Article

Pathogenicity of *Ustilago maydis* strains for production under controlled conditions

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Abstract

The wide pathogenic variability in strains of *Ustilago maydis* allows evaluation in hybrid and creole maize seeds for the production of huitlacoche. Therefore, the objective in this study was to evaluate the pathogenic capacity of *U. maydis* strains in the state of Aguascalientes, Mexico during the period 2014-2016 in hybrid and creole maize to produce huitlacoche under conditions controlled by the artificial inoculation method. Evaluating four strains of Aguascalientes and two witness strains originating in the State of Mexico, in three hybrids and three creole, con a completely random experimental design with three repetitions in 6x4 factorial arrangement. Thus, obtaining significant differences between commercial varieties and the three crosses evaluated, where cross 2 presented the highest pathogenicity with 59.6% (native) and 76.9% (hybrids) for severity index, in incidence with 82.2% (native) and 93.3% (hybrids), for grams per cob infected with 73.9 g (native) and 134 g (hybrids) and for yield per hectare it was 4.1 t ha^{-1} (native) and 7.7 t ha^{-1} (hybrids). The hybrid A7573 presented the highest susceptibility for the three crosses evaluated. Thus, concluding that the strains of the state Aguascalientes present pathogenicity, being a good option for the production of huitlacoche under controlled conditions.

Keywords: creole, greenhouse, hybrids.

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Introduction

The fungus *Ustilago maydis* [(D.C.) Corda] is a basidiomycete fungus known in the world to cause the disease called common maize (*Zea mays*) coal, this infection leads to the formation of large tumors that when developed on the cob are known as 'huitlacoche' or 'cuitlacoche' and are used in the preparation of different dishes (Ruíz and Martínez, 1998; 2001; Pataky and Chandler, 2003).

In Mexico, huitlacoche has been used since pre-Hispanic times as an exquisite food; however, the unattractive appearance of the disease in maize has attracted the attention of national and international diners, giving it added value (Bernal and Ramírez, 2011). In addition to noting that this fungus predominates glucose and fructose, it also contains carbohydrates, proteins, fats, minerals and vitamins such as riboflavin, niacin, biotin, and folic acid that constitute its nutritional value (Kealey and Kosikowski, 1981; Lizárraga, 1995).

Huitlacoche occurs in almost all maize-producing regions, although favorable climatic conditions for its development are very varied, some authors claim that the attack of this pathogen may be more severe in humid environments (relative humidity of 72-80%) temperate (temperature of 17-20 °C) (Villanueva *et al*., 1999; Martínez *et al*., 2005), other authors indicate that the attack is more common in warm areas (temperature of 26-34 °C) and moderately dry (Agrios, 2005).

The infection forms a dikaryon filament because of mating interactions. This process is controlled by *locus a* and *b*. The *locus a* regulates the formation of mating filaments or conjugation tubes, the transcription of proteins encoded by *locus b* comes into play after fusion to maintain the filamentous growth of infectious dikaryon and to promote the development of pathogens in the plant.

One aspect of mating in *U. maydis* is that there are multiple specificities in *locus b* with estimates ranging from 25 to 33 different types (Banuett, 1992; Banuett, 1995; Kronstad, 2003). *U. maydis* presents a genetic variability in the virulence of the fungus, this has a lot of lines or biotypes with different physiological and pathogenic characteristics. The main characteristic of this fungus is to be heterothallic which causes new hybridization biotypes to emerge in each new sexual generation, so a single damage is possible to isolate a range of haploid lines that differ in their pathogenicity.

It has also been shown that the growth index, color, spore production of colonies and other characteristics in culture medium can be modified by environmental fluctuations such as temperature, nutrition, vitamins, growth substances and chemical agents (Martínez *et al*., 2000; Martínez *et al*., 2005).

Current breeding programs are based on natural coal infection in field plots and removal of the most susceptible improvement lines in order to maintain blight resistance; however, numerous reports are available in the influence of the various inoculation techniques, the host development stage and environmental conditions for pathogenesis (Kealey and Kosikowski, 1981; Pataky, 1991).

