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Production of Cyclopiazonic Acid by *Penicillium commune* Isolated from Dry-Cured Ham on a Meat Extract–Based Substrate

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ABSTRACT

Penicillium commune, a mold frequently found on dry-cured meat products, is able to synthesize the mycotoxin cyclopiazonic acid (CPA). To evaluate the hazard due to CPA on such foods, the ability of *P. commune* to grow and produce CPA at water activities (a_w) in the range of 0.99 to 0.90 with a meat extract–based medium from 12 to 30°C was determined. CPA was quantified by high-pressure liquid chromatography and mass spectrometry. *P. commune* was able to grow at every a_w and temperature tested. The optimal environmental conditions for growth were 20 to 25°C, at 0.97 to 0.96 a_w, but the highest amount of CPA was produced at 30°C, 0.96 a_w. No direct correlation between growth rate and CPA production was assessed. Temperature seems to be the most important factor influencing CPA production. However, there was an interaction between temperature and a_w that significantly ($P < 0.001$) affected growth and CPA production. An a_w of 0.90 had a marked effect, depressing growth and CPA production. Meat extract–based medium proved to be an appropriate substrate for CPA biosynthesis by *P. commune* under a wide range of conditions.

Molds are common contaminants of fermented dry-cured meats, in which they find the appropriate conditions to outgrow bacteria, particularly in those of intermediate water activity (a_w) kept at room temperature. Most species found in fermented dry-cured foods are able to synthesize mycotoxins when grown under appropriate conditions. Thus, the ability of molds to produce toxins under the ecological conditions of these products needs to be established.

Penicillium commune has been reported as the most common natural producer of cyclopiazonic acid (CPA) *(7, 23).* This organism has been isolated from meat products, including dry-fermented sausages *(1)* and cured ham *(19, 22).* In the latter, *P. commune* has been detected during most of the ripening process as part of the normal surface flora, and all 15 studied strains showed toxicity to brine shrimp larvae and Vero cells, and 6 were mutagenic in the Ames test *(19).*

CPA is a toxic indole tetramic acid produced by some *Penicillium* and *Aspergillus* species isolated from several agricultural commodities *(13, 23).* Acute oral toxicity of CPA has been demonstrated in several animal species *(5, 21)* and also linked with Kodua poisoning in humans *(24).* CPA has shown immunosuppressive activity at low doses *(10),* causes necrosis of different tissues *(5),* acts as an inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum in vitro *(27),* and has been shown to be cytotoxic *(14)* and mutagenic in the Ames test *(29).* For these reasons, CPA should be considered as a potentially hazardous mycotoxin.

Ecological conditions, mainly temperature and a_w, are regarded as critical factors determining fungal growth and mycotoxin production. Conditions for mycotoxin production are generally more restricted than those for growth and can vary among different mycotoxins produced by the same species *(7).*

Furthermore, quantitative data about the effects of these factors on the physiology of fungi are essential to control their growth and predict their toxigenic activity. The effect of environmental factors on CPA production has been studied for different molds, including *P. commune* growing on substrates with a high carbohydrate content *(6, 8).* However, the ability to produce CPA on meat-based substrates is not known. CPA is derived from tryptophan *(11),* which is found as free amino acid in dry-cured ham *(3).* Therefore, the presence of *P. commune* in dry-cured meat products could lead to synthesis of CPA, and the production of this mycotoxin in a meat-based substrate should be established.

The aim of this work was to study the influence and possible interactions of temperature and a_w in the production of CPA by *P. commune* on a medium made up from meat extract to simulate meat products.

MATERIALS AND METHODS

Mold strain. The strain used was isolated from dry-cured ham *(19)* and characterized genotypically and phenotypically as *P. commune,* showing cytotoxicity against Vero cells *(4).*

Culture conditions. The ecological factors studied were temperature (12, 20, 25, and 30°C) and a_w at high, intermediate, and low values. *P. commune* was grown on several culture media based on simple compounds present in meat products as follows.

