Investigation note

First report of *Monascus purpureus* in corn silage, oats, triticale and lucerne

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Abstract

Monascus purpureus is used in the food industry as a natural pigment, mainly by Asian countries; however, this fungus produces a mycotoxin called citrinin, which causes diseases such as hepatotoxicity, teratogenicity, tumors and mutations, in mammals. This fungus was found in Serbia in corn and lucerne silage. The objective of this work was to identify the presence of the *Monascus purpureus* fungus in corn, oats, triticale and lucerne silage, the sampling was carried out in the periods of November December 2017 and April-May 2018, in the states of Jalisco, Zacatecas, Aguascalientes and Guanajuato of the Mexican Republic, silage isolation was carried out in the middle of Papa Dextrosa Agar (PDA), they were morphologically and molecularly identified. 63 different strains were obtained, seven were *Monascus purpureus*, one in lucerne, two in corn, two in oats and two in triticale. This research reports, for the first time, the presence of *Monascus purpureus* fungi in corn, oats, triticale and lucerne silages.

Keywords: fungi, identification, safety.

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In the 1960s the use of silage in the livestock industry increased considerably and became the method of forage preservation that is most used for feeding cattle (Cheli *et al*., 2013), this represents between 45-60% of diets in dairy cattle production systems throughout the world, as well as in the production of beef cattle in America and Europe (Adesogan, 2009; Millen *et al*., 2009; Tangni *et al*., 2013; Alpizar, 2015), the silage can be of different crops or forages such as lucerne, oats, corn, corncob mix or beet pulp (Tangni *et al*., 2013), the objective of this is to maximize the preservation of original nutrients from the forage crop, with a minimal loss in nutritional quality (Alonso *et al*., 2013). One of the problems presented by silage is contamination by bacteria and fungi, which cause the loss of dry matter and nutrients (Garon *et al*., 2006; Alonso *et al*., 2013), causing cattle to reduce their consumption , affecting milk production, daily weight gain performance (GDP) and animal health (Alpizar, 2015).

Carrillo (2003) indicates that fungal contamination in food can be before, during and after harvest, in transport and storage, since food is permanently in direct contact with spores of toxicogenic fungi, Reyes-Velázquez *et al*. (2008); Keller *et al*. (2012); Alpizar (2015), report that the contamination of corn silage in Mexico, Argentina, Brazil, Lithuania, Switzerland, Holland, Ireland and Denmark, is caused by fungi mainly of the genera: *Mucor* spp., *Penicillium* spp., *Aspergillus* spp., *Fusarium* spp., *Alternaria* spp., *Cladosporium* spp. and *Geotruchum* spp.

While other studies in Lithuania and Brazil have reported the presence of fungi of the genus: *Aspergillus* spp., *Rhizopus* spp. and *Penicillium* spp., in clover silages, ryegrass, sorghum, triticale, oats and pasture mix (Baliukoniene *et al*., 2012; Keller *et al*., 2012). Something very similar has been reported in corn and lucerne silages, where the contaminating fungi were: *Penicillium* spp., *Aspergillus fumigatus*, *Trichoderma viride*, *Geotrichum candidum*, *Paecilomyces variotii*, *Monascus ruber* and *Monascus purpureus* (Bočarov-Stančić *et al*., 2014).

Monascus purpureus is used in the pharmaceutical and food industry as a natural dye in Eastern culture and in Asian countries. This fungus grows at a temperature of 25 to 37 °C, with a maximum of 45 °C, it can grow in a wide range of pH, from 2.5 to 8 (Pineda-Insuasti *et al*., 2016). It is capable of producing citrinin, a mycotoxin that affects the kidney, but other target organs have also been reported, such as the liver and bone marrow, this mycotoxin is nephrotoxic, embryonic, fetotoxic, teratogenic and genotoxic causing damage to humans and animals (Flajs and Peraica. 2009; Bensassi *et al*., 2011; Wang *et al*., 2017).

The possible toxicological mechanisms of citrinin are located in the renal and hepatic mitochondria, causing atrophy in the interference of the electron transport process, in addition to the alteration of Ca^{2+} homeostasis and the generation of oxidative stress (Chia-Ding *et al*., 2014). The International Agency for Research on Cancer (IARC) classified citrinin in group 3 of carcinogens, due to limited evidence of its carcinogenicity in animals and there is no evidence for humans (Flajs and Peraica, 2009). On the other hand, Bočarov-Stančić *et al*. (2014), conducted studies on maize and lucerne silage in Serbia finding fungi of the *Monascus* genus in their samples. The objective of this research was to identify the presence of the *Monascus purpureus* fungus in corn, oats, triticale and lucerne silage.

