RESEARCH / INVESTIGACIÓN

Identification of maize kernel resistance proteins against *Aspergillus flavus* by a statistical approach: A predictive model of resistance capacity

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Abstract: Although kernel infection by *Aspergillus flavus* and pre-harvest aflatoxin contamination of *Zea mays* grain is a significant crop production problem, not only in Venezuela but also around the world, little progress has been made in identifying proteins and metabolic pathways associated with this pathogen resistance. Usually, a protein with a two-fold expression between control and condition is considered a biomarker of some phenomena, but we think it is essential to evaluate its contribution to resistance. That is why we decided to determine the behavior's resistance capacity in terms of expression levels of an identified protein of maize kernels infected with *A. flavus* by using a multivariate approach. In this work, we identify 47 of 66 differentially expressed spots with a remarkable contribution to resistance against the fungus *Aspergillus flavus*. We finally test this approach to know if it can be used as a predictive resistance model and probe it by including theoretical and experimental protein expression profiles of other inoculated maize lines.

Key words: Host resistance, resistance-associated proteins, *Zea mays*, *Aspergillus flavus*, comparative proteomics, resistance predictive model.

Introduction

The latest statistics released by the Food and Agriculture Organization of the United Nations (FAO), indicate that by the year 2011, maize was the second most produced and demanded vegetable item in the world, after sugar cane[1]. Fungi are the world's primary cause of maize crop loss because of their economic implications, which represents a substantial phytosanitary problem to be solved. In Venezuela, the major maize crop pathogen is *Aspergillus flavus*, with 35-45% of incidence. It is the cause of around 80% percent of kernel losses because it reduces not only the grain nutritional value, but also its presence usually causes the accumulation of highly toxic and carcinogenic secondary metabolites named aflatoxins[2–6].

In general, plants can respond to invasion by pathogens through the activation of a variety of defense strategies, which are induced by complex interactions between biochemical pathways in a coordinated manner[7,8]. Defense responses generally include several cellular changes due to variations of protein expressions involved in cellular metabolism, as the production of structural carbohydrates, structural proteins, signal transcription and translation, transporters, intracellular traffic, and production of antimicrobial compounds, among other processes[9,10]. However, there is not much research focused on the response mechanisms of maize against fungal infection. Despite being a long-term method, recurrent selection can decrease or even eliminate fungicide dependent cultures. Besides, it is a low cost method, so it is widely used in developing countries[11,12].

Resistant plant selection is made a long time ago and tries to relate phenotypical characteristics with genotypical ones. Kernel screening (KSA) assay is a resistance-evaluation method that consists of maize grain exposition to a pathogen concentration to determine its capacity to inhibit pathogen proliferation[13]. In this work, we modified this method to increase the *A. flavus* spores concentration to 4x10^6 spores/mL of *A. flavus*. We evaluated the kernel differential-expression proteins between high-production *A. flavus*-infection-susceptible and *A. flavus*-infection-resistant maize lines to determine plant resistance mechanisms. We extracted maize grain proteins using two different pH buffers, quantified them, and verified their integrity. Then we obtained two-dimensional patterns and compared them, detecting multiple protein-expression variations.

All the information obtained allowed the generation of similarity matrices that were used to study the relationships among maize genotypes investigated using different techniques of classification/ordination such as principal components analysis[14,15], Neighbor-joining clustering algorithm[16,17] and principal coordinates analysis[18]. The combination of these techniques allows two crucial facts: 1.- determining the proteins differential pattern expression that majorly contributes with resistance capacity by the interpretation of the variables responsible for the classification or ordination, which were identified by MALDI-TOF mass spectrometry, to construct a resistance response model of a maize-grain cell against *A. flavus* and 2.- developing a methodological strategy of data processing that is capable of predict the resistance level of other maize lines.

Materials and methods

Maize lines and fungal selection

We pre-selected eleven high-production maize lines developed initially by the International Maize and Wheat Improvement Center (CIMMYT) and adapted to Venezuelan lands, based on two characteristics, 1.- its high productivity and 2.- its resistance capacity against *A. flavus* determined by modified KSA assay[13]. After that, we selected four lines based on the grain color, two highly resistant (one white grain and one yellow grain) and two highly susceptible (one white grain and one yellow grain). We inoculated the seeds with 4x106 spores/mL of *A. flavus* and incubated each grain line for 7 days, at 30ºC and 90-100% humidity resistance-capacity evaluation (figure 1), and 20 h for protein-expression profile evaluations.

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A. flavus Ospino 1-B was the fungal strain used due to its high colonization power. It was grown on agar plates at 30 °C until the mycelium had covered all the plates, and the spores were collected by shaking the plate in sterile water. The spores were then counted in a Neubauer chamber to inoculate maize grains with the mentioned spore quantity.

Protein extraction

For protein extraction, each plant's whole grain was pulverized with liquid nitrogen, and aliquots of 100 mg were homogenized in 1 mL of extraction buffer mixed with 1X protease inhibitor mix (GE healthcare). Two different protein extraction buffers (pH=2.8 or pH=7.5) were used according to the protocol described by Campo *et al.*[19]. These aliquots were incubated for 1h with continuous slow stirring and then were centrifuged at 16.000 g for 5 min at 4°C. The supernatants' proteins were incubated at -20°C overnight, with two volumes of methanol, so the final alcohol concentration in the mixture was 70%. Then, all tubes were centrifuged at 16.000g for 5 min at 4°C, the supernatants were discarded and the pellets were washed twice with 70% ethanol at 4°C, after which, the tubes were centrifuged again, the supernatants were discarded, and all the precipitates were saved at -70°C until use. The protein pools were resuspended in 50 µL DeStreak buffer (GE Healthcare), quantified by the Bradford QuickStart® method (Bio-Rad), and SDS-PAGE verified their integrity. We carried out three independent infection experiments, with three protein extractions of the pulverized plant material, in order to explore the protein level variations associated with the biological material and the experimental techniques.

Two-dimensional gel electrophoresis

Nonlinear pH 3-10 isoelectric focusing dry strip gels (GE Healthcare) were rehydrated overnight in 250 µL rehydration solution (8M urea, 1% CHAPS, 20mM DTT, and 0.2% of 3-10 non/linear carrier ampholytes), along with 30 µg (analytical) or 100 µg (preparative) of proteins. Isoelectric focusing was performed at 20°C in an Ettam IPGphor (GE Healthcare), following the standard protocol recommended by the fabricant: gradual increase until 200V for 20 min, gradual increase until 450V for 15 min, gradual increase until 750V for 15 min, gradual increase until 2000V for 30 min, all gradual increases were performed being careful not to overcome 25 milliamperes. After isoelectric focusing, gel strips were equilibrated for 15 min in a buffer containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS containing 10 mg/mL DTT, followed for 15 min with the same buffer but containing 5 mg/mL iodoacetamide instead of DTT. For the second dimension, strips were loaded onto SDS polyacrylamide gels (12% acrylamide separating gel, 8x7x0.1 cm, width x height x thickness), then the top of the gel was sealed with 1% agarose solution, and finally, we ran two-dimentional gel electrophoresis at 200V for about 35 min, until the front run was about to go out of the gel. All gels were silver-stained with PlusOne Silver staining kit (GE Healthcare) which is compatible with mass espectrometry. Gel were digitized with an HP scanner and analyzed with the Phoretix 2D v-2004 (nonlinear Dynamics Ltd.) program.

