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The effect of water activity and temperature on the production of some mycotoxins

CENTRALE LANDBOUWCATALOGUS
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M. D. NORTHOLT

The effect of water activity and temperature on the production of some mycotoxins

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op vrijdag 7 december 1979
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

ISN 103194

The investigations described in this thesis were carried out in the National Institute of Public Health, The Netherlands, with financial support of the Chief Veterinary Inspectorate of Public Health.

BIBLIOTHEEK L.H.
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ONTV. TIJDSCHR. [illegible]

Cover: colony of Aspergillus ochraceus *NRRL3519 grown on malt extract sucrose agar medium with a water activity of 0.99 and incubated at 24 °C for 74 hours (7 ×).*

NN08201, 784

STELLINGEN

1. In tegenstelling tot de algemeen heersende opvatting kunnen mycotoxinen reeds tijdens het begin van schimmelgroei worden gevormd.

Dit proefschrift

2. Voor de bestudering van de vorming van mycotoxinen verdienen culturen op vaste voedingsbodems de voorkeur boven geschudde vloeistofculturen.

Dit proefschrift

3. De afwezigheid van penicillinezuur in beschimmelde droge worsten is niet een gevolg van ontleding maar van remming van de biosynthese van dit mycotoxine.

A. Ciegler, H. J. Mintzlaff, D. Weisleder en L. Leistner. 1972. Appl. Microbiol. 24: 114-119

4. Mycologisch onderzoek vooraf kan de trefzekerheid van chemisch onderzoek naar mycotoxinen verhogen.

5. Bij de beschrijving van een acute aflatoxinevergiftiging doet Graham Greene het waarnemingsvermogen van zijn hoofdpersoon onrecht aan.

Graham Greene. 1978. The human factor

6. Het opnemen van microbiologische eisen in de E.E.G.-richtlijn voor het gebruik van de „spinchiller" in pluimveeslachterijen is voor een goede hygiënische werkwijze niet noodzakelijk.

7. Het steriliseren van voorwaardelijk goedgekeurd vlees volgens de vleeskeuringswet levert slechts een beperkt houdbaar produkt op.

8. Het verschil in opbrengst tussen landbouwgewassen die geproduceerd zijn met stikstofkunstmest of met behulp van biologische stikstofbinding zal bij toenemende energieschaarste geen beletsel vormen om te kiezen voor de laatstgenoemde methode, vooral in de tropen.

9. Er bestaan onvoldoende gegevens over de eventuele kankerverwekkende eigenschappen van patuline.

H. Oswald, H. K. Frank, D. Komitowski en H. Winter. 1978. Fd. Cosmet. Toxicol. 16: 243-247

10. De toxiciteit van penicillia op semi-synthetische media behoeft geen indicatie te zijn voor de vorming van toxinen als deze schimmels bij de kaasbereiding worden gebruikt.

P. Lafont en J. Lafont. 1976. Fd. Cosmet. Toxicol. 14: 137-139

11. Het stellen van bakteriologische eisen aan water op kweekplaatsen voor schelpdieren biedt slechts een schijnzekerheid met betrekking tot de afwezigheid van microbiële gezondheidsrisico's.

S. M. Goyal, C. P. Gerba en J. L. Melnick. 1979. Appl. Environ. Microbiol. 37: 572-581

12. Uitgaande van het principe „de gebruiker betaalt" zou bruggeld niet bij de schippers, maar bij de weggebruikers geheven dienen te worden.

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BIBLIOTHEEK
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Acknowledgements

I would like to express my gratitude to Professor E. H. Kampelmacher for his guidance during the preparation of this thesis.

I would like to express my gratitude to the directorate of the National Institute of Public Health for giving me the opportunity and excellent facilities to prepare the thesis.

I would also like to express my appreciation to the head of the Laboratory, Dr. M. van Schothorst, for his encouragement and criticism.

I am much indebted to Dr. P. L. Schuller and Ir. H. P. van Egmond of the Laboratory of Chemical Analysis of Foodstuffs of the Institute for their co-operation with regard to the chemical analysis of the fungal cultures and their criticism. I also wish to thank the co-workers of this laboratory, Mr. W. E. Paulsch, Mr. E. A. Sizoo and Miss W. E. Deijll, for the analysis of the numerous cultures.

