Journal of Food Protection, Vol. 63, No. 2, 2000, Pages 231–236

Effects of Substrate, Water Activity, and Temperature on Growth and Verrucosidin Production by *Penicillium polonicum* Isolated from Dry-Cured Ham

FÉLIX NÚÑEZ, M. CARMEN DÍAZ, MAR RODRÍGUEZ, EMILIO ARANDA, ALBERTO MARTÍN, AND MIGUEL A. ASENSIO*

Higiene de los Alimentos, Facultad de Veterinaria, Universidad de Extremadura, Avenida de la Universidad s/n, 10071 Cáceres, Spain

MS 99-64: Received 17 March 1999/Accepted 29 July 1999

ABSTRACT

Penicillium polonicum, a common mold on dry-cured meat products, is able to produce verrucosidin, a potent neurotoxin. The ability of *P. polonicum* isolated from dry-cured ham to grow and produce verrucosidin from 4 to 40°C at water activities (a_w) of 0.99, 0.97, and 0.95 on malt extract agar (MEA) and a medium made up with meat extract, peptone, and agar (MPA) was evaluated. Verrucosidin was quantified by high-pressure liquid chromatography and mass spectrometry. *P. polonicum* was able to grow on MEA and MPA at all the a_w values tested from 4 to 37°C but not at 40°C. The optimal environmental conditions for growth were 20°C, 0.99 a_w on MEA and 20 to 25°C, 0.97 a_w on MPA, but the highest amount of verrucosidin was obtained at 25°C, 0.99 a_w in both media. No direct correlation between extension of mold growth and verrucosidin production was found. Temperature appears to be the most important factor ruling mycelial growth, whereas verrucosidin accumulation is mostly influenced by a_w. However, analysis of variance of the data showed that there was a complex interaction among all the environmental factors (medium, temperature, and a_w) that significantly ($P < 0.0001$) affected growth and verrucosidin production. The reduction of a_w to intermediates values of 0.95 has a stronger effect on growth on MEA than on MPA. Given that the meat-based medium proved to be an appropriate substrate for the biosynthesis of verrucosidin by *P. polonicum,* the ability of this mold to produce the toxin on meat products should be established.

Penicillium polonicum is frequently isolated from foods and feeds, since it is the most common species of the genus *Penicillium* group *Viridicata* in meat products *(6)*. This species has been isolated from the surface of dry-cured ham during most of the ripening process, and all the isolates showed a high toxigenic potential *(9)*.

It has been reported that 91% of isolates of *P. polonicum* produce verrucosidin, a potent neurotoxin *(6)* responsible for a neurological disease in cattle *(14)* and experimentally in mice *(3)*. It is considered the most potent cytotoxic compound in the group of tremorgenic mycotoxins and shows genotoxic effect *(3)*. However, there is little information on how the environmental conditions affect verrucosidin production by *P. polonicum.* Thus, a substantial knowledge of the characteristics of the fungal growth and verrucosidin production is needed to understand and control the interactions of the environmental conditions. Moreover, since *P. polonicum* colonizes meat products, its ability to produce mycotoxins on meat-based substrates should be established.

Environmental conditions, mainly temperature and water activity (a_w), are critical for fungal growth and mycotoxin production. Conditions for mycotoxin production are generally more restricted than those for growth and can vary between different mycotoxins produced by the same species *(4)*. Furthermore, quantitative data about the effects of these factors on the physiology of fungi are essential to control their growth and predict their toxigenic activities.

The aim of this work was to study the influence and possible interactions of temperature and a_w in determining the production of verrucosidin by *P. polonicum* isolated from dry-cured ham. Two agar media were used: malt extract agar (MEA) as an optimal substrate for growth and verrucosidin production and a medium made up of meat extract and peptone (MPA) as an approach to the composition of meat product.

MATERIAL AND METHODS

Mold strain. The mold strain used had been isolated from dry-cured ham and previously identified as *Penicillium aurantiogriseum,* according to morphologic characteristics *(9)*, but characterized as *P. polonicum* according to the profile of the secondary metabolites (unpublished data).