Comparative analysis of protein expression profiles

The protein expression profiles obtained from all maize lines were compared in two different analyses, white lines, and

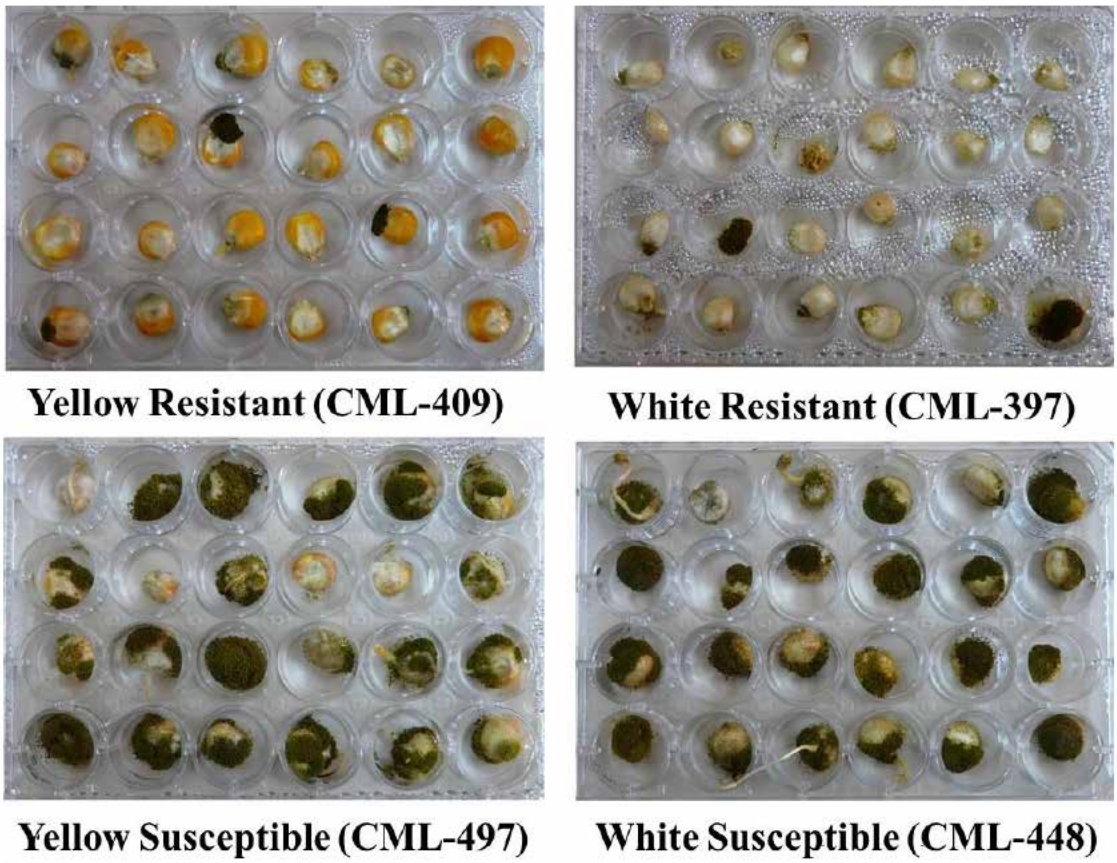

Figure 1. Characteristics of the chosen high-production maize lines in terms of their resistance or susceptibility to *A. flavus*. Kernel screening assay of the selected maize lines showing its resistance or susceptibility against *A. flavus* 4x10^6 spores /mL defined by fungus proliferation ratio in 7 days of infection.

yellow ones, between each other. We decided to compare just the profiles obtained from *A. flavus*-inoculated susceptible maize grain lines with those obtained from *A. flavus*-inoculated resistant maize grain lines. The two-fold differential-expression proteins detected by the Phoretix 2D program (Nonlinear Dynamics Ltd.) were selected for peptide sequencing.

Protein identification and database search

Proteins were identified by peptide mass fingerprinting using MALDI-TOF mass spectrometry (MS) performed with a PrOTOF 2000 (Perkin-Elmer Inc.) instrument, previously calibrated with trypsin-digested bovine serum albumin. For the analysis, 1µL of tryptic peptides were mixed with 1 µL of the matrix (α-cyano-4-hydroxycinnamic acid, 5mg/mL) and loaded on a stainless steel MALDI plate. Protein identification was carried out using MASCOT (Matrix Science Ltd.) or Protein Prospector software (v5.10.1, UCSF Mass Spectrometry Facility, University of California). Nonredundant NCBI (National Center for Biotechnology Information), MSDB, and Swiss-Prot (European Bioinformatics Institute Heidelberg, Germany) databases[20,21] were used for the search. The search parameters applied were described by Lee *et al.*[22]; they are a mass tolerance of 50 ppm with one incomplete cleavage allowed; alkylation of cysteine by carbamidomethylation was the only fixed modification; the variable modifications considered were acetylation of the *N*-terminus, oxidation of methionine, and the pyroGlu formation of *N*-terminal Gln.

Resistance response models

With all the available information and the additional one gathered in this work, we propose a whole maize cell resistance response model after 20 h of infection by *A. flavus*. This model was accomplished using bioinformatics interphases indexed in http://www.bioprofiling.de/index.html, which employs the information stored in NCBI and SwissProt databases[20,21]. These tools can be applied in just one plant, *Arabidopsis thaliana*, so the only way to use it was, placing this plant's accession number of homolog proteins instead of maize-identified proteins.

Statistical analysis for biomarkers determination and resistance prediction-method generation

The data of the 2D gels spots areas were used to determine the proteome-differences under the same conditions of infection, useful information to determine the differential-expression proteins more closely associated with maize-grain resistance cells against the fungus. We applied principal coordinates analysis (PCoA) to the binomial-transformed and mathematically size-adjusted by generalized Procrustes data matrices and projected on the principal coordinate plane to determine levels of resistance of the lines under study, given by its plane location. Cluster analysis was performed to evaluate the relationship between the proteins present in all the lines under study. We also applied Principal Components Analysis (PCA) to all original data matrices previously grouped by color and resistance capacity, eliminating extraction buffer variable by generalized Procrustes, to determine the spots that majorly contribute to resistance characteristic. We finally evaluated the statistical resistance-predictive method applied by using experimental and theoretical lines with characteristics of both resistant lines and evaluating them by principal coordinates analysis. All analysis was realized with the software InfoStat[23].