I wish to thank all my colleagues, especially Dr. Ir. S. H. W. Notermans, for inspiring discussions. I also wish to thank Miss P. S. S. Soentoro for identifying the fungal strains and Mr. E. Denegri, Mr. C. J. Heuvelman and Mrs. M. E. van Erne-van der Tol for their skillful technical assistance.

I am grateful to Dr. J. Harwig, Health and Welfare Canada, Food Protection Branch, Microbiology Division, Ottawa, for his suggestions and criticism. I thank Dr. G. M. D. Hogeweij for his contribution to the calculations of the model study. Special thanks go to my wife for the retrieval of literature and correction of the manuscript.

Finally I would like to express my thanks to my secretary, Mrs. B. E. Puyk-Blauwendraat, Mr. J. W. Weiss for documentation, the personnel of the typing pool, graphics department, photographer's studio, library, technical department, and other colleagues and friends who have helped me in any way.

Aan Hanneke

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2. Differences between *Aspergillus flavus* strains in growth and aflatoxin B_1 production in relation to water activity and temperature. J. Food Prot. 40 (1977) 778-781
3. Patulin production by some fungal species in relation to water activity and temperature. J. Food Prot. 41 (1978) 885-890
4. Penicillic acid production by some fungal species in relation to water activity and temperature. J. Food Prot. 42 (1979) 476-484
5. Ochratoxin A production by some fungal species in relation to water activity and temperature. J. Food Prot. 42 (1979) 485-490

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ening process are often also
unintended growth of molds, which may
ccur if no preventive measures are taken (6, 17, 19, 20).

Mycotoxin contamination of meat, eggs and milk may occur after feeding farm animals with contaminated rations. Transmission of aflatoxins and ochratoxin A have been demonstrated in dairy cattle, pigs and poultry (4, 12, 23) and is the cause of the extended contamination of milk and dairy products with aflatoxin M_1 (31, 33).

CONDITIONS LEADING TO MYCOTOXIN CONTAMINATION

Toxinogenic molds may invade agricultural produce during plant growth, during harvest and after harvest. Fusaria, which thrive under wet conditions, may infest growing plants and grow on the wet-stored product. Aspergilli and penicillia are mostly confined to the stored products, because of their adaptation to dry conditions. However, it has been established that *Aspergillus flavus* may also infect certain crops in the field. Since aflatoxin contamination of peanuts and corn is most studied (2, 13), the factors which favor aflatoxin contamination of these crops are described below.

The general view is that *Aspergillus flavus* may invade insect-damaged and mechanically-damaged plant tissue. Factors are insect population, amount of fungal spores in the field and the susceptibility of the plant depending on type of crop, variety and health. At harvest the crop may be exposed to mechanical damage and too rapid drying may cause cracks that serve as "porte d'entree" for molds. After harvest, accidental rewetting by condensation or leakage may permit mold growth. At all stages, and particularly during storage, moisture and temperature are important in the control of aflatoxin contamination. Time is also a factor, since time is required for processes such as spore germination, growth and mold penetration of substrate, and aflatoxin production. As far as is known these factors play a role in the etiology of contamination of agricultural commodities with other mycotoxins as well.

Water activity (a_W) has taken the place of moisture as the most useful expression of the availability of water for growth of micro-organisms (24). The a_W of a food is defined as the ratio of vapor pressure of the food and pure water. The a_W of a food product is low when the solute molecules bind water molecules. Then, water molecules are less free to escape from the surface of the food product into the vapor phase, and the vapor pressure is low. When bound, water molecules are not available for fungal growth. Therefore fungal growth is related to the a_W.

It can be concluded that a thorough understanding of the main factors which influence fungal growth and mycotoxin production can help in preventing mycotoxin contamination of foodstuffs. Prevention is preferable since destruction or removal of mycotoxins from food is not fully efficient. Moreover, when chemical treatment is applied, the food value of the product may be reduced and the formation of biologically active reaction products may be induced.

