molecule, while GLX-II catalyzes the formation of D-lactate from S-D-lactoylglutathione, allowing the regeneration of the molecule reduced glutathione (Turra *et al.*, 2015); In this way, the glyoxalase system could play an important role in plant tolerance to stress by recycling GSH and, therefore, maintaining the homeostasis of this molecule (Ghosh *et al.*, 2016; Hasanuzzaman *et al.*, 2017b).

To improve the response of plants to unfavorable growth conditions, it is necessary to know their response mechanisms to stress. In hybrid maize, the response of GLX-I was analyzed in samples that showed resistance to aflatoxin production by *A. flavus* (Chen *et al.*, 2004), however, the activity of this enzyme in native maize from Oaxaca raised with *A. flavus* has not been studied. Therefore, this research aimed to analyze the response of GLX-I to infection by *A. flavus* in coleoptiles of native maize from the state of Oaxaca.

Materials and methods

Samples of native maize from Oaxaca

Nine samples of 4 races from different collection points of native maize from Oaxaca donated by Flavio Aragón Cuevas, researcher at the Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias en Oaxaca (INIFAP) were analyzed. Table 1 describes the samples.

Table 1. Samples of native maize from the state of Oaxaca evaluated.

Sample	Race	Color	Place of origin
1	Bolita	Belatove	Central valleys
2	Bolita	Yellow	Central valleys
3	Bolita	Yellow	Central valleys
4	Bolita	Blue	Central valleys
5	Conejo veloz	White	Costa
6	Bolita	White	Central valleys
7	Costeño	White	Costa
8	Zapalote	Purple	Costa
9	Bolita	Blue	Central valleys

Aspergillus flavus strain

For the infection tests, an *A. flavus* strain isolated from dried chili peppers marketed in Oaxaca and characterized in the Food Laboratory of the Tecnológico Nacional de México, Oaxaca campus for its aflatoxin production was used. An aliquot of 0.1 mL of a concentrated solution of spores was inoculated on plates with potato-dextrose agar (PDA) medium and incubated for 7 days at 28 ± 2 °C in a Labolan brand incubator. The spores were recovered in water containing 0.01% triton and stored at 5 °C until used.

Obtaining maize coleoptiles

To obtain maize coleoptiles, the procedure reported by Varapizuela *et al.* (2019) was used. Thirty seeds of each sample were disinfected by immersion in a 70 % ethyl alcohol solution for 10 minutes and rinsed three times with sterile distilled water. Five grains of each sample were placed in a Petri dish with a cotton bed and moistened filter paper, being this an experimental unit. Three plates were used as healthy controls inoculated with 50 µL of sterile water and three more were inoculated with 50 µL of an *A. flavus* spore suspension of 4×10^6 spores.mL⁻¹ to each grain. The plates were incubated at 28 ± 2 °C for 7 days.

Extraction and quantification of total proteins

Protein extraction was performed by maceration with a 1:2 (w/v) ratio using the extraction buffer reported by Chen *et al.* (2007) with some modifications, which contained 0.25 M NaCl, 50 mM Tris-HCl pH 8.0 and 14 mM β-mercaptoethanol and centrifuging the samples at 12,000 rpm for 15 minutes. The supernatants obtained were quantified in triplicate by the Bradford technique using a BSA (bovine serum albumin) standard curve and stored at -20 °C in a Frigidaire® freezer until used.

GLX-I enzyme activity assay

The glyoxalase I (GLX-I) enzymatic activity of maize coleoptile extracts from healthy and *A. flavus*-infected samples was determined according to the assay reported by Chen *et al.* (2004). The assay mixture contained 100 mM sodium phosphate buffer pH 7.5, 3.5 mM

methylglyoxal (MG), 1.7 mM reduced glutathione (GSH), and 16.0 mM magnesium sulfate in a final volume of 1 mL. The mixture was transferred to a quartz cell and incubated at ± 27 °C for 1 minute. The reaction began by adding the enzymes of the extracts obtained by maceration (0.02 mL of crude extract or extract boiled for 10 minutes at 100 °C as control) and the formation of S-D-lactoylglutathione thioester was monitored by measuring the increase in absorbance at 240 nm in a SmartSpec™ 3000 spectrophotometer (BIO-RAD) at 20 minutes. The absorbance was converted to a molar amount using the molecular coefficient of 3.370 µmol for S-lactoylglutathione. One unit of absorbance is defined as 1 µM S-lactoylglutathione.min⁻¹. The assay was performed in triplicate for each extract.

Analysis of data

Total protein quantification data were analyzed based on the mean and standard deviations (means and standard deviation of three replicates and three values per replicate). Data analysis of GLX-I activity was determined by analysis of variance and Tukey's statistical test using Minitab17 software. The samples that presented a statistically significant difference in the enzymatic activity assay were analyzed by Reverse Transcriptase PCR.