Results and Discussion

Maize lines selection

Guided by the mentioned characteristics, we pre-selected 11 maize lines (Table 1), and then we selected 4 maize lines from this initial selection: 2 resistant and 2 susceptible to *A. flavus* infection. White grain lines were selected with a close relationship between them, both having the same germplasm source, known as Tuxpeño 1. In contrast, yellow grain lines were deliberately selected with different germplasm sources (CML-409 is derived from "Antigua Veracruz" population, while CML-497 is derived from RCY source). The maize-grain-lines-resistance levels were confirmed by KSA assay (figure 1), modified as mentioned.

N°	Maize line	Color	Characteristic	Adjusted means	Real means
1	CML-493	Yellow	Resistant	1,21	0,84
2	**CML-397**	**White**	**Resistant**	**1,23**	**0,91**
3	CML-274	White	Resistant	1,29	0,69
4	CML-46	White	Resistant	1,30	0,91
5	**CML-409**	**Yellow**	**Resistant**	**1,31**	**1,22**
6	CML-176	White	Witness	1,46	1,35
7	CML-159	White	Susceptible	1,72	2,06
8	CML-247	White	Susceptible	1,77	2,69
9	**CML-497**	**Yellow**	**Susceptible**	**1,80**	**2,95**
10	**CML-448**	**White**	**Susceptible**	**1,80**	**3,43**
11	CML-7	White	Susceptible	1,86	3,19

The adjustment was made to compare the results of all lines that were not obtained simultaneously. All values were adjusted to guarantee data normality by the function $\sqrt[2.55]{Colonization(Z)+3}$, where Z is the real mean value obtained. In the cases of equal adjusted mean values, resistance is determined by real mean values.

In bold letters, we show the four selected maize lines for protein identification through comparative proteomics.

Table 1. Modified KSA assay results of pre-selected maize lines against A. flavus grain infection. These results are ordered by resistance capacity 7 days post-infection. Adjusted values by a mean test (5%) of three replicates.

Although CML-493 was the most resistant maize line, it was not used because it is a quality protein maize (QPM) line, and we neither used CML-7 maize line even though it was the most susceptible line, because its yield in tons per hectare in Venezuela is far lower than the one of CML-448 maize line.

Evaluation of studied maize lines proteomes

We decided to study the relationship between the four evaluated proteomes and determine if there is any proteome expression-pattern difference in the proteins with both types of lines, yellow and white. For this, we used a data matrix using only proteins present in at least one of the white lines and one of the yellow maize lines, resulting in 224 spots. Generalized Procrustes adjusted this data matrix to eliminate the extraction method factor and maintaining only the factors, grain color, and resistance capacity. We made a cluster analysis of the adjusted matrix, using calculating the square Euclidean distance and Ward. We demonstrate that the proteomes obtained from the two resistant lines, under infection conditions for 20 hours, are more similar than those with their same color-susceptible lines. Furthermore, the relationship between resistant lines is stronger than the relationship between susceptible lines. This demonstrates that, under infection, both resistant lines show a similar response mechanism (Figure 2).

Determination of major resistance-contributing proteins

Using these 224 spots matrices, we made a PCA to determine the proteins (variables) that majorly contribute to each maize line's centroids position and, consequently, to resistance capacity. In this way, we determined that 66 proteins majorly contribute to resistance (figure 3). In this figure, we show the 66 proteins spot number, separated by colors where spots in green are the spots that majorly contribute to the YR line position, in blue are the spots that majorly contribute to the WR line position, and in red are the spots that majorly contribute to the position of both resistant lines. We then decided to identify these significant resistance-contributing proteins.

Protein identification by peptide-mass fingerprinting and sequencing

MALDI-TOF MS. carried out identification of the two-fold up or down-regulated proteins. Selected spots were subjected to in-gel tryptic digestion. MALDI-TOF MS analysis and mass spectra were searched against the protein sequence databases. 47 of the 66 selected spots were identified by peptide mass fingerprinting (PMF) (Table 2). Two to three peptides were sequenced from each spot for protein identification. Nineteen proteins remained unidentified, some of them because the homology score was too low to consider it as that protein, others because they did not show significant homology to any known protein sequence. This is an exciting matter for further investigations. We consider it essential to remark some of the identified proteins, like NADPH HC toxin reductase (NHCTR), whose function is to inactivate HC toxin, a cyclic tetrapeptide *Helminthosporium carbonum*, by carbonyl group reduction[24]. Hm1 was the first considered resistance gene cloned and evaluated, proving the toxin's encoded protein activity[25]. HC toxin is a potent histone deacetylase inhibitor[26]. Histone deacetylase eliminates histones, acetyl groups, transforming the lysine and arginine amino groups into amides, diminishing their DNA affinity, allowing separation and posterior transcription. Thus, HC toxin inhibits the separation between histones and DNA, inhibiting protein transcription, probably resistance proteins[27]. At the transcriptional level, we determined an overexpression in the resistant lines after infection, exceptionally the white resistant (WR) line that reaches higher relative-expression values. Besides, there are no significant changes in the white susceptible (WS) line after infection, and we could not detect any expres-