In the periods of November-December 2017 and April-May 2018, the 'W' technique was used for the taking of the samples (Bautista and Santos, 2004), a total of 16 silage samples of corn, oats, triticale and lucerne were obtained, from four locations for each entity, Aguascalientes, Zacatecas, Guanajuato and Jalisco. With the use of the aforementioned technique, a kilogram of five points of the silage was taken, which were homogenized obtaining a total of five kilograms and a subsample of 5 g was extracted, they were made cuts of approximately 5 mm, obtaining 12 subsamples per sample, they were disinfected with a 3% sodium hypochlorite solution for one minute, rinsed three times with sterile distilled water, allowed to dry, inoculated in 20 mL petri dishes with acidified PDA medium (200 µL lactic acid 85% per liter), were incubated at 28 ±2°C for seven days.

Subsequently, the mycelial growth of the inoculated silo samples was observed; purification of each fungus was performed, in acidified PDA medium and incubating at 27 °C, during 8 days in which the reproductive structures necessary for the identification of the fungus develop. Portions of mycelium were taken with spores, stains were performed, placed on a slide where there was already a drop of the blue cotton dye and placed on the covers object, then they were observed under a microscope and identified based on the keys taxonomies of Barnett and Hunter (1998).

By means of the PCR-ITS technique, molecular identification was performed, the isolated strains were extracted with DNA using the modified Doyle and Doyle method (1990). The extraction product was run on a 1% agarose gel by electrophoresis. Subsequently, the polymerase chain reaction (PCR) method was developed for the internal regions transcribed ITS1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3'), using Taq & GO Matermix (1.5 mM $MgCl_2$, 200 µM DNTP's) following manufacturer recommendations (MP®); 0.5 µL of ITS1 at 20 µM; 0.5 µL of ITS4 at 20 µM; 1 µL of problem DNA set to 100 ng and sterile double-distilled water to volume to 15 µL.

The PCR reaction conditions were: 1 cycle of initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 95 °C for 10 seconds, 30 alignment cycles at 57 °C for 30 s, 30 extension cycles at 72 °C for 2 min and 1 final extension cycle at 72 °C for 5 min. The amplification was visualized on a 1% agarose gel by electrophoresis. The PCR product was sequenced to the Macrogen laboratory in Maryland, United States of America.

The samples of silage of lucerne, corn, oats and triticale underwent a macroscopic evaluation to identify possible abnormalities in their appearance, observing that the silages were compact and presented a hard consistency that made cutting difficult, in the evaluation tones were observed intense red as seen in Figures 1 and 2, which show similar tones to those reported for *Fusarium* spp.

Of the 16 samples obtained from the silages, 63 different strains of fungi were isolated, of which 11.1% (7/63) corresponded to the fungus *Monascus purpureus*. Seven strains of *Monascus purpureus* fungi were obtained, two in corn silage, one in lucerne, two in triticale and two in oats representing 3.17, 1.58, 3.17 and 3.17% respectively.

Figure 1. Sample of corn silage where you can see the hue of the fungus and caking of the silage.

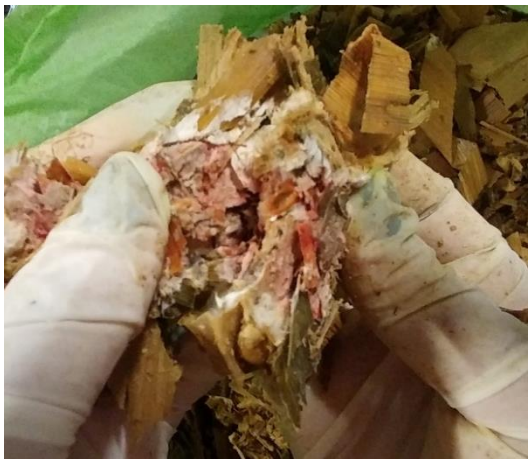

Figure 2. Sample of corn silage where the caking of the silage is observed.

Also, in the cultures that were made of each of the samples, a range of colors could be observed that were from white, cream, orange, intense red to a red color in the mycelium as observed on the obverse of the boxes Petri (Figure 3), while on the reverse the colorations were orange to red as seen in Figure 4.

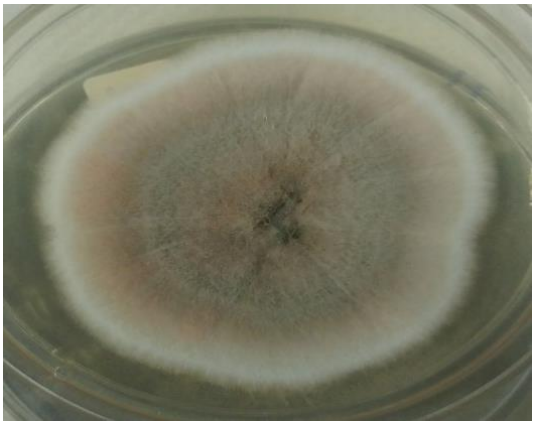

Figure 3. In the mycelium the different shades of the fungus are observed.