Analysis of GLX-I gene expression by Reverse Transcriptase PCR

For the extraction of RNA from maize coleoptiles, the Guanidine Thiocyanate method (Chomczynski and Sacchi, 1987) was used and the samples were stored at -20 °C until use. The GLX-I oligos designed by José Luis Hernández Morales belonging to the Tecnológico Nacional de México/Instituto Tecnológico de Oaxaca were used based on the sequences reported in genbank with No. NCBI AY241545, Forward (5' GGATAGTGAAGCCTCGAAGG 3') and Reverse (3' GCATTACTACATCCTAGCACAGC 5'), using as positive control the oligos of the Mac1 maize actin gene (Baker *et al.*, 2009), Forward (5' GTGACAAATGGCACTGGAATG 3') and Reverse (5' GACCTGACCATCAGGCATCT 3'), No. NCBI J01238, both with a molecular weight of 900 bp. For the reaction, the Quiagen kit for RT (Reverse Transcriptase) was used following the supplier's instructions and a concentration of 250 ng of maize coleoptile RNA. The reaction was carried out in a BIO-RAD MyCycler thermal cycler with a reverse transcriptase at 50 °C, 30 minutes, initial PCR activation at 95 °C, 15 minutes and 35 cycles of: denaturation at 94 °C, 30 seconds, alignment 52 °C, 30 seconds, extension 72 °C, 45 seconds, ending with a final extension cycle at 72 °C for 10 minutes. The PCR products were analyzed on 2% agarose gels. Densitometry analysis of GLX-I expression in the agarose gel was performed using ImageJ software version 1.8.0 (<https://imagej.net/software/imagej/>), which calculates the area and statistics of the pixels of defined sections (Schneider *et al.*, 2012). The extraction was performed in triplicate.

Results and discussion

Obtaining maize coleoptiles

Physiological differences were found between the growth of healthy maize coleoptiles and those infected with *A. flavus*. Healthy coleoptiles showed greater elongation, light yellow color with hyaline parts, as well as greater thickness (figure 1a).

All samples of infected coleoptiles presented smaller size, brown to black pigmentation and wilting (figure 1b), symptoms like those reported by Varapizuela *et al.* (2019), where the infection produced by *Aspergillus parasiticus* in coleoptiles of native Oaxacan maize presented wilting and chlorosis. This may be since the fungus uses the coleoptiles as a substrate for its development.

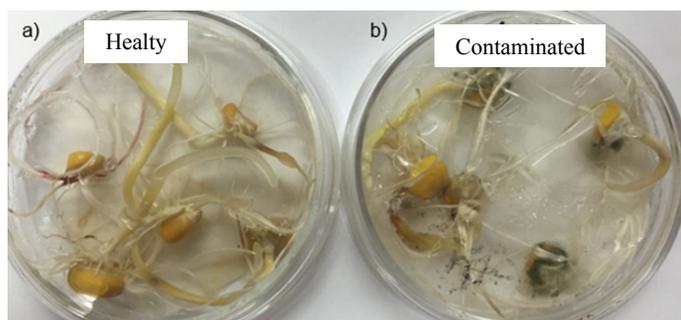


Figure 1. Growth of coleoptiles of maize native to Oaxaca from sample 1 of the bolita race. a) Healthy coleoptile. b) Coleoptile infected with *Aspergillus flavus*.

Total protein quantification

Samples 2, 3, 5 and 9 showed a significant decrease in the concentration of total proteins in relation to healthy coleoptiles (figure 2). Sadiq *et al.* (2011), reported a decrease in protein for rice coleoptiles when the seeds are germinated under anoxic conditions, however, there was an increase in specific stress response proteins. The overproduction of reactive oxygen species and cytotoxic compounds can lead to the malfunction of the synthesis of structural proteins for plant development, but they can trigger the response of specific enzymes to counteract the damage caused (Bania and Mahanta, 2012; Li, 2016).

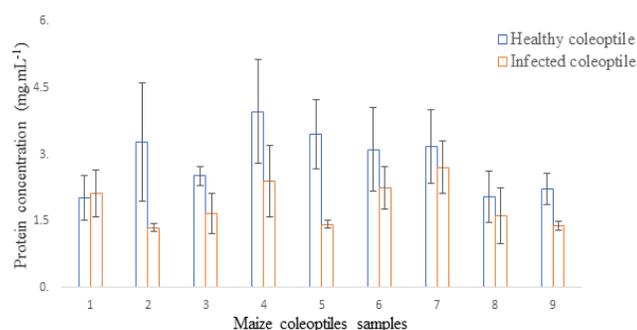


Figure 2. Quantification of total proteins of the extracts of healthy maize coleoptiles and those infected with *Aspergillus flavus*.