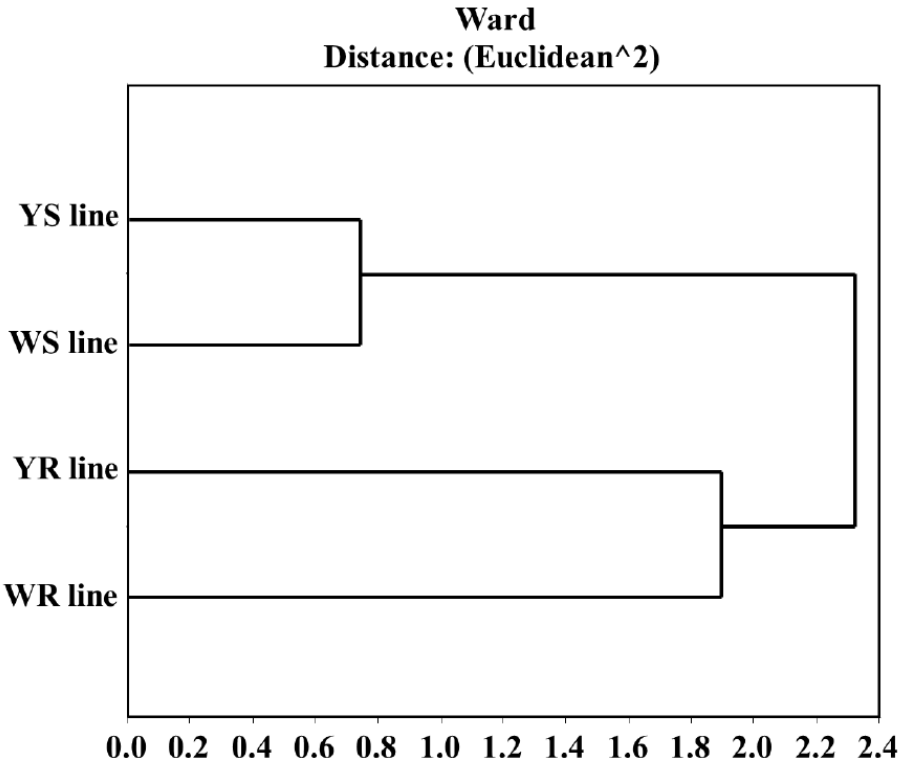

Figure 2. Cluster diagram of the four studied lines inoculated (20h) with A. flavus. This was made using the 224 spots detected in at least one yellow line and one white line. It shows a stronger relationship between the resistant lines' proteome with each other than between maize-grain color. Results obtained with InfoStat v 2012.

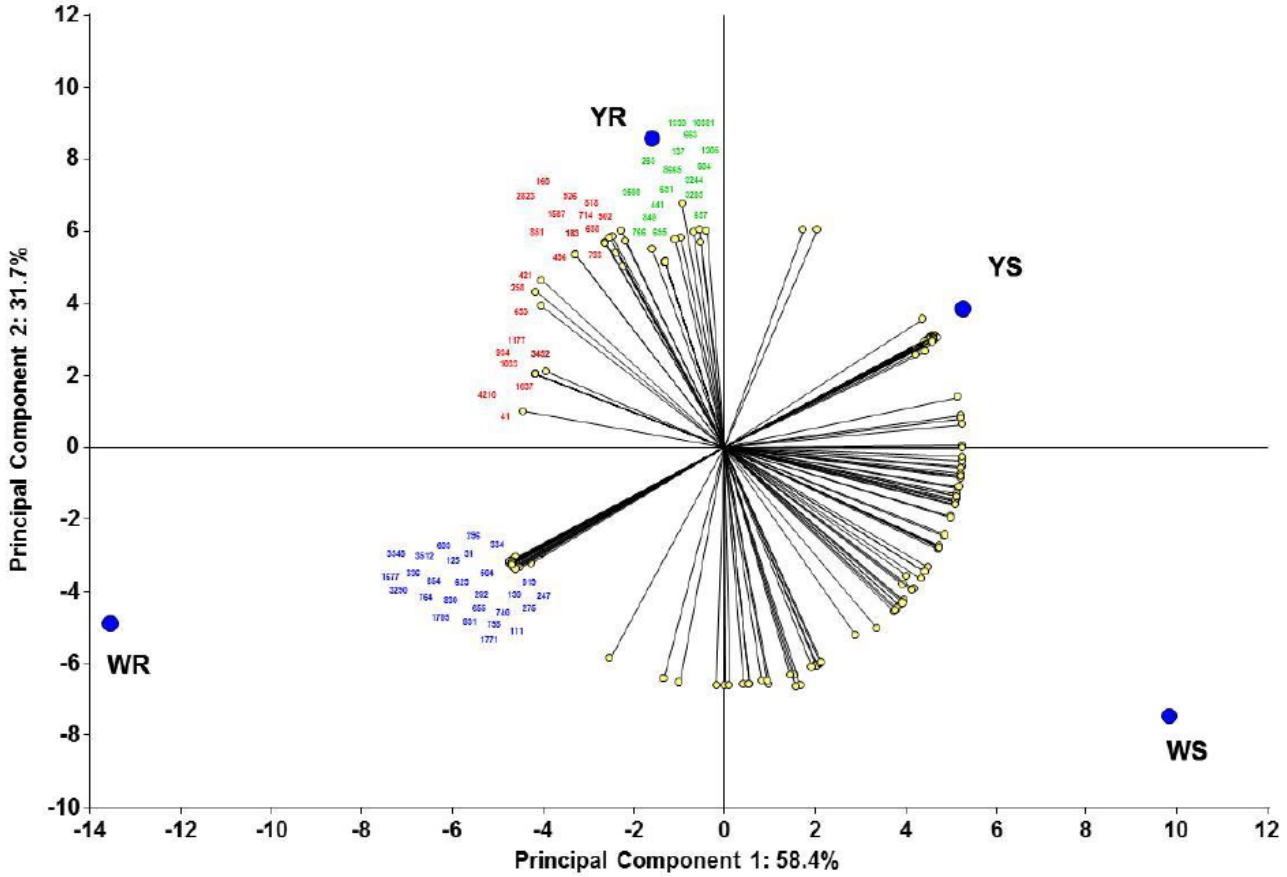

Figure 3. Principal components analysis to determine resistance-contributing proteins. Statistical analysis made using InfoStat v2012[23].

sion level in the yellow susceptible (YS) line (data not shown or see supplementary data or figure S1). This protein showed an overexpression on white-grains resistant-maize line with respect to the susceptible one at the translational level and was not detected in yellow lines.

NADPH HC toxin reductase overexpression in WR line was detected in this work, a fascinating result, because it is known that this enzyme responds almost exclusively to the fungus *Helminthosporium carbonum*, due to a coevolution phenomenon between the two organisms[28]. This result indicates that *A. flavus* somehow stimulates maize grains response mechanisms, similar to those activated by *H. carbonum*, perhaps because of a similar infection mechanism. In *H. carbonum*, the toxin synthesis depends on a complex locus of five genes that encodes, among other things, promoter proteins and HC-toxin synthase (Genbank: M98024.2)[29]. We compared the HC-toxin synthase sequence of *H. carbonum* with *A. flavus* (taxid:5059) and found a mRNA sequence coding for a protein with 97% of homology NRRL3357, nonribosomal peptide synthase Pes1 (XM_002380451.1). This suggests the existence of a similar toxin-synthesis mechanism between the two fungi, which provokes an overexpression of NADPH HC toxin reductase. This work is the first report of overexpression of this enzyme caused by *A. flavus*.

We also detected four proteins involved in the shikimate pathway, phospho-2-dehydro-3-deoxyheptonate aldolase 2 (PDDA2), myb-like transcription factor (myb-P), chalcone synthase (ChS), and α-1,4-glucan-protein synthase (α-GS), which describe their importance in the resistance phenomena. Several proteins involved in the catabolism, phosphoglycerate kinase (PGK), xylose isomerase (XI), soluble inorganic pyrophosphatase (SIPP), glyceraldehyde 3-phosphate dehydrogenase cyt 1 (GAPDHc1), fructose bisphosphate aldolase (FBA), are required for energy production due to the high demand to face the pathogen.