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TABLE 1. *Diameter (mm) of* P. commune *colonies growing on MPA for 14 days at different temperatures and* a_w

Temperature (°C)	a_w 0.99	0.97	0.96	0.90
12	28 ± 1.1 A, 1[a]	27 ± 0.8 AB, 1	25 ± 1.1 A, 1	9 ± 1.9 A, 2
20	27 ± 3.3 A, 1,2	28 ± 6.7 AB, 1,2	30 ± 4.5 AB, 2	23 ± 1.6 B, 1
25	25 ± 0.8 A, 1	31 ± 4.3 A, 2	34 ± 4.9 B, 2	22 ± 5.3 B, 1
30	15 ± 1.2 B, 1	24 ± 1.4 B, 2	20 ± 3.4 C, 1,2	10 ± 2.1 A, 3

[a] For a given a_w (column), values not followed by the same letter are significantly different ($P < 0.05$). For a given temperature (row), values not followed by the same number are significantly different ($P < 0.05$).

High a_w (0.99): The medium was composed of meat extract peptone agar (MPA) containing 2.5% meat extract (Scharlau Chemie, Barcelona, Spain), 0.25% peptone (Difco Laboratories, Detroit, Mich.), and 2% agar (Schalau Chemie). Intermediate a_w (0.97, 0.96): (i) Adding a 5% NaCl, a common level in dry-cured hams, moved a_w down to 0.97 and (ii) a 20% meat extract was incorporated to reach the concentration of nitrogen compounds found in dry-cured ham ($a_w = 0.96$). Low a_w (0.90): To bring a_w down to values usually found in dry-cured ham without a big impact on the concentration of meat components, a 20% glycerol was further added.

These values of temperature and a_w are in the range of those reached during processing of dry-cured ham. Inoculum was prepared by growing the mold on malt extract agar at 25°C for 7 days. Conidia were harvested by washing the surface of incubated malt extract agar with sterile water containing 0.1% Tween 20. The resulting conidial suspension was used as inoculum at three points per plate. A total of 9 plates containing MPA were inoculated for every set of conditions and processed as three batches (three plates each).

Cultures were grown on standard petri dishes containing approximately 20 ml of medium. Plates were incubated at the specified temperatures for 14 days in polyethylene bags to prevent moisture loss.

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

Colony measurement and mycotoxin extraction. The diameter of 27 colonies per set of conditions was measured before mycotoxin extraction.

The content of three 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 International, Maidstone, UK). Then the filtrate was evaporated in a rotatory evaporator model VV2000 (Heidolph, Kelheim, Germany) at 40°C. The residue was resuspended in 5 ml of chloroform, filtered through a 0.45-μm pore size nylon membrane (MSI, Westboro, Mass.), and evaporated to dryness under a gentle stream of nitrogen. The extracts were stored at 4°C in the dark until required and resuspended in 200 μl of acetonitrile just before liquid chromatography (LC) analysis. Extractions from each medium and culturing condition were done in triplicate.

Analytical detection and confirmation of CPA. Five microliters of each extract was analyzed by LC 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 were (i) 100% water and (ii) 0.05% trifluoroacetic acid in acetonitrile. To achieve separation of metabolites, the flow rate was set at 0.8 ml/min and the following gradient was performed: initial 10% of 0.05% trifluoroacetic acid in acetonitrile for 0.5 min, linear change to 70% of 0.05% trifluoroacetic acid in acetonitrile for 20 min, 70% of 0.05% trifluoroacetic acid in acetonitrile for 10 min, linear change to 90% of 0.05% trifluoroacetic acid in acetonitrile for 2 min, 90% of 0.05% trifluoroacetic acid in acetonitrile for 5 min, linear change to 99% of 0.05% trifluoroacetic acid in acetonitrile for 1 min, 99% of 0.05% trifluoroacetic acid in acetonitrile for 10 min, linear change to 10% of 0.05% trifluoroacetic acid in acetonitrile for 2 min, and kept at this percentage for 5 min. Detection of CPA was done by atmospheric pressure chemical ionization mass spectrometry (MS) on a Finnigan LCQ (Finnigan, San Jose, Calif.). 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 CPA by LC-MS revealed a linear relationship ($r^2 = 0.9959$) between detector response and amount of CPA standard (Sigma-Aldrich Química S.A., Madrid, Spain) from 1 to 50 ng. The full MS spectra data were acquired and processed using Navigator version 1.1 software. Under the described conditions, CPA eluted at 25 min as a protonated molecular ion of *m/z* 337.