In the artificial production of huitlacoche there is the artificial inoculation technique on commercial hybrids of sweet maize and open pollination varieties (Martínez *et al*., 2000) this technique has been developed since the XVIII century (Walter, 1935). In different parts of Mexico, the pathogenicity of some strains of *U. maydis* and the susceptibility and resistance in maize varieties have been evaluated; however, for the state of Aguascalientes there is no information on the pathogenicity of wild strains, as well as susceptibility and resistance in maize varieties adapted in the estate.

Therefore, the objective of this research was to evaluate different strains of *Ustilago maydis* from Aguascalientes state to infect maize plants under controlled conditions with the artificial inoculation method.

Materials and methods

Location of the experimental site

Greenhouse of 80 m^2, located in the Center of Agricultural Sciences of the Autonomous University of Aguascalientes; Jesús María, Aguascalientes (21.9668018, -102.3676961, 1 880 meters above sea level). During the period of infection, the controlled conditions within the greenhouse, was a temperature between 26-32 °C and relative humidity around 70%.

Plant material

Three strains of *Ustilago maydis* of creole maize and three maize strains of commercial varieties (AS822 [Aspros], A7573 [Asgrow] and H-383 [INIFAP]).

Crossing of the strains

Laboratory of Biotechnology and Food Functionality of the Center for Basic Sciences of the Autonomous University of Aguascalientes. 27 strains collected in different municipalities of Aguascalientes state were isolated and crossed through the Guevara (1999) procedure during the period 2014-2016. Subsequently, 4 pairs of strains from the state of Aguascalientes and 2 pairs of strains from the State of Mexico were chosen (Table 1) for inoculation in maize plants. The inoculum was established at a concentration of $10x10^6$ sporidia for each of the pairs of strains.

Table 1. Strains to evaluate in greenhouse with artificial inoculation method.

Crosses	Strains	Locality	Municipality	State
1	1B	Providence	Aguascalientes	Aguascalientes
	3B	UAA Posta	Jesús María	
2	13B	Francisco Villa	Asientos	
	16B			
3 Witness*	ch1			Mexico
	Q318			

*= cross of strains checked for infection in maize plants.

Experimental design

360 black bags were placed with approximately 15 kg of soil each in five double grooves with 36 pots each, the experimental design was completely random with three repetitions in 6x4 factorial arrangement. Factor A were maize varieties: Creole 1 (C1), Creole 2 (C2), Creole 3 (C3), AS-822, Aspros (AS), A-7573, Asgrow (AW) and H-383, INIFAP (IP) (Table 2). Factor B was cross 1, cross 2, cross 3 and without inoculating (Table 2).

Table 2. Varieties of maize used in greenhouses.

Characteristic	Creole 1 (C1)	Creole 2 (C2)	Creole 3 (C3)	AS822, Aspros (AS)	A7573, Asgrow (AW)	H-383, INIFAP (IP)
Locality	Paredes*	Ejido del Bajío*	Ejido Peñuelas*	Certified seed		
Municipality	San José de Gracia	Rincon de Romos, Ags.	Aguascalientes			
State of origin	Aguascalientes	Zacatecas	Aguascalientes	Jalisco	Bajío	Aguascalientes
Hybrid type	-	-	-	Triple	Triple	Simple
Cycle	-	-	-	Intermediate	Early intermediate	Intermediate
Planting conditions	Temporary	Temporary	Temporary	Good Temporary Irrigation	Irrigation	Good temporary Irrigation
Flowering days	75-80**	80-85**	80-85**	77-80	60-70	80-85
Days of maturity	130-136**	115-120**	115-120**	142-148	100-110	110-115
Plant height (m)	2.3-2.5**	1.3-1.5**	2-2.1**	2.15-2.25	2.1-2.5	3
Cob height (m)	1.9**	90-110**	1.4-1.5**	1.4-1.55	1.1-1.5	-
Grain type	White	White	Yellow	Semi-jagged white	White	Semi-jagged white

*= seed collection location. **= data obtained in the field and/ interview with the producer, all are approximate data.