IMPORTANT FUNGI AND THEIR MYCOTOXINS

The fungal species of the genera *Aspergillus*, *Penicillium* and *Fusarium* are the most common toxinogenic molds. The following mycotoxins have been found in nature; aflatoxins, sterigmatocystin, ochratoxins, citrinin, patulin, penicillic acid, zearalenone and T-2 toxin. Only a few mycotoxins are investigated intensively in this study. (i) Aflatoxins and (ii) ochratoxin A were the mycotoxins of choice because of their frequent occurrence in agricultural products and their implication in natural cases of mycotoxicity (26). (iii) Patulin has been chosen for its frequent occurrence in apples and apple products, which is an important food in The Netherlands (15, 32, 34). (iiii) Penicillic acid has been chosen since *Penicillium cyclopium*, a potential producer of this mycotoxin, has been determined as the dominant fungal species in a large number of foods (15, 17, 18, 20). Little work has been done to examine foodstuffs for the presence of penicillic acid. The results of the current investigation might indicate under what conditions foodstuffs become contaminated with penicillic acid, which is necessary to take preventive measures. Moreover, they may help the analytical chemists to find mycotoxin-contaminated foodstuffs.

AIM AND SEQUENCE OF THE INVESTIGATION

Preventing mold from growing and producing m
cotoxins requires knowledge of the conditions und
which each of the toxinogenic molds becomes acti
In this investigation the relation between fu
growth and mycotoxin formation and the factors
ter activity and temperature is studied. Various fu
species known to produce mycotoxin were t
Optimal substrates for production of the part
mycotoxin were used to determine the lowest
ble water activity and temperature at which
toxins may be produced.

In article 1 a water activity measurement
described which has been designed for accur
surements. The first study encompasses the
production by *Aspergillus parasiticus* at diff
ditions of water activity and temperature.
is gained with both liquid cultures and su
res. Because of its reproducibility and res
mold growth in practice, the surface cult
red and used in the following investigatio

In article 2 attention is paid to differe
Aspergillus flavus strains in their respo
conditions of water activity and temper

In articles 3, 4 and 5 the results of si
tions with patulin, penicillic acid an
respectively, are described and discu
of water activity and temperature o
duction is studied with semi-syntheti
also with some natural substrates.

In the last experimental sectio
effect of water activity and temper
toxin production rate and mycot

time of the four mycotoxins is described. For this a calculation model is developed to exclude the influence of mycelial weight and growth time on the outcome of the experiments.

In the chapter Discussion the results of articles 1-5 are discussed in the light of the results described in the last experimental section. In a final discussion the practical implications of the results with respect to safeguarding of foods from mycotoxin contamination are considered.

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Effect of water activity and temperature on the mycotoxin production rate and mycotoxin production lag time

ABSTRACT

The effect of water activity (a_W) and temperature on the mycotoxin production rate (MPR) and mycotoxin production lag time (L) was investigated for the production of aflatoxin B_1, patulin, penicillic acid and ochratoxin A by *Aspergillus flavus*, *Penicillium expansum*, *Penicillium martensii* and *Aspergillus ochraceus*, respectively. For nearly all experiments the amount of mycotoxin produced could be correlated linearly with mycelial mass and production time. The values of MPR and L were estimated from the increase of mycotoxin produced and mycelial dry weight of agar cultures, using a newly developed calculation model. A decrease in a_W or temperature below the optimum value for mycotoxin production decreases the MPR of aflatoxin B_1 and patulin, whereas neither the MPR of penicillic acid nor the MPR of ochratoxin A was effected within the a_W and temperature range tested. The MPR values of the two *A.flavus* strains tested differed by a factor 19. The L values varied from 1 to 36 h depending on the type of mycotoxin and the condition of a_W and temperature. An aeration experiment indicated that the production of patulin and penicillic acid was more sensitive to lack of oxygen than the production of aflatoxin B_1 and ochratoxin A.