Additionally, we detected chaperone proteins, motor proteins, ATP synthesis proteins and transmembrane transporters, and some hypothetical and unknown functional proteins.

Response model of a maize-kernel cell against *A. flavus* at 20 hours infection

All the information gathered was used to develop a response model that describes the processes that we consider provide the highest corn-kernels resistance-levels against *A. flavus*. The ORFs whose role is not confirmed yet, were not included in the response model. Figure 4 shows the model which summarizes what occurs within the cell, including proteins reported in other studies[19,30–37]. It can be noted that an immediate response mechanism takes place (left side of the model), where an overexpression of several proteins responsible for reactive-oxygen species (ROS) production occurs. These ROS are involved both in the direct attack and in cell damaged wall repair, but they are highly toxic for host cells, because they cause membrane pores, protein damage and conglomeration among other events. Therefore, the activation of proteins related to ROS degradation can be expected as was observed. Chaperone proteins that protect the native proteins folding were also detected, and the subexpression of proteins related to the degradation of other proteins indicates that in resistant-plants maize-grains, the protection of proteins already synthesized is favored instead of protein degradation and resynthesis, which supposes lower energy cost. We found that energy is not wasted unnecessarily. However, energy requirements increase proportionally because of infection, which sti-

Spot N°	Protein name	Mass (kDa)/pI		Score	Coverage (%)	Acc. N°	Specie	Resistant vs. Susceptible	
		Theoretical	Experimental					Yellows	Whites
926	A49-like RNA polymerase I associated factor family	8,9/ 49,070	7,96/ 30.573	68 (a)	33	EU968493.1	*Zea mays*	0	+2,644±0,18
496	Acetolactate synthase1	5,8/59.730	5,9/63.590	68 (a)	19	NP_001151761	*Zea mays*	+2,746±0,26	+1,422±0,03
275	Alcohol deshydrogenase 1	6,3/ 40.904	6,43/ 43.156	96 (a)	37	AAF43977	*Zea mays*	+2,01±0,06	+2,21±0,08
3244	Aldose reductase	6,2/ 31.131	7,95/ 58.066	149 (c)	30	ACG28331.1	*Zea mays*	+2,5280,06	0
3285	Aldose reductase	6,2/ 31.131	8,1/ 28.106	209 (c)	44	ACG28331.1	*Zea mays*	+5,292±0,23	0
830	Alpha-1,4-glucan-protein synthase	5,8/41.691	5,7/38.194	55 (a)	14	NP_001105598	*Zea mays*	R	R
831	Alpha-1,4-glucan-protein synthase	5,8/41.691	5,7/38.840	62 (a)	23	NP_001105598	*Zea mays*	0	R
714	Aquaporin PIP1-5	8,3/30.933	8,2/34.743	58 (a)	25	NP_001105131	*Zea mays*	+2,134±0,18	0
658	ATP synthase β chain	5,9/59.057	5,2/51.793	58 (a)	17	NP_001151807	*Zea mays*	+1,082±0,16	+2,032±0,12
137	ATP synthase CF0 A subunit	5,3/27.334	5,4/28140	56 (a)	28	NP_043019.1	*Zea mays*	-1,017±0,19	-2,25±0,26
111	Chalcone synthase C2	6,7/41.588	6,4/41.547	66 (a)	21	NP_001142246	*Zea mays*	-1,166±0,04	-2,208±0,03
818	DIBOA-glucoside dioxygenase BX6	6,2/41.628	6,1/40.240	80 (a)	26	NP_001105100	*Zea mays*	+3,761±0,10	+2,005±0,01
296	Fructose-bisphosphate aldolase, cytoplasmic isozyme	7,5/39.036	7,6/41.348	61 (a)	21	NP_001105336	*Zea mays*	0	+2,677±0,13
629	Glutathione transferase	6,2/ 25.119	6,3/ 25.602	78 (a)	47	CAA73369	*Zea mays*	-4,242±0,61	+2,698±0,31
258	Glyceraldehyde-3- phosphate dehydrogenase, cytosolic 1	6,5/36.600	6,4/36.347	52 (a)	18	NP_001105413	*Zea mays*	+2,123±0,02	+3,005±0,43
31	Heat shock protein 70	5,3/63.193	5,2/69.705	42 (a)	15	CAA27330	*Zea mays*	-2,008±0,16	+2,288±0,04
41	Heat shock protein 82	5,0/82.125	5,1/75.398	68 (a)	21	NP_001135416.2	*Zea mays*	-2,946±0,13	0
125	thioredoxin peroxidase (Hypotetical protein OsI_01349)	6,11/ 24.167	5,9/ 28.858	38 (a)	26	EEC70391.1	*Oryza sativa* (indica)	+4,194±0,66	+3,507±0,60
8665	Hypothetical protein Loc100276485	5.5/49.741	5,6/24731	47 (a)	21	NP_001143733.1	*Zea mays*	-2,189±0,08	-4,218±0,11
421	Kinesin heavy chain	8,09/ 17.317	8,15/ 15.427	46 (a)	35	AAK91823.1	*Zea mays*	+2,538±0,04	0
3508	Kinesin heavy chain	8.09/ 17.317	8,35/ 15.141	44 (a)	33	AAK91823.1	*Zea mays*	+3,129±0,04	+1,538±0,18
3512	Kinesin heavy chain	8.09/ 17.317	7,95/ 15.281	53 (a)	32	AAK91823.1	*Zea mays*	+6,934±0,24	0
334	Lon protease homolog 2, peroxisomal	7,7/97900	7,6/100.212	58 (a)	18	NP_001105903	*Zea mays*	+1,683±0,04	-2,032±0,04
336	Lon protease homolog 2, peroxisomal	7,7/97900	7,7/100.212	65 (a)	20	NP_001105903	*Zea mays*	+1,281±0,05	-2,968±0,31
603	Myb-related protein P	9,9/17.332	9,3/17.817	49 (a)	32	P27898-2	*Zea mays*	+3,856±0,28	+5,255±0,62
733	NADPH adrenodoxin oxidoreductase	7,31/ 54.533	7,10/ 50.178	46 (a)	36	ACG45856	*Zea mays*	0	-2,046±0,06
247	NADPH HC toxin reductase	5,45/ 27.100	7,25/ 26.588	33 (a)	34	AAM78363	*Zea mays*	0	+2,890±0,07
654	NADPH-dependent oxidoreductase	7,6/ 40.581	6,3/ 25.135	44 (a)	34	NP_001149289.1	*Zea mays*	-1,099±0,09	+2,182 ±0,80
653	Phospho-2-dehydro-3-deoxyheptonate aldolase 2	6,2/ 21.892	5,35/ 26.287	76 (a)	25	EU974270	*Zea mays*	-1,223±0,26	+2,252±0,38
504	Phosphoglycerate kinase	5,65/ 42.470	5,50/ 45.746	40 (a)	35	NP_001142404	*Zea mays*	+1,466± 0,14	+3,241± 0,38
740	Phosphoglycerate kinase	5,65/ 42.470	5,30/ 46.061	43 (a)	37	NP_001142404	*Zea mays*	+1,180± 0,01	+2,380±0,26
1567	Proteasome subunit alpha type 3	5,9/ 27.535	5,73/ 27.816	48 (a)	35	ACG48183	*Zea mays*	-5,920±0,87	S
441	Serine acetyltransferase 1	6,4/33.902	7,9/37.418	33 (a)	18	ACG35209.1	*Zea mays*	-1,623±0,10	-2,032±0,09
657	Soluble inorganic pyrophosphatase	5,3/24.524	5,5/25.133	55 (a)	25	NP_001104889	*Zea mays*	-1,676±0,16	-2,92±0,11