Statistical analysis. The effect of each environmental factor on growth and CPA production was evaluated by two-way analysis of variance (ANOVA) together with the interactions between temperature and a_w using the general linear model procedure included in SAS software *(26)*. Least squares regression model *(26)* was applied to the response surface plots to test for trends of growth and CPA production.

RESULTS

Growth and CPA production at high a_w (0.99). Growth of *P. commune* was very similar from 12 to 25°C, but slower at 30°C (Table 1). CPA was produced from 12 to 30°C (Table 2), showing higher yields at 25 and 30°C.

Growth and CPA production at intermediate a_w (0.97, 0.96). The pattern for growth of *P. commune* at 0.97 and 0.96 a_w (Table 1) was similar to that at 0.99 a_w, with the slowest growth at 30°C ($P < 0.05$). Likewise, CPA production was bigger at the higher temperature (Table 2). At the same time, the highest CPA yield for every temperature tested was reached at 0.96 a_w.

Growth and CPA production at low a_w (0.90). Growth of *P. commune* at 0.90 a_w was the lowest for every temperature tested, with minimum values at 30 and 12°C (Table 1). Maximum CPA production at 0.90 a_w was obtained not at the higher temperatures, but at 20°C (Table 2).

TABLE 2. *CPA (ng/plate) by* P. commune *growing on MPA for 14 days at different temperatures and* a_w

Temperature (°C)	a_w 0.99	0.97	0.96	0.90
12	42 ± 3.0 A, 1[a]	24 ± 5.2 A, 1	122 ± 5.6 A, 2	ND[b] A, 3
20	32 ± 5.3 A, 1	30 ± 1.1 A, 1	83 ± 2.0 A, 2	65 ± 7.0 B, 1,2
25	163 ± 13.0 B, 1	56 ± 9.9 A, 2	229 ± 16.2 B, 3	38 ± 9.9 BC, 2
30	180 ± 20.5 B, 1	310 ± 9.6 B, 2	618 ± 13.6 C, 3	24 ± 6.5 C, 4

[a] For a given a_w (column), values not followed by the same letter are significantly different ($P < 0.05$). For a given temperature (row), values not followed by the same number are significantly different ($P < 0.05$).
[b] ND, not detected.

The most restrictive conditions for CPA production on MPA were found at 0.90 a_w, 12°C, where CPA could not even be detected.

Interactions between ecological conditions on growth and CPA production. The ANOVA for growth at the different culture conditions (Table 3) showed that both a_w and temperature affected growth of *P. commune* ($P < 0.001$). The two-factor interaction ($a_w \times$ temperature) was also highly significant ($P < 0.001$).

Similarly, the two single factors (a_w and temperature) were highly significant ($P < 0.001$) in influencing CPA production by *P. commune* (Table 3), and the two-factor interaction significantly affected CPA accumulation ($P < 0.001$). However, the most important factor influencing growth was a_w (variance ratio = 94), whereas the most important factor influencing CPA production was temperature (variance ratio = 981).

DISCUSSION

Mycelial growth of *P. commune* from 12 to 30°C is a temperature- and a_w-dependent process (Table 3), with maximum development at intermediate temperatures (20 and 25°C) and a_w (0.97 and 0.96). Growth is not significantly affected in the whole range of ecological conditions tested (Fig. 1). Only when both limiting factors are combined, i.e., low a_w together with high or low temperatures, colony diameter drops sharply. However, ripening of some meat products, such as dry-cured ham, takes place typically above 12°C with an a_w of more than 0.90 on the surface in the first stages of processing *(3, 20, 30).* This ability to grow at a fast rate on a wide range of ecological conditions on a meat extract–based medium is consistent with its frequent detection on dry-cured ham at different ripening times *(19).*

The main interest for the growth of *P. commune* at intermediate a_w relies on its toxigenic activity *(19).* Similar to mycelial growth, *P. commune* produced CPA in a meat extract–based medium (MPA) at a wide range of temperatures (12 to 30°C) and a_w (0.99 to 0.90), except when both factors were at the lowest values (12°C, 0.90 a_w).