The strain used had shown toxic effects against brine shrimp larvae, Vero cells, antimicrobial activity against *Staphylococcus aureus*, and mutagenicity in the Ames test *(9)*. The factors studied were temperature (4, 12, 20, 25, 30, 37, and 40°C) and a_w (0.99, 0.97, and 0.95) in two media, MEA (2% malt extract, 2% glucose, 0.1% peptone, 2% agar) and MPA (2.5% meat extract, 0.25% peptone, 2% agar). Inoculum was prepared by growing the mold on MEA at 25°C for 7 days. Conidia were harvested by washing the surface of incubated MEA with sterile water containing 0.1% Tween 20. The resulting conidial suspension was used as inoculum at three points per plate. A total of 12 plates were inoculated for every set of conditions and processed as three batches (four plates each).

* Author for correspondence. Tel: 34–927–257125; Fax: 34–927–257110; E-mail: masensio@unex.es.

TABLE 1. *Diameter of colonies and verrucosidin production (μg/plate) by* Penicillium polonicum *growing on malt extract agar (MEA) and meat extract peptone agar (MPA) at 0.99 a_w for 14 days at different temperatures*

Temperature (°C)	Diameter (mm)		Verrucosidin production (μg/plate)	
	MEA	MPA	MEA	MPA
4	11 ± 1.9 A, 1[a]	18 ± 0.8 A, 2	ND[b]	ND
12	46 ± 2.1 B, 1	29 ± 0.9 B, C	4.46 ± 1.18 A, 1	0.04 ± 0.03 A, 1
20	57 ± 1.9 C, 1	40 ± 1.0 C, 2	21.80 ± 4.62 B, 1	13.39 ± 0.22 B, 2
25	52 ± 1.8 D, 1	39 ± 1.4 C, 2	39.74 ± 5.33 C, 1	18.60 ± 0.79 C, 2
30	17 ± 1.6 E, 1	22 ± 1.9 D, 2	8.40 ± 1.25 A, 1	1.19 ± 0.23 A, 2
37	6 ± 2.6 F, 1	10 ± 1.5 E, 2	ND	ND
40	NG[b]	NG	—	—

[a] For a given medium (column), values followed by different letters are significantly different ($P < 0.05$). For a given temperature (row), values followed by different numbers are significantly different ($P < 0.05$).
[b] ND, not detected; NG, no growth.

Culture conditions. The a_w of both MEA and MPA was 0.99. To get the a_w down to 0.97, 5% of NaCl was added in both media, and to reach 0.95 a_w, an additional 20% of either malt extract (for MEA) or meat extract (for MPA) was added.

Cultures were grown on standard petri dishes containing approximately 20 ml of agar medium. Plates were incubated at the specified temperatures for 14 days in polyethylene bags to avoid changes in media a_w.

The a_w of fresh and incubated media was measured with a dew point metter (FA-st/1 GBX, Dillon, France).

Colony measures and mycotoxins extraction. The diameter of 36 colonies per set of conditions were measured before mycotoxins were extracted.

The content of four petri dishes was transferred to a plastic bag and macerated with 150 ml of chloroform in a Stomacher Lab Blender 400 (Seward Medical, London, UK) for 4 min. After 1 h, the slurry was twice filtered through anhydrous sodium sulfate with Whatman no. 1 filter paper (Whatman Int., Maidstone, UK). Then the filtrate was evaporated on a rotary evaporator model VV2000 (Heidolph, Kelheim, Germany) at 40°C. The residue was resuspended in 5 ml of chloroform, filtered through a nylon membrane with 0.45 μm of pore size (MSI, Westboro, Mass.), and evaporated to dryness under a stream of nitrogen. The extracts were stored at 4°C in darkness until required and then resuspended in 200 μl of acetonitrile just before high-pressure liquid chromatography analysis. Extractions from each medium and every treatment were carried out in triplicate.