Germination and inoculation

The seeds were placed in running water for 24 hours before planting to promote early germination. four drip irrigations of 15 min each were scheduled at the germination stage for 15 days, irrigation was suspended for 15 days and restarted in the vegetative stage with the same programming, once the baby corn was inoculated it was increased to six irrigations of 15 min each.

The inoculation was performed with a hypodermic syringe, injecting 2 ml of inoculum corresponding to the treatment, was inoculated by the channel of the styles when they had a length of 1 to 5 cm and before pollination occurred. After inoculation, the management of maize plants was 4 irrigations per day of 15 min each. In addition to considering during the period of infection, the application of spray water (1 min a day) on the foliage, in order to increase relative humidity and promote the growth of the fungus.

Variables evaluated

22 days after inoculation, the following assessments were performed: severity index (IS): proportion of the corncob covered by galls formed by the fungus. Five severity grades were taken into account: IS1 (0%), IS2 (25%), IS3 (50%), IS4 (75%) and IS5 (100%) (Madrigal *et al*., 2010).

Incidence percentage (PI)

Number of baby corn infected with some degree of severity divided by the number of inoculated baby corn multiplied by 100 (Madrigal *et al*., 2010).

Grams per infected cob (GMI)

Total weight in grams of the shelled huitlacoche of all infected corncob divided by the number of corncobs infected by the fungus (Madrigal *et al*., 2010).

Yield per hectare in tons (RHt)

It was obtained by multiplying the density of maize population by GMI (Madrigal *et al*., 2010).

Statistical analysis

All results were analyzed using InfoStat software version 2017 for Windows and expressed as average ± deviation standard (n= 3). One-way variance analysis (Anova) was used, applying Tukey analysis ($p< 0.05$) for means with significant differences. In the same software with the average values, a principal component analysis (PCA) was carried out, to observe differences and similarities of the analyzed samples.

Results and discussion

In the severity index (IS), it can be observed that there are significant differences between treatments, where the treatments of the creole 1varieties (94.7 ±1.3), AS (90.7 ±9.3) and Asgrow (89.3 ±10.7) with cross 2 were those that had the highest values of severity index, while the treatments of the creole 2 variety with cross 2 (42.7 ±17.6) and with cross 1 (48 ±4.6) were the ones that presented the lowest values of IS. In Figure 1, huitlacoche galls formed in cob can be observed through artificial production of the different treatments evaluated.

