CULTURE CHARACTERISTICS, GROWTH KINETIC AND CITRININ PRODUCTION BY *PENICILLIUM CITRINUM* THOM ISOLATES RECOVERED FROM ARGENTINEAN CORN

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ABSTRACT

Citrinin production ability in a liquid medium, the macroscopic morphological characters and growth rate on agarized medium were observed on thirty-seven Penicillium citrinum *Thom isolates recovered from freshly harvested corn in the main production area in Argentina. It was shown that the percentage of strains with toxin ability production was higher among the isolates, which grew at lower rates. No correlation was found between the citrinin production ability and the macroscopic morphological characters observed. It is likely the occurrence of citrinin in the corn harvested in Argentina.*

RESUMEN

Se determinó la capacidad de producción de citrinina en medio líquido en 37 cepas de Penicillium citrinum *Thom aisladas de maíz recién cosechado en la región maicera argentina y se observaron distintos caracteres culturales macroscópicos de las colonias y se midió la velocidad de crecimiento radial en medio agarizado. El porcentaje de cepas con capacidad de producir citrinina fue mayor entre las cepas que crecieron a una velocidad menor. No se encontró correlación alguna entre la capacidad de producir citrinina y los caracteres macroscópicos observados. Es probable la aparición de esta micotoxina en el maíz en Argentina.*

INTRODUCTION

Citrinin (Figure 1) is the most common natural contaminating toxin of *Penicillium* species in cereals [1]. This mycotoxin was isolated for the first time in 1931 by Hetherington and Raistrick [2] from a liquid medium culture filtrate of *Penicillium citrinum* Thom. Citrinin was originally isolated as an antibiotic but its utility, as such, was negated due to its nefrotoxicity [3]. This toxin was associated with the endemic porcine nephropathy in the Balkans [4]. It has been suggested that this illness can be caused by the ingestion of contaminated feeds with ochratoxin A and citrinin. The renal damage observed in the affected pigs is comparable to those induced experimentally in pigs and rats [5-6]. Synergistic effects of citrinin have also been observed with other mycotoxins, like ochratoxin A and penicillic acid. Regarding the occurrence of this mycotoxin, it has been found as natural contaminant of wheat, rye and barley [7] and in corn used as animal feeds [8]. Citrinin can occur under different

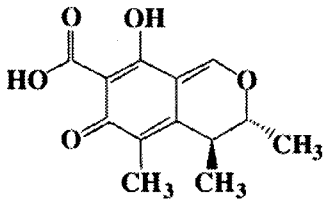

FIGURE 1. Chemical structure of citrinin.

environmental conditions and substrates and it appears jointly with other mycotoxins like patulin and ochratoxin A. Corn has been shown as an appropriate substrate for citrinin production.

Citrinin analysis is not simple, there are not validate quantitative methods for most of the natural substrates because this toxin is unstable. The informed values are possibly inferior to the real existent ones in the grains.

Penicillium citrinum was frequently isolated in Argentinean corn as contaminant [9], and González [9] observed macroscopic morphological differences in the *P. citrinum* colonies developed in agarized medium.

The objectives of this work are: (i) to study the capacity of citrinin production in liquid medium by *P. citrinum* isolates recorded from Argentinean corn to evaluate the possibility that this cereal can be contaminated by this metabolite, (ii) to determine the possibility that some cultural macroscopic characters and/or the kinetic behavior were associated with the toxigenic ability, with the purpose to help the quick identification of potentially toxigenic isolates.

MATERIALS AND METHODS

Microorganism. *37* isolates of *P. citrinum* from corn harvested at the main production area in Argentina [9], were tested. The conidial inocula were prepared as described by González *et al.* [10].

Toxigenic ability. To determine the citrinin production capacity, two steps procedure was performed. First the isolates were inoculated in Czapek liquid medium (Merck cat. 5460) and incubated under appropriate conditions for the mycotoxin accumulation. The following step was the toxin extraction from culture and its later detection. Portions of 100 ml of Czapek liquid medium were placed in 250 ml Erlenmeyer and sterilized at 121°C for 15 minutes. Once cool they were inoculated with the conidia suspension and incubated at 25°C for 7 days in the darkness [11]. Concluded the incubation the media coloration was observed, since the citrinin in Czapek liquid medium produces a yellow tonality. Citrinin pigment was obtained as an intense yellow precipitate by acidification of the medium with hydrochloric acid 1N [12]. Citrinin production was confirmed as follow: the yellow precipitate was re-dissolved in 40 ml of chloroform and mechanical and shook for one hour. The extract was separated by filtration and the residual extracted three times with 40 ml of chloroform shaking 30 minutes each time. The combined extracts were evaporated to dryness and re-dissolved in 250 μl of chloroform. The thin layer chromatography (TLC) was used for detect the mycotoxin [11]. The silica gel plates (G 60 of 0.25 mm) were used at room temperature, in a non-saturated cube. The development system used was benzene: glacial acetic acid: acetone (90:10:9) and the toxin was detected by visual comparison with the citrinin standard (Sigma cat. C1017) under U.V. light of 366 nm. This toxin produces a spot with intense greenish yellow fluorescence and a characteristic tail.