A_w and temperature also affected CPA concentration (Table 3, Fig. 2), and the two-factor interaction was also significant (Table 3). Temperature was the single parameter having more influence on CPA production on MPA, as it has been shown on carbohydrate-containing media *(8).* This fact contrasts with those reported for mycotoxin biosynthesis by *Aspergillus flavus* and *Aspergillus parasiticus (9, 17)* and for verrucosidin synthesis by *Penicillium polonicum (18),* where a_w is the most important single factor for mycotoxin accumulation. It does not seem that 5% NaCl has a big impact on CPA production below 25°C. However, an additional 20% meat extract resulted in an increase in CPA production at every temperature, which cannot be explained

TABLE 3. *ANOVA for radial growth and CPA production by* P. commune *on MPA*

Source	df[a]	Radial growth Mean square	F[b]	CPA production Mean square	F
a_w	3	1,058	94	74,128	705
Temperature	3	891	79	103,126	981
$a_w \times$ temperature	9	117	10	31,186	297

[a] df, degrees of freedom.
[b] F, variance ratio. $P < 0.001$.

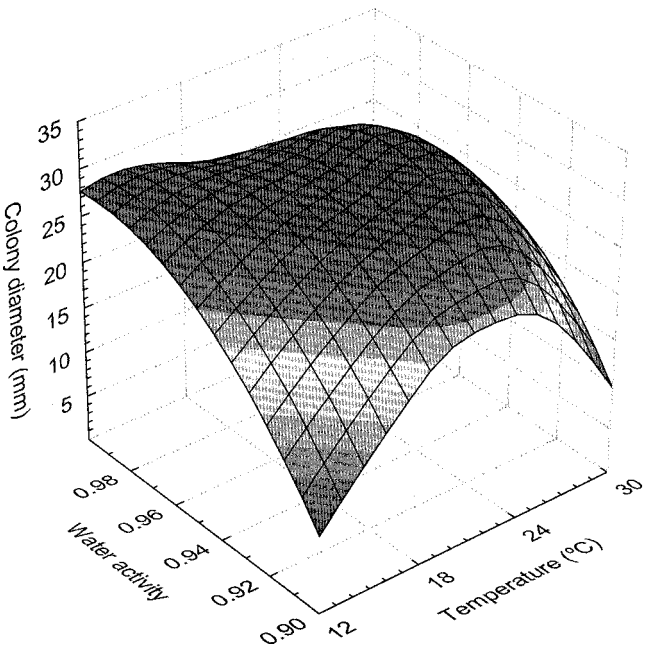

FIGURE 1. *Response surface plot of mycelial growth of* P. commune *(colony diameter in millimeters) as affected by temperature and* a_w *on MPA.*

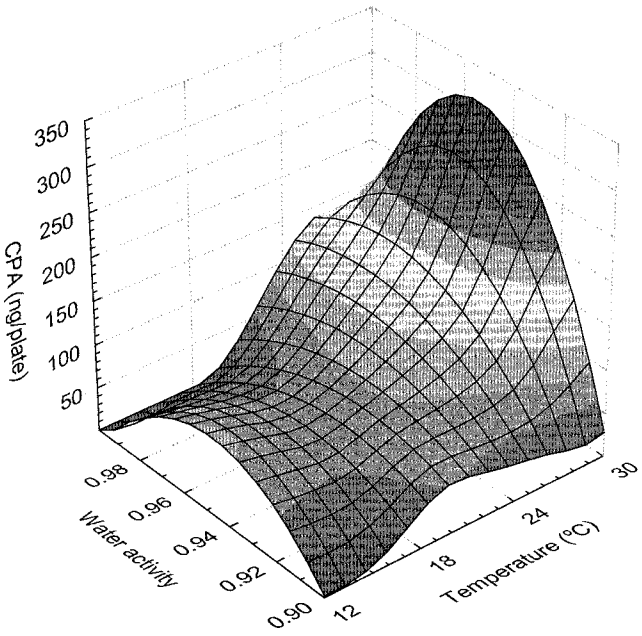

FIGURE 2. *Response surface plot of mycelial growth of CPA production by* P. commune *(nanograms per plate) as affected by temperature and a_w on MPA.*