Table 2. Summary of the proteins identified by MS from fungal-infected grains by both buffers.

663	Soluble inorganic pyrophosphatase	5,3/24.524	5,6/24.561	55 (a)	23	NP_001104889	*Zea mays*	-1,307±0,07	-4,355±0,05
764	Superoxide dismutase [Cu-Zn] 4AP	6,640/ 15.071	5,60/ 17.071	165 (c)	28	P23346.2	*Zea mays*	0	+3,944±0,21
766	Superoxide dismutase [Cu-Zn] 4AP	6,640/ 15.071	6,03/ 17.488	631 (c)	35	P23346.2	*Zea mays*	-38,253±2,98	+4,370±0,40
263	Uncharacterized protein LOC100191853	6,43/ 39.826	6,62/ 35.839	53 (b)	39	NP_001130749	*Zea mays*	+13,731±1,34	+2,638±0,26
292	Uncharacterized protein LOC100216703	9,1/ 39.743	9,1/ 45.311	35 (a)	33	NP_001136580	*Zea mays*	0	+3,642±0,06
819	Uncharacterized protein LOC100273276	5,4/ 43.226	5,4/ 42.889	61 (a)	37	NP_001141189	*Zea mays*	-16,559±1,27	+1,276±0,14
655	Uncharacterized protein LOC100383799	9,08/ 39.744	6,4/ 25.091	55 (a)	61	NM_001176433.1	*Zea mays*	0	+3,0±0,16
502	Unknown protein ACL54769.1	5,8/40.462	5,6/41.564	67 (a)	23	ACL54769	*Zea mays*	+3,635±0,24	+1,633±0,04
691	T-complex protein 1 subunit delta (TcP1Sδ).	6.78 / 57.733	5,8/ 58.243	869 (c)	43	ACN26238.1	*Zea mays*	-46,14±1,56	-4,91±0,09
695	Xylose isomerase	5,4 / 54.381	5,73/ 52.663	37 (a)	32	ACG35698	*Zea mays*	-3,202±0,07	-2,552±0,63
130	Ypt homolog1	5,60/ 23.311	5,10/ 27.228	44 (a)	42	NM_001112076.1	*Zea mays*	0	+2,178±0,06
840	Ypt homolog2	5,80/ 22.400	5,69/ 26.010	37 (a)	61	NP_001105441.1	*Zea mays*	+1,371±0,27	+4,044±0,06
758	Zein-beta precursor	8,1/19.916	7,5/17.563	39 (a)	38	NP_001105739.1	*Zea mays*	R	+2,968±0,22

Table 2. Summary of the proteins identified by MS from fungal-infected grains by both buffers.

mulates an increase of catabolic-proteins expression and an increase of ATP synthesis in chloroplasts and mitochondria, and an energy waste necessary for proteins synthesis involved in defense against ROS species, as well as of proteins involved in synthesis of molecules used for direct attack and in cell wall-repair and reinforcement, both in affected cells and in neighbor ones, which stops the infection advance. Our study differs from others where the point of view is from the fungus and not from the maize's response[38,39].

Statistical analysis for correlating proteome with resistance capacity

All original data matrices of the proteins with differential expression patterns were analized to correlate spots present in both yellow and white grain maize obtained by both buffers, so we regrouped all information, making irrelevant the extraction buffers just focus on grain color and resistance capacity. This was accomplished by transformation to presence/absence (binomial), and we applied generalized Procrustes methodology. Distance matrices generated were submitted to PCoA (figure 5). In this figure we noted that due to the matrices characteristics, 266-483 variables (spots) depending on the evaluated matrix and only in 4 cases (white resistant line proteome, white susceptible line proteome, yellow resistant line proteome, and yellow susceptible line proteome) PCoA could collect 97.6% of the information in the two firs axis, noticing that in axis X is the information concerning the grain color and in Y axis, the resistance capacity (figure 5). We can also observe that due to their distances from the centroid to the Y axis, WS (CML-448) and WR (CML-397) are the most susceptible and the most resistant lines, respectively, which corroborates the results of the modified KSA assay (table 1).

We think that the PCoA generates a statistical plane used as a predictive model of resistance capacity against *A. flavus*.

Testing the predictive statistical model of resistance

To evaluate the proposed statistical predictive model, we perform a principal coordinates analysis including not only the studied maize lines proteomes behavior, but also by creating two theoretical maize lines, a theoretical white resistant line (ThWR) which has the spot behavior of WR plus the protein patterns identified in YR that could bring resistance but absent in the WR line (figure 6A); and a theoretical yellow resistant line (ThYR), which has the spot behavior of YR plus the protein patterns identified in WR, that could bring resistance but absent in the YR line (figure 6B). Specifically, for the ThWR we added to its theoretical proteome, the behavior of the two isoforms of aldose reductase, Hypothetical protein Loc100273276 and aquaporin PIP1-5 obtained from yellow lines. For the ThYR case we added to the theoretical proteome, the behavior of NADPH HC toxin reductase, Phospho-2-dehydro-3-deoxyheptonate aldolase 2, the isoforms of Lon protease homolog 2, peroxisomal, NADPH adrenodoxin oxidoreductase Fructose bisphosphate aldolase, the isoforms of Phosphoglycerate kinase, ATP synthase β chain and the HSP70 obtained from the white lines. Statistical analysis demonstrates two important aspects; in first place, the metabolic model of maize kernel-cell response to the infection of *A. flavus* is very accurate, and in the second place, they corroborate the predictive power of the model developed in this work. Although ThWR receives no statistical gain in resistance when we incorporate the factors absent from it but present in YR, it demonstrates the predictive power of statistical analysis developed since the ThWR is located towards the white lines. For the case of the ThYR, we can see that the line remains located on the side of the yellow lines, which confirms the capacity of the predictive statistical model. Furthermore, by ThYR location in coordinates plane, we can infer that this hypothetical line would be more resistant than its original line (YR), which corroborates the contribution of the protein's behavior obtained from WR to resistance.