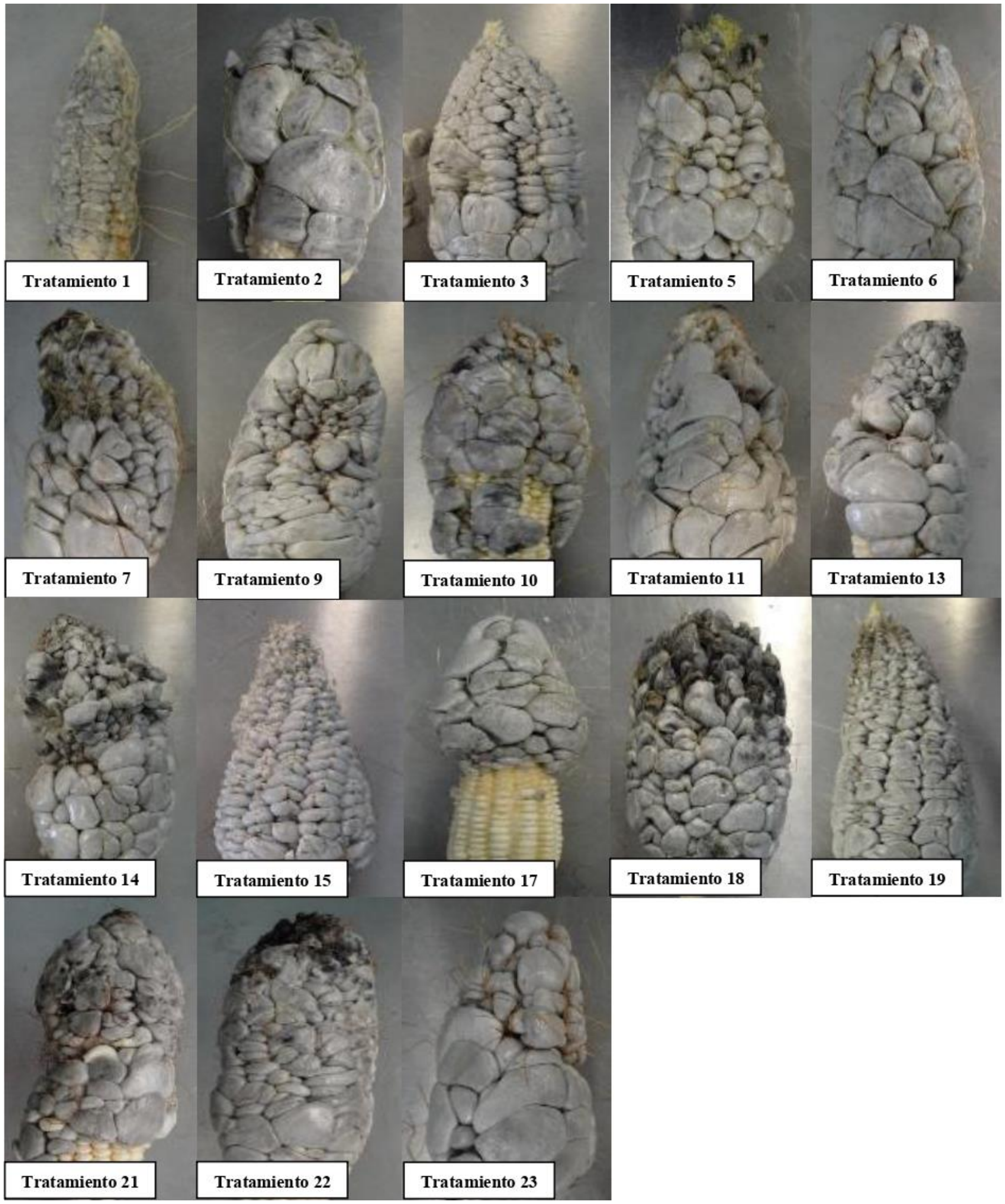

Figure 1. Huitlacoche galls in cob through artificial production of the different treatments evaluated in the period 2014-2016.

In the incidence percentage (IP), it is observed that there are significant differences between treatments, where the variety C3 with cross 2 and the variety C1 with the cross had a higher PI (100%), while the variety C2 with cross 2 obtained a lower IP (60 ±23.1) (Table 3).

Table 3. Variety-cross evaluated for huitlacoche production.