by a_w reduction from 0.97 to 0.96. The difference could be due to the amount of meat extract in the medium. Since tryptophan is the precursor of CPA *(11),* increasing the concentration of meat extract would provide more substrate for CPA synthesis. Moreover, the stimulating effect of meat extract could be related to the fact that the strain of *P. commune* studied had been isolated from dry-cured ham and the amount of nitrogen compounds on MPA, with 22.5% meat extract, is estimated to be similar to that found on dry-cured ham *(3).* However, decreasing a_w to 0.90 depressed the biosynthesis of CPA except at 20°C. The only difference with the medium at a_w 0.96 is 20% glycerol, but very little effect of this compound on CPA production is expected. In fact, CPA production at 0.90 a_w is similar to that at 0.97 a_w, both at 20 and 25°C.

The influence of each ecological condition (a_w or temperature) on CPA production seems to be stronger at the optimum value of the other factor. Thus, the influence of temperature was stronger at high and intermediate a_w and that of a_w at higher temperatures (Table 2).

These results reveal a poor correlation between mycelial growth and CPA production. The ecological conditions for maximum toxin yield (30°C, 0.96 a_w) differ from those for maximum mycelial growth (20 and 25°C, 0.97 and 0.96 a_w). Thus, CPA production increases under suboptimal growth conditions, as has been reported for other mycotoxins *(2, 18, 28),* but it is favored by the extra 20% meat extract.

On media containing a high level of carbohydrates (yeast extract agar and Czapek yeast extract agar), Gqaleni et al. *(8)* found optimum CPA production at a lower temperature (25°C) and higher a_w (0.996), and the yield was also higher than the one obtained here on MPA *(6, 8).* Although differences in CPA production could be due to the natural variability and different origin of isolates *(6, 12),* media composition may play an important role, as has been shown for verrucosidin production by *P. polonicum (18).*

The interaction of ecological factors (a_w and temperature) is critical for *P. commune* growth and CPA production on MPA (Table 3), as other multifactorial studies have demonstrated for fungal growth and mycotoxin production for a variety of molds and substrates *(8, 15, 18).* Perhaps none of these factors has an overriding effect on growth and mycotoxin production, but each one contributes to the final outcome.

The low mycotoxin production at 0.90 a_w and the lower a_w reached on the surface of some dry-cured meat products at the end of ripening process *(20, 30)* contribute to the overlooking of this hazard. However, slow production of CPA during the long ripening of these products could be cause for concern in the fully ripened product. Leistner *(13)* reported that CPA is the major mycotoxin produced by penicillia isolated from mold fermented sausages, suggesting that cured meats should be surveyed for this mycotoxin. Similar results have been obtained with growth and verrucosidin production by *P. polonicum* isolated from dry-cured ham *(18).* For this, the ability of different molds to produce toxins on meat products, particularly on dry-cured ham, should be studied.

The hazard for CPA and verrucosidin could be overcome by lowering ripening temperature below 12°C. However, the biochemical reactions that take place during ripening would become too slow and any positive contribution from the mold population *(16, 25)* would be minimized. Therefore, appropriate starter cultures of molds should be used to avoid the presence of mycotoxin-producing molds in meat products, keeping the positive effect of microorganisms.

The present study demonstrates that *P. commune* produced CPA on a meat-based substrate, with an extremely low level of carbohydrates in a wide range of a_w and temperature. These ranges of ecological factors are reached in some fermented meat products during the ripening process *(3, 20, 30).* Thus, the presence of *P. commune* on this kind of meat product could be a hazard for human health, which could be prevented using starter cultures.

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