Analytical detection and confirmation of secondary metabolites. Five microliters of each extract was analyzed by high-pressure liquid chromatography on a Supelcosil LC-18 column (25 cm long, 4.6 mm inside diameter, and 5 μm particle size) (SUPELCO, Bellafonte, Pa.) in a Hewlett Packard series 1100 apparatus (Hewlett Packard, Palo Alto, Calif.). Mobile phases A and B were composed of 100% water and 0.05% trifluoroacetic acid in acetonitrile, respectively. To achieve the separation of metabolites, the flow rate was set at 0.8 ml/min and the following gradient was performed: initial 10% B for 0.5 min, linear change to 70% B in 20 min, 70% B for 10 min, linear change to 90% B in 2 min, 90% B for 5 min, linear change to 10% B in 2 min, and kept at this percentage for 5 min. Detection of verrucosidin was done by atmospheric pressure inonization–mass spectrometry on a Finnigan LCQ (Finnigan, San Jose, Calif.) equipped with an atmospherical pressure (chemical) ionization interface. Positive ions were detected under the following conditions: vaporizer temperature, 450°C; sheath gas flow rate, 60 arbitrary units; auxiliary gas flow rate, 20 arbitrary units; discharge current, 5 μA; capillary temperature, 150°C; capillary voltage, 0 V; and tube lens offset, 0 V. The calibration curve for verrucosidin by high-pressure liquid chromatography–mass spectrometry revealed a linear relationship ($r^2 = 0.9929$) between detector response and amount of verrucosidin from 0.1 to 100 ng. The full mass spectrometry spectra data were acquired and processed using Navigator version 1.1 sp1 software.

Confirmation of identity of verrucosidin was done by ion spray mass spectrometry analysis using parent-daughter analyses of extracts obtained from mold cultures and by comparison with the mass spectrum of verrucosidin standard by using *m/z* 417 (protonated molecular ion). Verrucosidin standard was kindly supplied by Dr. L. Leistner (Institute for Microbiology, Toxicology and Histology, Kulmbach, Germany).

Statistical analysis. The effect of each environmental factor on growth and verrucosidin production was evaluated by three-

TABLE 2. *Diameter of colonies and verrucosidin production (μg/plate) by* Penicillium polonicum *growing on malt extract agar (MEA) and meat extract peptone agar (MPA) at 0.97 a_w for 14 days at different temperatures*

Temperature (°C)	Diameter (mm)		Verrucosidin production (μg/plate)	
	MEA	MPA	MEA	MPA
12	44 ± 3.0 A, 1[a]	33 ± 1.7 A, 2	1.21 ± 0.38 A, 1	2.56 ± 0.34 A, 1
20	50 ± 2.6 B, 1	45 ± 2.5 B, 2	5.90 ± 1.15 AB, 1	1.28 ± 0.09 A, 1
25	33 ± 3.4 C, 1	44 ± 2.0 C, 2	6.69 ± 1.68 B, 1	4.18 ± 0.56 A, 1
30	17 ± 1.0 D, 1	28 ± 1.8 D, 2	4.16 ± 0.54 AB, 1	2.32 ± 0.60 A, 1

[a] For a given medium (column), values followed by different letters are significantly different ($P < 0.05$). For a given temperature (row), values followed by different numbers are significantly different ($P < 0.05$).

TABLE 3. *Diameter of colonies and verrucosidin production (μg/plate) by* Penicillium polonicum *growing on malt extract agar (MEA) and meat extract peptone agar (MPA) at 0.95 a_w for 14 days at different temperatures*

Temperature (°C)	Diameter (mm)		Verrucosidin production (μg/plate)	
	MEA	MPA	MEA	MPA
12	35 ± 3.3 A, 1[a]	33 ± 1.4 A, 2	0.86 ± 0.18 A, 1	0.28 ± 0.09 A, 1
20	40 ± 3.0 B, 1	35 ± 1.1 B, 2	1.30 ± 0.23 A, 1	0.09 ± 0.04 A, 1
25	29 ± 3.2 C, 1	38 ± 1.6 C, 2	0.05 ± 0.01 A, 1	0.81 ± 0.16 A, 1
30	24 ± 2.2 D, 1	28 ± 0.7 D, 2	0.84 ± 0.23 A, 1	0.01 ± 0.00 A, 1

[a] For a given medium (column), values followed by different letters are significantly different ($P < 0.05$). For a given temperature (row), values followed by different numbers are significantly different ($P < 0.05$).

way analysis of variance, with the interactions among temperature, a_w, and media, using the General Linear Model procedure included in SAS software *(12)*.

Least-squares regression model was applied to the response surface plots to test for trends of growth and verrucosidin production *(12)*.

RESULTS

Growth and mycotoxin production at high a_w (0.99). *P. polonicum* grew at all temperatures from 4 to 37°C on either MEA or MPA media (Table 1). The maximum growth at 0.99 a_w was reached on MEA at 20°C, followed by 25 and 12°C.

Verrucosidin was produced from 12 to 30°C in both media (Table 1) but was not detected at 4 and 37°C. The highest production of verrucosidin at 0.99 a_w (39.7 μg/plate) was obtained at 25°C in MEA. The production of verrucosidin was high at 25 and 20°C in both media, showing a deep drop at 12 and 30°C.