Tto.	Variety	Crosses	IS (%)	PI	GMI	RHt
1	C1	1	57.3 ±7.4ab	73.3 ±13.3ab	32.9 ±11.6de	1.6 ±0.8cd
5	C2	1	48 ±4.6b	66.7 ±6.7ab	53.1 ±16.6cde	2.3 ±0.9cd
9	C3	1	65.3 ±1.3ab	93.3 ±6.7ab	91.5 ±17.1bcde	5.1 ±1.1abcd
13	AS	1	81.3 ±7.1ab	93.3 ±6.7ab	96.2 ±16.5abcde	5.5 ±1.2abcd
17	AW	1	78.7 ±11.6ab	93.3 ±6.7ab	122 ±31abcde	7 ±2.1abcd
21	IP	1	49.3 ±7.4b	80ab	76.2 ±9.2cde	3.7 ±.4bcd
2	C1	2	68 ±6.1ab	86.7 ±6.7ab	75.5 ± 1.7cde	3.8 ± 0.4bcd
6	C2	2	42.7 ±17.6b	60 ±23.1b	51.4 ±33.1cde	2.6 ±0.2bcd
10	C3	2	68 ±6.1ab	100a	94.7 ±12.8bcde	5.7 ±0.8abcd
14	AS	2	74.7 ±4.8ab	93.3 ±6.7ab	93.3 ±12.5bcde	5.3 ±1abcd
18	AW	2	81.3 ±10.9ab	93.3 ±6.7ab	178.8 ±65.6abc	10.5 ±4.6abc
22	IP	2	74.7 ±2.7ab	93.3 ±6.7ab	129.9 ±15.3abcde	7.2 ± 0.7abcd
3	C1	3	94.7 ±1.3a	100a	131.4 ±5.9abcde	7.9 ± 0.4abcd
7	C2	3	76.0 ±4ab	80ab	159.9 ±7abcd	7.7 ± 0.3abcd
11	C3	3	74.7 ±8.1ab	86.7 ±6.7ab	165.9 ±24.6abcd	8.8 ± 1.9abcd
15	AS	3	89.3 ±10.7a	93.3 ±6.7ab	163.4 ±23.5abcd	9.3 ± 1.9abc
19	AW	3	90.7 ±9.3a	93.3 ±6.7ab	230.9 ±39.4a	13.2 ± 3a
23	IP	3	73.3 ±11.4ab	86.7 ±6.7ab	216.1 ±63.2ab	11.7 ± 4.2ab
4	C1	SN	0c	0c	0e	0c
8	C2	SN	0c	0c	0e	0c
12	C3	SN	0c	0c	0e	0c
16	AS	SN	0c	0c	0e	0c
20	AW	SN	0c	0c	0e	0c
24	IP	SN	0c	0c	0e	0c

Means with the same letters are statistically equal, according to the Tukey test ($p< 0.05$); honest significant minimum difference. Tto= treatment; IS= severity index in percentage; PI= percentage of incidence; GMI= grams per infected cob; RHt= yield per hectare in tons. SN= without inoculating (control).

In the results of the grams per infected cob (GMI) variables and in yield per hectare in tons (RHt) the behavior of the samples was the same, in both variables there are significant differences between treatments, where the treatment of the AW variety with cross 3 presented a higher yield per cob (230.9 ±39.4) and per hectare (13.2 ±3), while the treatment of the variety C1 with cross 1 presented the lowest yields per cob (32.9 ±11.6) and per hectare (1.6 ±0.8) (Table 3).

The yields obtained with the Asgro2 hybrid inoculated with cross 3 (13.2 t ha^{-1}) approximate the yields obtained by Martínez *et al*. (2000), where they report that, in experimental and commercial open pit trials, they reached a maximum production of 14 t ha^{-1}. However, cross 1with INIFAP hybrid (3.7 t ha^{-1}) and variety creole 3 with cross 1 (5.1 t ha^{-1}) are close to those reported by Madrigal *et al*. (2010) where they obtained that the hybrid 30G40 presented the highest yield with 4 786.2 kg ha^{-1}.

Although, Martínez *et al*. (2000) obtained a better yield of the fungus per infected plant (279.7 g) compared to the results of this research (230.9 g), the difference between the two results can be influenced by the varieties used in both investigations. The incubation period of the fungus varies from 12 to 25 days, in greenhouse can present a faster incubation period (7 to 16 days) and slower in the field (17 to 19 days) (Thakur *et al*., 1989; Pope and McCarter, 1992; Valverde *et al*., 1993; Carroll, 1994; Pataky *et al*., 1995).

This is consistent with what was obtained in the results for the GMI variable (Table 3) since the incubation and growth period of the fungus varied for all treatments, some had lower production and other varieties had optimal development of the fungus at 22 days of incubation.

The results obtained in creole seeds in the incidence percentage variable (PI) were 77.8-88.9%, while in the severity index variable in percentage (IS) it was between 56.9-81.8%, in turn the variable grams per infected cob (GMI) resulted in 59.2- 152.4 g and the yield per hectare in tons (RHt) was 3-8.1 t ha^{-1} (Table 3), these results vary with respect to those reported by Valdez *et al*. (2009).