Radial growth measurements. 10 µl of conidial suspension of each isolate of *P. citrinum* (c.a. 10^5 conidia/ml) were dispensed in the center of Petri's dishes (eight replicates) containing malt agar (Merck cat. 5439) and incubated at 25°C for one week. Every 24 h perpendicular diameters were recorded at the linear growth phase of the mold. The initial colony diameter was determined from the circular inoculum. The diameters were adjusted to a function of first degree; by this mean the linear coefficient took as estimate of the growth rate in this moment.

Colony observations. In the same colonies, where linear growth was measured, after 14 days of incubation at 25°C, the following characters onto malt agar were tabulated: color, texture, furrows and bands [13]. This agarized medium was used to simulate the behavior of the mold growing on the cereal surface.

Statistical analyses. Rank correlation: To establish the relation between the radial growth rate ($X=Kd$) and the citrinin production capacity measured as toxicogenic isolates percentage (Y), the measure of correlation given by Spearman ρ was calculated and non parametric correlation Spearman test was applied. Spearman's Rho (ρ) is defined as:

$$\rho = \frac{\sum_{i=1}^{n}\left[R(X_i)-\frac{n+1}{2}\right]\left[R(Y_i)-\frac{n+1}{2}\right]}{n(n^2-1)/12} \qquad [I]$$

where $R(X_i)$ and $R(Y_i)$ represents the rank of X and Y respectably, and n is the samples size. The Sperman rank correlation test was used to test for independence between X and Y. The statistic used was T, defined as [14]:

$$T = \sum_{i=1}^{n}\left[R(X_i)-R(Y_i)\right]^2 \qquad [II]$$

RESULTS AND DISCUSSION

The citrinin production ability of the *P. citrinum* isolates is shown in Table 1. It can be seen that 35 % of the isolates had toxigenic capacity. According to the observed macroscopic morphological characters in agar malt, the beige and green colored colonies were prevalent and the gray and yellow were less frequent. The velvety was the most common texture, followed by cottony and only one strain presented a woolly aspect. The presence of furrows, as well as bands showed no tendency, similar frequencies were observed on the appearance or absence of those characters.

The Chi square test was performed to determine the independence of citrinin production and the macroscopic cultural characters expressed. For the cases in which the relationship between bands or texture and citrinin were considered the Fisher exact test was preferred. The data were classified according to two criteria, one the citrinin production and other the morphological character. In the results of the independence test of the characters in function of the citrinin production, the hypothesis proposed of independence is not rejected (Table 2) for any case of 5% significance levels (p value ≥ 0.05).

The calculated Spearman coefficient correlation between Kd and the citrinin-producing isolates percentage ρ was -0.79 (Table 3). If the measure of correlation is negative, then the larger values of X tend to be paired with the smaller values of Y. Then the hypotheses to test were H_o: the X_i and Y_i are mutually independent; H_1: there is a tendency for the smaller values of X_i to be paired with the larger

TABLE 1. Macroscopic morphological characters and citrinin production in *Penicillium citrinum* strains isolated from Argentinean corn. CIM: Centro de Investigación en Micotoxinas; -: negative observation; +: positive observation.