Growth and mycotoxin production at intermediate a_w (0.97, 0.95). On MEA the growth was poorer as a_w decreased, except at 30°C, where the maximum growth was reached at 0.95 a_w (Tables 2 and 3). However, on MPA the maximum growth at all temperatures assayed was always reached at 0.97 a_w. Even at 12 and 30°C, *P. polonicum* grew faster at 0.95 than at 0.99 a_w.

At these intermediate a_w, the best growth was still recorded on MEA at 20°C, being faster at 12°C than at 25°C. On MPA, growth at 20°C was similar to that at 25°C (0.95 a_w) or even lower (0.97 a_w).

Verrucosidin was detected in all samples at least at 10 ng/plate (Tables 2 and 3). Yields at 0.97 and 0.95 a_w were lower than those at 0.99 a_w when mold was grown at 20 and 25°C ($P < 0.05$) but showed no significant difference when grown at 12 and 30°C ($P \geq 0.05$).

Interactions between ecological conditions on growth and verrucosidin production. The analysis of variance for growth at the different culture conditions (Table 4) showed that all single factors tested (i.e., medium, temperature, and a_w) affected the growth of *P. polonicum* ($P < 0.0001$). Also, all two- (medium plus temperature, medium plus a_w, and temperature plus a_w) and three-factor interactions (medium plus temperature plus a_w) were highly significant ($P < 0.0001$). The most important single factor was temperature ($F = 8684.63$).

All three single factors (medium, temperature, and a_w) were highly significant ($P < 0.0001$), influencing verrucosidin production by *P. polonicum* (Table 4). Furthermore, all the two- and three-factor interactions also significantly affected verrucosidin accumulation ($P < 0.0001$). Among the single factors tested, a_w had the highest effect ($F = 491.30$).

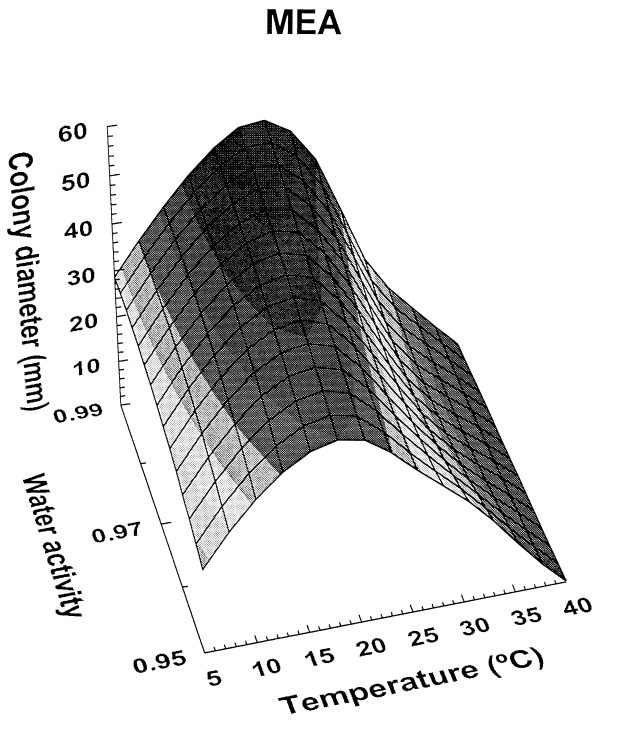
DISCUSSION

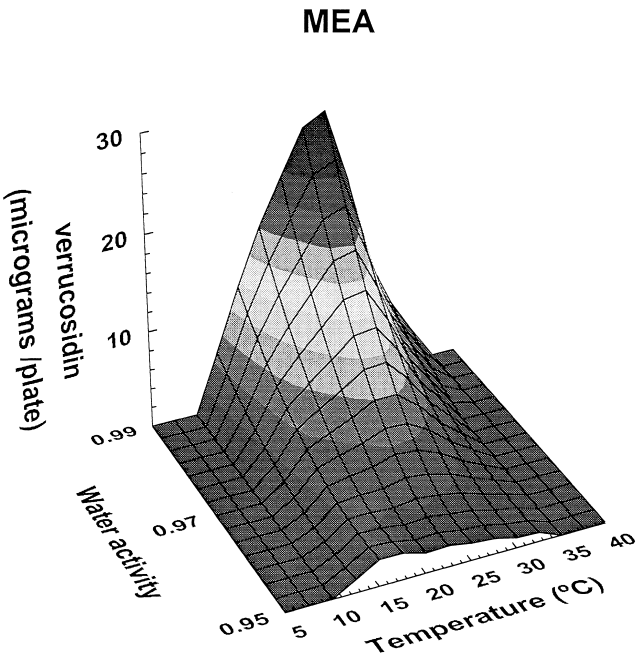
Mycelial growth of *P. polonicum* from 4 to 37°C is a temperature-dependent process (Table 4), with maximum development at 20 and 25°C (Table 1). The remaining ecological factors tested, i.e., media composition and a_w, do not have such a strong, independent effect on growth of *P. polonicum*, as shown by the *F* value (Table 4). The optimum a_w for growth changes with temperature and media composition. On MEA the best growth was obtained at 0.99