INTRODUCTION

The objective of the investigation

In the articles the effect of water activity (a_W) and temperature on mycotoxin production and growth was studied. The results described, however, have their limitations, since the amount of mycotoxin in the cultures was determined at the time that the colonies reached a diameter of 3 cm. In this way the amount of mycotoxin was dependent on the radial growth rate of the colonies. Moreover the amount of mycotoxin could be in proportion to the amount of mycelium within the colonies. Therefore the differences between the amounts of mycotoxin produced at different conditions of a_W and temperature might be explained partly by the influence of radial growth rate and mycelial density.

The objective of the investigation described in this chapter is to determine the effect of a_W and temperatures on merely the mycotoxin producing enzymes complex, without the influence of the other factors.

Therefore two variables of cultures grown at different conditions of a_W and temperature were estimated: the mycotoxin production rate (MPR), which is defined as the amount of mycotoxin produced per mg mycelial dry weight and per hour, and the mycotoxin production lag time (L), which is the period of time that elapses until the newly formed mycelium starts producing mycotoxin.

To meet the objective, a calculation model is developed, which is based on the suppositions that the mycelium produces mycotoxin at a constant rate, and that every piece of mycelium takes part in the production of mycotoxin and produces mycotoxin at the same rate. The experiments include the determination of mycotoxin and mycelial dry weight of a number of agar cultures incubated for different periods of time and at different conditions of a_W and temperature.

The experiment was undertaken with fungal species which produce different mycotoxins, i.e. aflatoxin B_1, patulin, penicillic acid and ochratoxin A. Moreover two *Aspergillus flavus* strains, a low and a high producer of aflatoxin B_1, were compared. As the incubation of cultures took place in polyethylene bags, the possible limitation of oxygen supply was investigated. To check the accuracy of the determination of mycelial dry weight, the amount of mycelium was also determined by measuring the volume of colonies, which provided further information about changes of hyphal density due to the cultural conditions.

Fungal colony growth

To understand the calculation model and the suppositions made, a general description of colony growth and fungal metabolism is given.

After germination of a fungal spore on the surface of an agar medium, one or more hyphae grow out. The circular form of the fungal colony is established as a result of apical growth and branching of the hyphae. During the formation of the colony, the fungus develops aerial and submerged hyphae (Fig. 1). Growth of a colony can be considered from two points of view, growth at the margin and growth within the colony. As described in the articles for a number of aspergilli and penicillia, the growth at the margin takes place at a constant rate. Within the colony, growth slows down progressively. A maximal hyphal density is attained gradually, there being a gradient between the central mass of maximum density and the margin (9). Below the surface of the colony, hyphal density decreases exponentially with depth (15). The margin of the colony consists of newly formed hyphae which form metabolites essential for growth like proteins, nucleic acid, lipids and carbohydrates, commonly called primary metabolites. More distal portions of the hyphae are progressively older and, in a period of transition, the metabolism changes from what is called primary to secondary