Where they obtained in 15 genotypes of creole maize variations in the percentages of infection in cobs from 31 to 92% (average 72%), for the percentages of coverage of the fungus on the cob ranged from 46 to 97%, with an average value of 77% and huitlacoche weight per cob, it varied considerably on maize lines (80 to 450 g) and yields of approximately 15 t ha^{-1}.

Martínez *et al*. (2000) evaluated 100 fungus isolations in an experimental hybrid, selecting 12 of them for their vigor, presenting on average 190 g of the fungus per infected plant, and 135.24 g per inoculated plant (8.11 t ha^{-1}), severity index of 36.82 and an incidence of 70.64%.

Of the 300 families of maternal half-siblings evaluated (1998) by inoculation with the mixture of the 12 selected isolations (1997), 16 susceptible families were chosen, which had on average 154.97 g of the fungus per infected plant and 112.88 g per inoculated plant (6.67 t ha^{-1}), 76.67% incidence and 34.82 severity index, as well as 14 families resistant (0% incidence) to the fungus attack. The results obtained in IP, IS, GMI and RHt in hybrid varieties inoculated with cross 2 exceed those obtained by these authors (Table 3).

Principal components analysis (PCA)

A principal components analysis (PCA) was performed, taking into account the results obtained from all measured variables: severity index (IS), incidence percentage (PI), yield per cob (GMI) and yield per hectare in tons (RHt), to compare the main characteristics of treatments of the varieties C1, C2, C3, AS, AW and IP with crosses 1, 2 and 3.

Where, it was observed that principal component one (CP1) and two (CP2) explained 91.1% and 8.3% of the total variance, respectively. The combination of states of two-components by a multivariate ordering Biplot can explain 99.4% of the accumulated variability of variables

(Figure 1) and this was considered to be high enough for reliable differentiation between treatment characteristics. El value of the cophenetic correlation (1 000) indicated that the variable reduction made was adequate.

In Figure 2, it can be seen that the treatments more related to each variable are located closer to the arrow that represents said variables, in this way it can be observed that there is a grouping of treatments, this grouping was between the uninoculated varieties (GSN: orange oval) and those that were inoculated (GI: red oval), in this way it can be observed that the uninoculated varieties had the lowest values in all variables analyzed: IS, PI, GMI and RHt.