TABLE 4. *Analysis of variance for radial growth and verrucosidin production by* P. polonicum

Source	Radial growth			Verrucosidin production		
	df[a]	Mean square	*F*[b]	df	Mean square	*F*
Medium	1	391	101	1	25453384	103
Temperature	6	33622	8685	5	34233713	138
a_w	2	1744	450	2	121544827	491
Medium–temperature	6	2311	597	5	2785556	11
Medium–a_w	2	3471	897	2	21182177	86
Temperature–a_w	6	1324	342	6	30005538	121
Medium–temperature–a_w	6	553	143	6	3245349	13

[a] df, degrees of freedom.
[b] *F*, variance ratio. *P* value <0.0001.





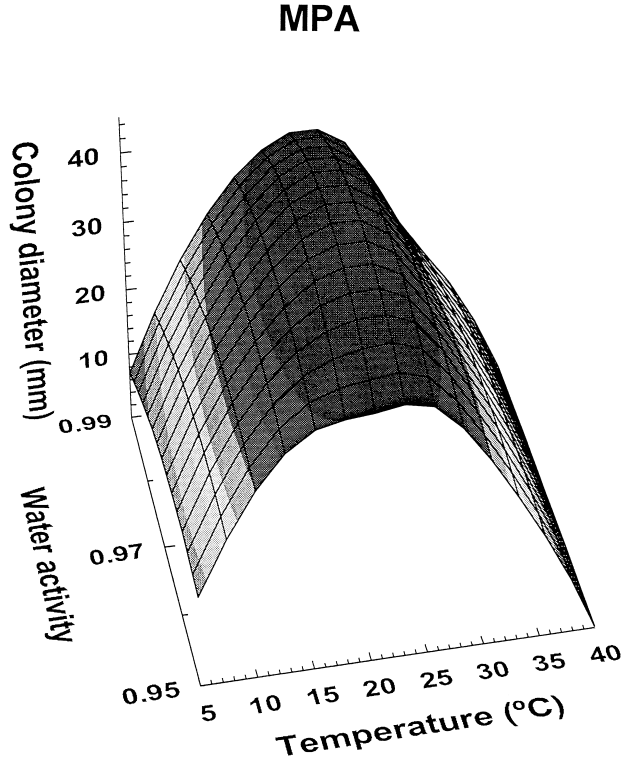


FIGURE 1. *Response surface plot of mycelial growth of* P. polonicum *(colony diameter in millimeters) as affected by temperature and* a_w *on MEA and MPA.*

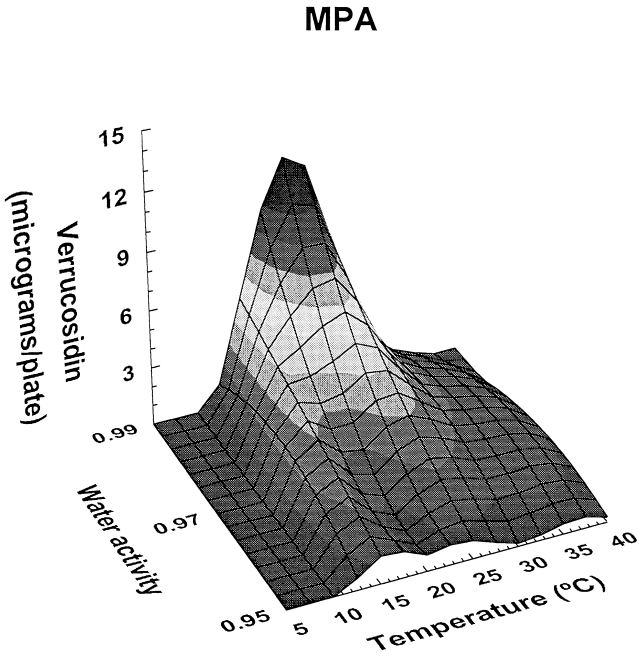


FIGURE 2. *Response surface plot of verrucosidin production by* P. polonicum *(micrograms per plate) as affected by temperature and* a_w *on MEA and MPA.*