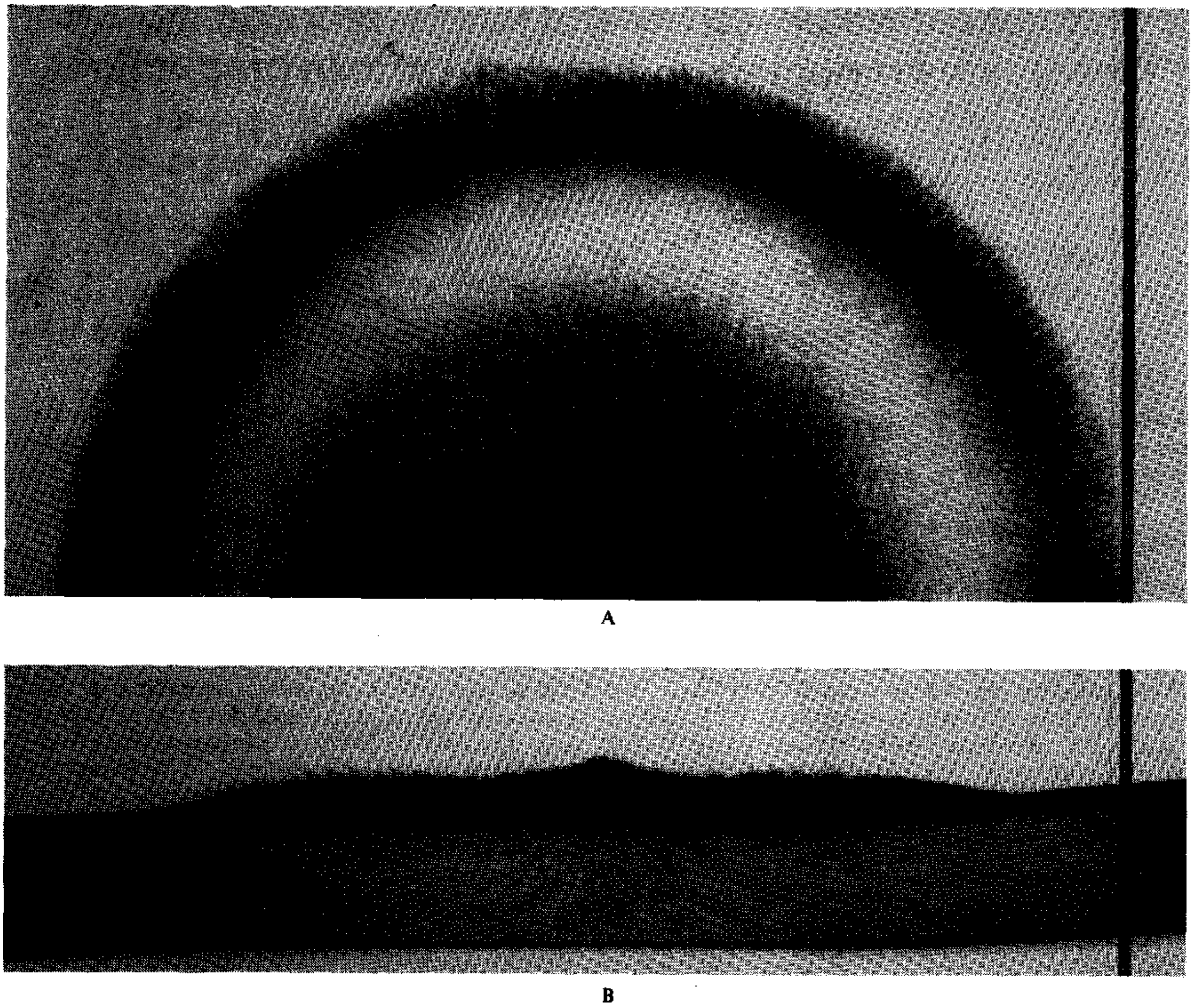

Figure 1. *A colony of* Penicillium expansum *RIV11 on malt extract sucrose agar medium of 0.99 a_w and grown at 24°C for 75 hours (6 ×). A: View from above of the aerial mycelium. B: Transverse view of the submerged mycelium.*

metabolism. Secondary metabolites, such as mycotoxins, have no apparent significance in fungal growth or physiology (7). Detroy and Hesseltine (6) found that the transition period of *Aspergillus parasiticus* is marked by a decrease of protein synthesis and RNA synthesis. The most commonly agreed upon postulate suggests that secondary metabolites are formed when large amounts of primary metabolic precursors, such as acetate, malonate, pyruvate, and amino acids, accumulate (7). This postulate seems of value for aflatoxins, patulin, penicillic acid and ochratoxin A since the main precursor of these mycotoxins is acetate and for penicillic acid also malonate (1, 7, 12, 13). It should be noted that as long as the hyphae at the margin grow, which applies to the fungal cultures in the present investigation, the colony consists of juvenile and aged mycelial mass.

MATERIALS AND METHODS

Organisms and spore suspensions

Fungal strains were chosen which demonstrated a high production of mycotoxin in the experiments described in the articles. For the production of aflatoxin B_1, patulin, penicillic acid, and ochratoxin A these were *Aspergillus flavus* ATCC15517, *Penicillium espansum* RIV11, *Penicillium martensii* RIV159, *Aspergillus ochraceus* NRRL3519, respectively. Moreover an experiment was carried out with the aflatoxin-producing *Aspergillus flavus* RIV104, showing a lower optimum temperature than *Aspergillus flavus* ATCC15517, as was described in article 2.