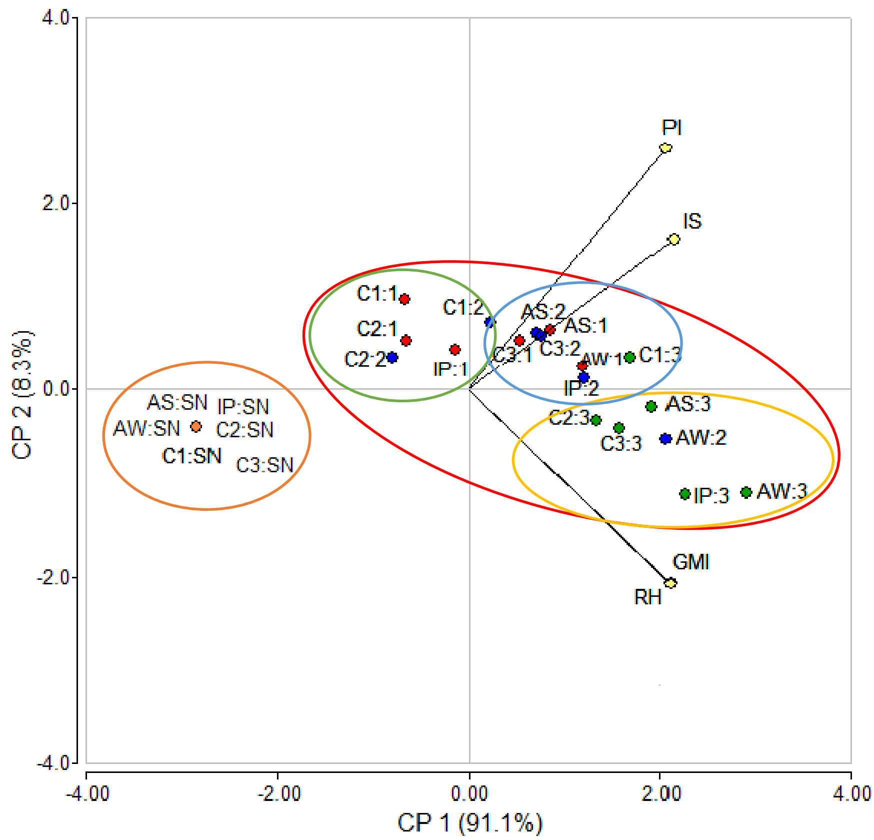

Figure 2. Biplot of multivariate ordering of treatments for the production of huitlacoche in the period 2014-2016. Orange point= uninoculated treatments; red point= varieties inoculated with cross 1; blue point= varieties inoculated with cross 2; green point= varieties inoculated with cross 3; yellow point= determined variables. Orange oval= group formed by uninoculated treatments (GSN); red oval= group consisting of inoculated treatments (GI); yellow oval= group 1 (G1), blue oval= group two (G2); green oval= group 3 (G3).

However, in the group consisting of varieties that if they were inoculated with different crosses (GI: red oval), it can also be observed that there is the formation of three groups, grouped mainly according to the cross used for inoculation.

Group one (G1: yellow oval) consists of treatments of the AW variety inoculated with cross 2 and C2, C3, AS, AW and IP varieties inoculated with cross 3, where, this group of treatments present a higher yield per cob (GMI) and per hectare (RHt), otherwise to group three (G3: green oval) consisting of the treatments of C1, C2 and IP varieties inoculated with cross 1 and C1 and C2 varieties inoculated with cross 2, which were the treatments that had the lowest values of GMI and RHt, as well as IS and PI.

However, group two (G2: blue oval) consisting of the treatments of C3, AS and AW varieties inoculated with cross 1, C3, AS and IP varieties inoculated with cross 2 and C1 variety inoculated with cross 3, was the group of treatments that presented intermediate values in all the variables analyzed, since they presented good GM1 and RH, but not larger than those of G1, while for IS and PI values, both G1 and G2 had similar values.

The crosses used are made up of only two strains for each cross, where G2 had the largest PI (93.3%) and G1 obtained the highest values for IS (84.4%), GMI (203.4 g) and RHt (11.4 t ha^{-1}). The largest infection percentage was obtained with the variety Bida-54 and the inoculum of ten strains (78.8%), followed by the inoculum with four strains in the same maize variety (52.8%). The Bengal variety, obtained an infection level of 27% and huitlacoche yield of 6.3 t ha^{-1}.

The significant differences between the results are consistent with the authors Christenses (1963); Villanueva *et al*. (1999); Pataky and Chandler (2003), where they mention that there is a pathogenic variation of the fungus, the infection is based on the ability of the pathogen, the susceptibility of the host and its interaction with the environment and that for each variety of maize there is a ratio of optimal temperatures and humidity for the development of *U. maydis.*

The strains evaluated from the state of Aguascalientes had high IS values compared to some authors mentioned, which agrees with Calderón (2010), where it reports that the IS value generated by each strain of huitlacoche is not entirely related to the variety or color of maize, indicating that even though the varieties of white maize are the most susceptible, this rather depends on the virulence of the strain.

Conclusions

The strains of the state of Aguascalientes (1B, 3B, 13B and 16B) had a severity index in high percentage, presenting a high pathogenicity and an option for the production of huitlacoche in the state of Aguascalientes. The hybrids had greater susceptibility with the three crosses evaluated compared to creole varieties. It is important to observe the behavior of each variety and with this establish a homogeneous incubation period in each variety to obtain a higher production of huitlacoche.

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