a_w. However, on MPA growth recorded at 0.97 a_w was faster than at 0.99 at every temperature tested. On this medium, growth at 12 and 30°C was faster at 0.95 than at 0.99 a_w, and little effect was observed at 20 and 25°C. Thus, the reduction of a_w to intermediate values of 0.95 has a stronger effect on MEA than on MPA (Fig. 1). Also, at 0.99 a_w *P.*

polonicum grew better at extreme temperatures, such as 4, 30, and 37°C, on MPA than on MEA. It seems then that replacement of malt extract and glucose (in MEA) for meat extract (in MPA) does not improve the efficiency for growth of *P. polonicum* when the ecological conditions are close to the optima, but it does at suboptimal conditions. The better growth at that condition may be related to the greater availability of nitrogen compounds on MPA. The total nitrogen concentration on MPA, according to the supplier data (Oxoid, Unipath, Basingstoke, UK), is 10 times that on MEA. This positive effect of meat extract on *P. polonicum* growth at suboptimal conditions could be related to the origin of the isolate, given that the strain used was obtained from dry-cured ham, a meat product of intermediate a_w.

However, the real interest in the growth of *P. polonicum* at intermediate a_w relies on its toxigenic activity *(9)*. Among the identified metabolites of this species *(6)*, verrucosidin has been described as a potent neurotoxin that shows cytotoxicity and genotoxicity at a nanomole range concentration *(3)*. Since the production of secondary metabolites is strongly influenced by the ecological conditions, the ability of *P. polonicum* to synthesize verrucosidin is of higher interest than just its ability to grow.

The amount of verrucosidin detected on MEA incubated for 14 days at 25°C, 0.99 a_w (39.7 μg/plate) was well in the range of 0 to 250 μg/plate reported for 266 strains of *P. aurantiogriseum* from different origins and cultured in the same conditions *(2)*.

As mycelial growth, the amount of verrucosidin seems to be influenced by the complex interaction of medium composition, temperature, and a_w (Fig. 2). At 0.99 a_w, verrucosidin production is strongly depressed when incubation temperature is taken away from 25°C. As a consequence, this toxin was not detected after 14 days of incubation at 4 or 37°C (Table 1). However, the influence of temperature on verrucosidin production seems to be limited to the highest a_w, since little if any effect was found below 0.99 a_w (Tables 2 and 3). Decreasing a_w to 0.95 depressed the biosynthesis of verrucosidin, but this toxin was still detected in both media at all the temperatures where verrucosidin was produced at 0.99 a_w.

As has been described for aflatoxin biosynthesis by *Aspergillus flavus* and *Aspergillus parasiticus (5, 8)*, temperature is the most important single factor for mycelial growth, whereas that for mycotoxin accumulation is a_w (Table 4).

Verrucosidin production on MEA was higher than on MPA at 0.99 a_w at all the temperatures tested. No significant difference was found at 0.97 or 0.95, which might be due to the low production levels at these a_w values.

These results reveal that there is no correlation between growth and quantity of toxin produced. The ecological conditions for maximum toxin yield on both media (25°C, 0.99 a_w) differ from those for maximum mycelial growth (20°C on MEA and 0.97 a_w on MPA). Thus, verrucosidin production increases at suboptimal growing conditions, as has been reported for other mycotoxins *(1, 13)*.

Multifactorial studies have demonstrated that a_w and temperature are interdependent and their interaction is the most critical determinant for fungal growth and mycotoxin production for a variety of molds and substrates *(5, 7)*. *P. polonicum* growth and mycotoxin production were also ruled by a complex interaction among medium, temperature, and a_w (Table 4). Probably, none of the factors studied has an overriding effect on growth and mycotoxin production, but every single one contributes to the final outcome.