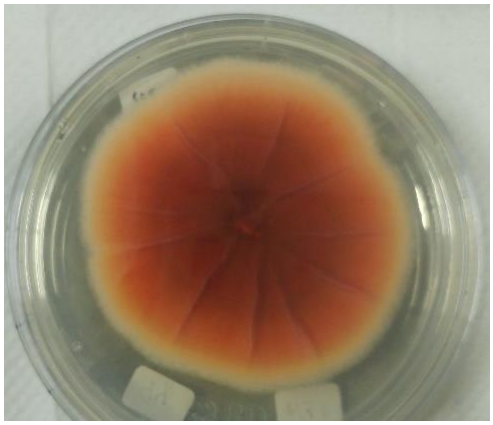

Figure 4. The intense red that characterizes the *Monascus* fungus is observed.

Microscopically, it was possible to observe ascocarp with thin walls and round conidia chains (Figure 5), in parallel, it was also possible to observe ascospores inside the ascocarp (Figure 6).

Pineda-Insuasti *et al*. (2016), indicate that the *Monascus* fungus easily grows in agro-industrial waste such as corn husk or oil palm leaf, coupled with the growth conditions: temperature, pH, humidity and concentration of nutrients (nitrogen, zinc, manganese and iron) cause the fungus to develop and spread in the field, which leads to one of the main problems of contamination of silage (Kim *et al*., 2002).

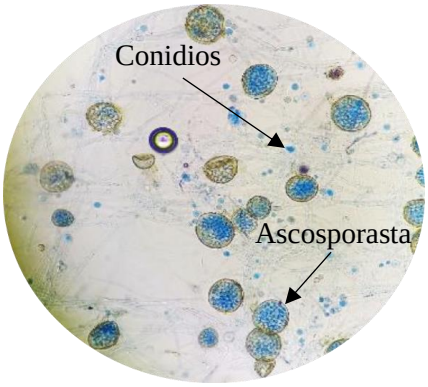

Figure 5. Microscopic view of the *Monascus* spp. fungus, the ascospore structures and conidia characteristic of this fungus (40x objective) are observed.

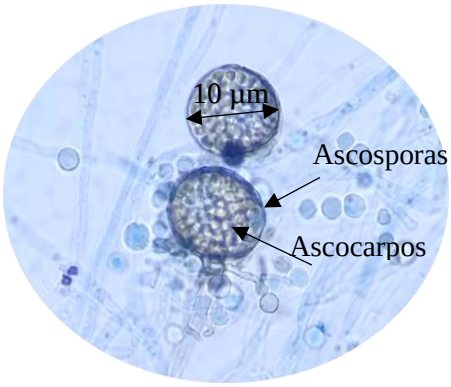

Figure 6. Microscopic view of the fungus *Monascus* spp., a better definition of the ascospore structures, ascocarp and size characteristic of this fungus (100x objective) is observed.

Bočarov-Stančić *et al*. (2014), conducted studies on maize and lucerne silage in Serbia finding fungi of the genus *Monascus* in their samples. Garon *et al*. (2006), report the presence of *Monascus* in maize silage in France, they made the sampling of maize silage in the months of November, December and February, data that coincide with our sampling of different types of silage and the presence of the *Monascus* fungus in corn, lucerne, triticale and oat silages.

The sequences obtained from the molecular analysis were compared with the sequences reported in the database of the gene bank of the National Center for Biotechnology Information NCBI of the USA (www.ncbi.nlm.nih.gov/), using the BLAST program, where it was obtained that 95.58% of the isolated samples showed similarity to the *Monascus purpureus* fungus (Table 1).

Table 1. Molecular characterization of the isolates of the sequences reported in the gene bank with the intergenic sequences (STIs) of the rDNA genes, present in the silages of lucerne, corn, oats and triticale.

Isolated	Species	Number access	IS (%)
1	*Monascus purpureus*	KY828906.1	95.58
2	*Monascus purpureus*	MK087167.1	95.58
3	*Monascus purpureus*	MK087147.1	95.58
4	*Monascus purpureus*	GQ503882.1	95.58
5	*Monascus purpureus*	MK621210.1	95.06
6	*Monascus purpureus*	MK087172.1	95.06
7	*Monascus purpureus*	MG576116.1	95.58

Isolated= 1 lucerne silage; 2, 3= corn silage; 4, 5= oat silage; 6, 7= triticale silage.

Conclusions

This research reports the presence of *Monascus purpureus* in corn, oats, triticale and lucerne silages from the states of Aguascalientes, Guanajuato, Jalisco and Zacatecas.

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