We decided to make a final test of the model incorporating the proteome behavior obtained from the other two pre-selected maize lines (CML-46 and CML-247) at the same time (figure 7). In these results, we can see that both lines locate not

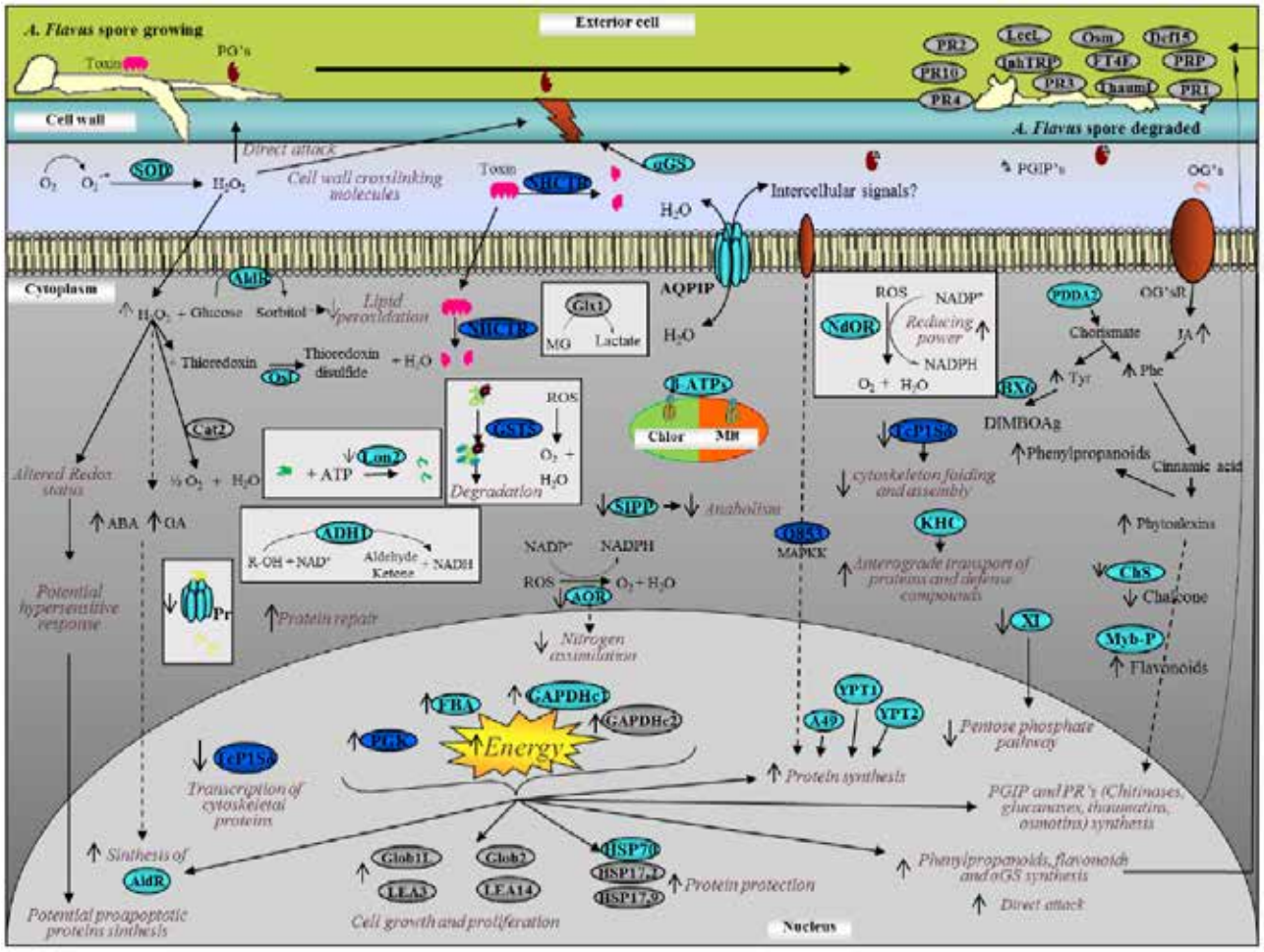

Figure 4. Response model of maize kernel cell against *A. Flavus* at 20 hours post-infection. The model was constructed using the characteristics that we consider to provide the highest maize kernel cells resistance against infection with *A. flavus*. In gray, we show the most relevant proteins reported; in light blue, proteins with only their translational behavior; and in dark blue, proteins with transcriptional and translational behavior.

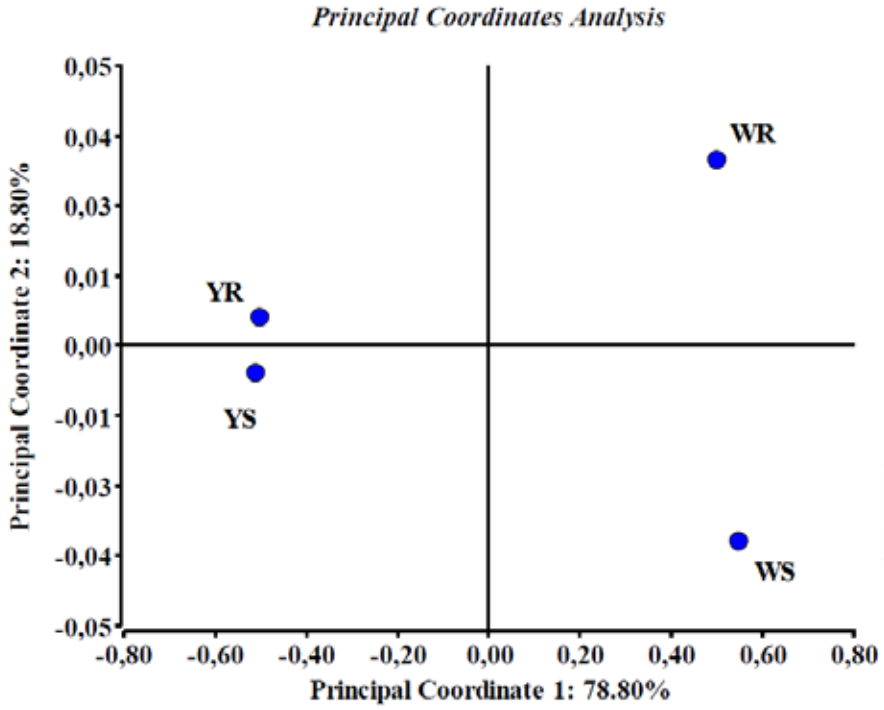

Figure 5. Principal coordinate analysis of the studied maize lines proteomic expression patterns. Points are centroids of each studied maize line. Note that in axis X is the information concerning the grain color and in Yaxis, the resistance capacity. Results obtained with InfoStat v 2012[23].