CIM#	Colour	Texture	Furrows	Bands	Citrinin	CIM#	Colour	Texture	Furrows	Bands	Citrinin
4222	Beige	Velvety	-	+	-	4333	Beige	Velvety	+	+	-
4223	Beige	Velvety	-	+	-	4334	Beige	Velvety	+	+	-
4224	Beige	Velvety	-	+	-	4335	Beige	Velvety	+	+	-
4225	Beige	Velvety	-	+	-	4336	Green	Cottony	+	-	-
4226	Beige	Velvety	-	+	-	4337	Green	Velvety	+	+	+
4319	Green	Velvety	-	+	-	4338	Green	Cottony	+	-	+
4320	Green	Velvety	-	+	-	4339	Grey	Cottony	+	+	+
4321	Beige	Velvety	-	+	-	4340	Grey	Cottony	-	+	+
4322	Beige	Velvety	-	+	-	4341	Grey	Cottony	-	-	+
4323	Green	Velvety	-	+	-	4342	Grey	Cottony	-	+	+
4324	Beige	Velvety	-	+	-	4343	Green	woolly	+	+	+
4325	Beige	Cottony	+	+	-	4344	Green	Cottony	-	+	+
4326	Beige	Cottony	+	+	-	4345	Yellow	Velvety	+	-	+
4327	Green	Velvety	+	-	-	4346	Beige	Velvety	-	+	+
4328	Beige	Cottony	+	+	-	4347	Beige	Velvety	-	+	+
4329	Green	Cottony	+	-	-	4348	Beige	Velvety	+	+	+
4330	Green	Velvety	+	+	-	4349	Green	Velvety	+	+	+
4331	Green	Cottony	+	-	-	4350	Green	Velvety	-	+	+
4332	Beige	Velvety	+	+	-						

TABLE 2. Independence test of macroscopic morphological characters and citrinin production. DF: degree of freedom; a "chi square"; b Fisher exact test

Character	DF	Value	Probability	Significance level $\alpha = 0,05$
Colour [a]	1	0.542	0.462	non significant
Texture [b]	1	3.625	0.163	non significant
Bands [b]	1	0.221	0.638	non significant
Furrows [a]	1	0.217	0.641	non significant

TABLE 3. ρ Spearman calculation. *X*: radial growth rate, *Y*: toxicogenic isolates percentage

X	*R(X)*	*Y*	*R(Y)*
0.0106	1	77	8
0.0139	2	40	5
0.0174	3	42	6
0.0214	4	25	3
0.0258	5	53	7
0.0291	6	39	4
0.0335	7	17	2
0.0370	8	7	1

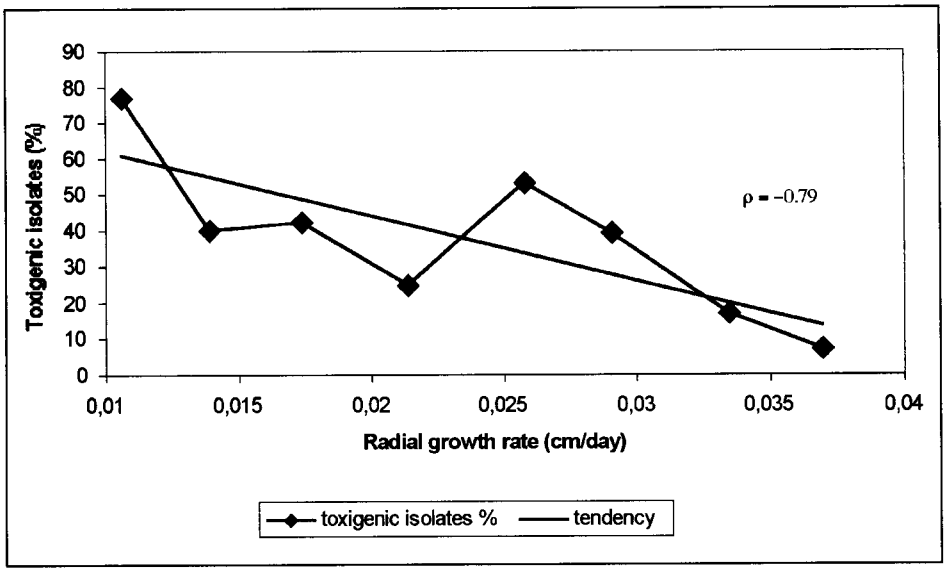

FIGURE 2. *Penicillium citrinum* radial growth rate and citrinin producing strains percentage.

values of Y_i, and vice versa. At the 0.05 level of significance, the quintile of T is 32 [14]. The calculated T was 150, and because exceeds 32, the null hypothesis (H_o) was rejected.
It may be concluded therefore that a negative tendency exists in the relationship between the growth rate and the percentage of citrinin producing isolates (Fig. 2), those that grew fast were not citrinin producers.

There are in the literature data of the kinetic growth of *P citrinum* in function factors as the inoculum size, temperature and water activity, and also on of citrinin production kinetic in connection with water activity, temperature, and different substrata [15]. This is the first study that relates the growth rate with the percentage of citrinin producing isolates.

As a third part of *P citrinum* isolates had toxigenic ability, citrinin contamination of corn is possible in the main production area in Argentina.

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