The present study demonstrates that *P. polonicum* is able to produce a large amount of verrucosidin on a meat-related substrate, with an extremely low level of carbohydrates. The low verrucosidin production at 0.95 a_w and the lower a_w reached in most dry-cured meat products *(11)* contribute to overlooking this hazard. However, the long ripening of these products, which for dry-cured ham particularly is extended for several months, can make the presence of *P. polonicum* on meat products pose a hazard to human health. Because of this reason, its ability to produce toxins on meat products, particularly on dry-cured ham, should be studied.

The hazard could be overcome by lowering the ripening temperature to some point below 12°C. However, the biochemical reactions that take place during ripening would become too slow and would eliminate any positive contribution of the mold population *(10)*. Therefore, appropriate starter cultures of molds should be used to prevent colonization of meat products by mycotoxin-producing molds.

ACKNOWLEDGMENT

This work was supported by a grant from the Spanish Comisión Interministerial de Ciencia y Tecnología (ALI95–0237).

REFERENCES

1. Bacon, C. W., J. G. Sweeney, J. D. Robbins, and D. Burdick. 1973. Production of penicillic acid and ochratoxin A on poultry feed by *Aspergillus ochraceus*: temperature and moisture requirements. Appl. Microbiol. 26:155–160.
2. El-Banna, A. A., and L. Leistner. 1987. Quantitative determination of verrucosidin produced by *Penicillium aurantiogriseum.* Microbiol-Aliments-Nutr. 5:191–195.
3. Fink-Gremmels, J., A. Henning, and L. Leistner. 1991. Verrucosidin—a *Penicillium* toxin, toxicological aspects. Acta. Vet. Scand. Suppl. 87:457–458.
4. Frisvad, J. C., and R. A. Samson. 1991. Filamentous fungi in foods and feeds: ecology, spoilage, and mycotoxin production, p. 31–68. *In* D. K. Arora, K. G. Mukerji, and E. H. Marth (ed.), Handbook of applied mycology: foods and feeds. Marcel Dekker, Inc., New York.
5. Gqaleni, N., J. E. Smith, J. Lacey, and G. Gettinby. 1997. Effects of temperature, water activity, and incubation time on production of aflatoxins and cyclopiazonic acid by an isolate of *Aspergillus flavus* in surface agar culture. Appl. Environ. Microbiol. 63:1048–1053.
6. Lund, F., and J. C. Frisvad. 1994. Chemotaxonomy of *Penicillium aurantiogriseum* and related species. Mycol. Res. 98:481–492.
7. Marín, S., V. Sanchis, R. Sáenz, A. J. Ramos, I. Viñas, and N. Magan. 1998. Ecological determinants for germination and growth of some *Aspergillus* and *Penicillium* spp. from maize grain. J. Appl. Microbiol. 84:25–36.
8. Northold, M. D., C. A. Verhulsdonk, P. S. Soentoro, and W. E. Paulsch. 1976. Effect of water activity and temperature on aflatoxin production by *Aspergillus parasiticus.* J. Milk Food Technol. 39:170–174.
9. Núñez, F., M. M. Rodríguez, M. E. Bermúdez, J. J. Córdoba, and M. A. Asensio. 1996. Composition and toxigenic potential of the mould population on dry-cured Iberian ham. Int. J. Food Microbiol. 32:185–197.

10. Rodríguez, M. M., F. Núñez, J. J. Córdoba, M. E. Bermúdez, and M. A. Asensio. 1998. Evaluation of proteolytic activity of microorganisms isolated from dry cured ham. J. Appl. Microbiol. 85:905–912.
11. Rodríguez, M. M., F. Núñez, J. J. Córdoba, C. Sanabria, M. E. Bermúdez, and M. A. Asensio. 1994. Characterization of *Staphylococcus* spp. and *Micrococcus spp.* isolated from Iberian ham throughout the ripening process. Int. J. Food Microbiol. 24:329–335.
12. SAS Institute Inc. 1997. SAS/STAT user's guide. SAS Institute Inc., Cary, N.C.
13. Shih, C. N., and E. H. Marth. 1974. Some cultural conditions that control biosynthesis of lipid and aflatoxin by *Aspergillus parasiticus.* Appl. Microbiol. 27:452–456.
14. Wilson, B.J., C. S. Byerly, and L. T. Burka. 1981. Neurologic disease of fungal origin in three herds of cattle. J. Am. Vet. Med. Assoc. 179:480–481.