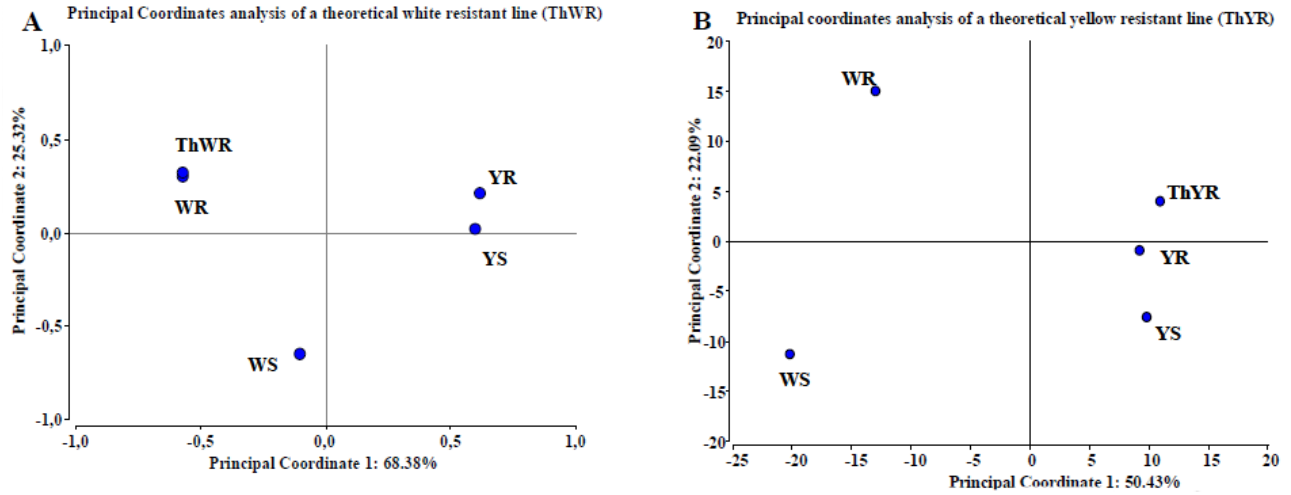

Figure 6. Test of PCoA predictive model using theoretical white (A) and yellow (B) maize lines. In (A) we observe no gain of resistance of the ThWR line than WR line; the analysis collects 93.7% of the information. In (B) we observe a real gain of resistance capacity of ThYR line compared to YR line when we include the behavior of some proteins from WR line that are absent in YR. The analysis collects 72.52% of the information. Results obtained with InfoStat v 2012[23].

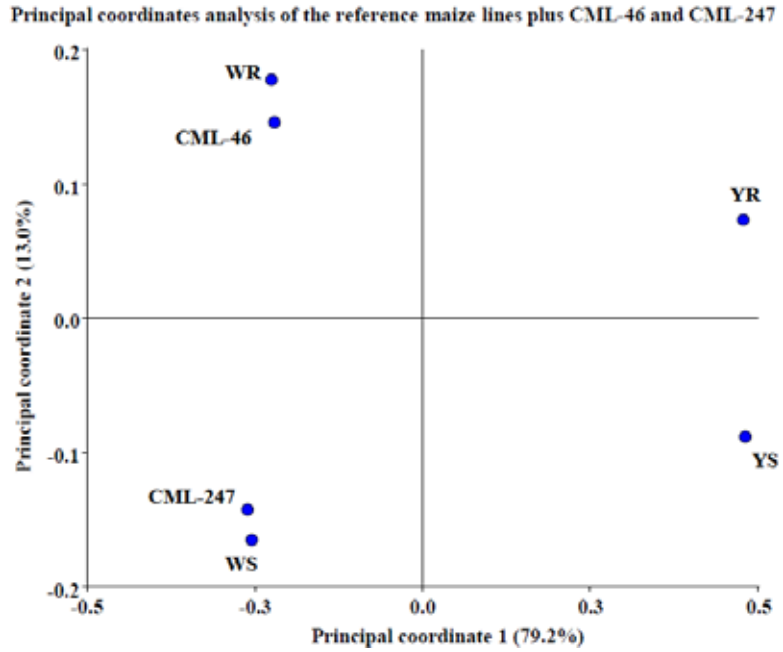

Figure 7. Test of PCoA predictive model using two more maize lines proteomic behavior. We observe that both lines locate not only on the white side of the coordinates plane but also on the coordinates plane, corroborating the statistical analysis predictive capacity. In two planes, the analysis collects 92.2% of the information. Results obtained with InfoStat v 2012[23].

only on the white side of the coordinates plane, but also their position concerning Y axis, which is the axis that collects the information relative to resistance capacity. This corroborates the statistical analysis predictive capacity and demonstrates its strong relationship with KSA results (table 1). KSA results reported CML-46 line to be slightly more resistant than YR (CML-409), but not as much as WR (CML-397) line, and on the other hand, CML- 247 was reported to be more susceptible than YS (CML-497) but less than WS (CML-448). These are the same results obtained by proteomics and PCoA analysis developed in this work.

Conclusions

This work exhibits a global view of the interrelations between the maize-kernel cell-proteins that occurs in response to infection with *A. flavus*, in an easy-to-understand response model of an individual cell. Besides, with the statistical analysis developed, it was possible to determine which proteins contribute to resistance capacity. Principal coordinates analysis allowed us to generate a statistical plane that can be used as a predictive method of other maize lines' resistance capacity. Cluster analysis showed that the proteomes obtained from the two resistant lines under infection conditions are more similar between each other than between susceptible lines. Furthermore, this relation is closer than the relationship between susceptible lines. This demonstrates that under infection, both resistant lines show a similar response mechanism. This work is the first known report of overexpression of NADPH HC toxin reductase stimulated by *A. flavus* infection. The presence of a similar enzyme to HC toxin synthetase from *H. carbonum* in the genome of *A. flavus*, detected by sequence homology, suggests the existence of a similar toxin and corroborates this result. We finally present an overview of the response mechanism of maize kernels to infection with A. flavus, and propose the use of these statistical methods of resistance capacity determination, using proteins expression pattern for a recurrent selection process to choose the most resistant maize lines after each sowing event, always preserving the required productivity levels.

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