GLX-I enzyme activity

In Tukey's analysis of GLX-I activity (table 2), it was found that samples 1 and 5 had a statistically significant higher enzymatic activity in infected coleoptiles compared to healthy ones, while sample 9 had higher enzymatic activity in the healthy coleoptile than in the infected one. This result could indicate that the activity of the enzyme is not always related to the changes or stress suffered by the plant. Chen *et al.* (2004), evaluated the enzymatic activity of GLX-I in 6 samples of healthy hybrid maize inoculated with *A. flavus*, reporting that only in one there was a significant difference in the enzymatic activity in maize inoculated with the fungus compared to the control without inoculate, whose data related to the response of this enzyme with resistance to aflatoxin production, these results coincide with those reported in this investigation. On the other hand, Ben *et al.* (2018) reported an increase in the activity of the glyoxalase system in sorghum plants, when there is

a stress generated by an excess or a deficit of nitrogen in the plant, unlike that reported by Borysiuk *et al.* (2018), where the excess of nitrogen in *Arabidopsis* plants excessively increased the levels of methylglyoxal, inhibiting the capacity of the detoxifying pathway of the glyoxalase system and generating severe damage to proteins. Resistance to stress generated by biotic or abiotic factors in plants is a mechanism that encompasses many enzymes that counteract the damage generated by the stress produced, which depend on the type of plant and growth conditions to present activity (Hasanuzzaman *et al.*, 2017a; Hasanuzzaman *et al.*, 2017b; Kaur *et al.*, 2017).

Table 2. Enzymatic activity of glyoxalase I (GLX-I) in maize coleoptiles.

Sample	$\mu\text{mol } S\text{-lactoylglutination.mg}^{-1} \text{ of protein.min}^{-1}$	
	Healthy coleoptile	Infected coleoptile
1	0.507 ^{bd}	0.772 ^a
2	0.267 ^{cd}	0.668 ^{cd}
3	0.498 ^{ac}	0.649 ^{bd}
4	0.302 ^{ad}	0.454 ^{bd}
5	0.415 ^{ab}	0.609 ^{cd}
6	0.473 ^{ab}	0.637 ^{ab}
7	0.436 ^{ab}	0.386 ^{bd}
8	0.684 ^{ab}	0.653 ^{bd}
9	0.559 ^{ac}	0.551 ^d

*Values followed by the same letter do not differ significantly according to Tukey's statistical test ($p \leq 0.05$).

Analysis of GLX-I expression by RT-PCR

Samples 1 and 5 showed more than double the expression of the gene in infected coleoptiles compared to samples from healthy coleoptiles. On the other hand, sample 9 of infected coleoptiles showed lower expression of the gene compared to the healthy coleoptile, however, the difference was not significant (figure 3).

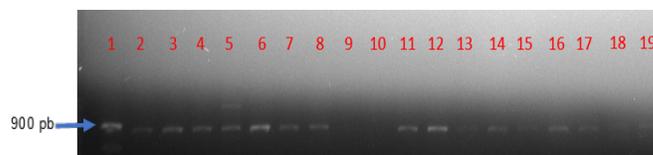


Figure 3. RT-PCR products of the GLX-I gene from healthy maize coleoptiles and those infected with *Aspergillus flavus* in 2% agarose gel. Lane 1, amplified actin gene (900 bp) in healthy sample 1. Lanes 2 to 4, shows 1 healthy. Lanes 5 to 7, sample 1 infected. Lanes 8 to 10, shows 5 healthy. Lanes 11 to 13, sample 5 infected. Lanes 14, to 16, shows 9 healthy. Lanes 17 to 19, sample 9 infected.

Alvarez-Gerding *et al.* (2015), analyzed the overexpression of glyoxalase I and glyoxalase II genes in transgenic samples of reed *Phragmites australis* compared to wild type when stressed by salinity. The transgenic samples increased the expression of the glyoxalase I and II genes, also reducing the physiological damage caused in the plant compared to the wild type. According to what was reported by Fountain *et al.* (2010), in maize samples, the expression of the GLX-I gene did not show significant changes in expression, since the physiological stress induced by drought was

not enough to stimulate significant changes. The maize coleoptiles analyzed in this work that presented higher enzymatic activity and greater gene expression were more resistant to infection, unlike those that presented low enzymatic activity and gene expression, since these changes can be affected by factors such as the environmental ones, this is because some samples of the same race present different susceptibility.

The resistance of plants to the stress generated by biotic or abiotic conditions is a very important issue, since knowing better the resistance mechanisms will help to improve the treatments to which they are subjected to increase their quality and production. Several detoxifying pathways are involved in this process, which are comprised of many enzymes that are responsible for reducing and cushioning the damage generated by cytotoxic compounds generated by adverse growth conditions (Ghosh, 2017; Zhou *et al.*, 2018). Recent studies have shown that resistance to infection by *A. flavus* in maize is a trait controlled by multiple genes, however, the behavior of these genes reported in hybrids is still unknown in native lines that are considered a great source of genomic wealth and that have adapted to different growth conditions such as temperature, altitude, humidity, among others, prevailing from generation to generation (Rajasekaran *et al.*, 2019). It is important to know the natural response mechanisms of plants to stress, to find varieties that are resistant to unfavorable growth conditions, minimizing production losses and contamination of seeds by fungi and damage to the health of those who consume the products.

Conclusions

Faced with the infection of Oaxacan maize coleoptiles by *Aspergillus flavus*, there was a decrease in total proteins and an increase in the glyoxalase I response in both gene expression and enzymatic activity in samples 1 and 5.

The high activity of the enzyme and expression of the gene participate as a response to the infection of native maize, while low concentrations of the enzyme and low expression to susceptibility. However, the results should be analyzed with subsequent resistance or susceptibility tests.

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