Cultures grown for 7-10 days at 24°C on malt extract agar (Oxoid) were washed with an aqueous solution of 6 g of sodiumheptadecyl-sulfate/l (Tergitol-7, BDH) to prepare spore suspensions of ca. 10^6 spores per ml.

Cultural method

To investigate the influence of a_W and temperature on the production rate of a particular mycotoxin, three (four in case of patulin) experiments were carried out under different conditions of a_W and temperature. For each experiment 48 cultures on malt extract sucrose agar medium (Oxoid) were used. Various a_W conditions were achieved by adding sucrose. Agar plates with 20 ml of agar medium per plate were prepared as described in the appendix. The a_W measurements were performed with a recently developed device using a MBW dewpoint instrument type DP4 (8). The scheme of features of the device is similar to the device used previously and which is described in article 1.

Each culture consisted of two inoculated agar plates with three colonies each and one non-inoculated agar plate which served as control for the determination of the a_W after incubation. Agar plates of all cultures were inoculated within one hour with an inoculation needle dipped in a spore suspension. The three plates were enclosed in a 0.3 liter polyethylene bag (gauge 0.04 mm). The maximal oxygen permeability of the bag at 0 and 37°C was 1.5 and $1.6.10^{-2}$ g O_2 per h, respectively. The a_W of three control agar plates of each series was measured after incubation. The accuracy of the determination of the average a_W was 0.002 a_W. The maximal variation of the incubation temperature was 0.3°C.

Two cultures were used for the determination of the germination time. Therefore, the diameters of the colonies were measured daily. The germination time was obtained by extrapolating the regression line of the average diameter to the X-axis.

At regular intervals of time and until the colonies reached a diameter of 3 cm, four cultures were taken from the incubator. After the total surface area of the colonies of each culture was determined by measuring the diameter of the colonies, two cultures were used for the determination of mycelial dry weight and two cultures were used for the determination of the amount of mycotoxin. Moreover at intermediate times, extra pairs of cultures were taken for determination of mycelial dry weight. Besides, two one-colony cultures were used for measurement of the total volume of culture colonies.

To investigate whether a decrease of the mycotoxin production rate at the end of the incubation period might be due to lack of oxygen, four cultures of each fungal strain were grown at particular conditions of a_W and temperature. One pair of cultures, one of which was enclosed in a polyethylene bag and the other culture was aerated for 1 min twice a day, was incubated until the mycotoxin production decreased. Such another pair was incubated longer until the average diameter reached the size of 3 cm. The cultures were examined for the amount of mycotoxin.

Determination of mycelial dry weight

The mycelial dry weight was determined according to the method of Sutton and Starzyk (14) by placing the agar-mycelial contents of two petri dishes in a beaker of boiling distilled water for 4 min. The content of the beaker was then filtered through a Buchner funnel by using pre-dried and pre-weighed 9 cm Whatman no. 114 filter paper. The beaker and the mycelial mat on the filter paper were rinsed with 400 ml of boiling distilled water. The filter paper with the mycelium was dried in a 70°C forced-air drying oven for 24 h. The dry sample was placed in a desiccator with silica gel for 6 days and then weighed. The end weights were corrected for the loss of soluble substances from the filter paper, which was assessed by rinsing five pre-dried and pre-weighed filter papers with 400 ml of boiling distilled water and weighing after drying. The loss of paper substances amounted to 3 ± 0.6 mg. In each experiment, at least 18 cultures were sampled during the course of time for the determination of mycelial dry weight.

Determination of colony volume

The total volume of culture colonies is the sum of the aerial and the submerged mycelium volume. The aerial mycelium volume was determined by photographing a side view of one colony at regular time intervals, using a Zeiss Tessovar camera set at 2.5 fold magnification. Figure 2-A is an example of a photograph made at a particular incubation time. To estimate the volume the colony height was divided into 2-4 sections which determined 2-4 cylinders (Fig. 2-B). The total volume of the cylinders was used as an estimate for the aerial mycelium volume.

To estimate the submerged mycelium volume, a transverse section of 2 mm width was cut from a colony with a similar diameter as the colony used for the photograph of aerial mycelium. After staining in a lactophenol cotton blue solution (4) the section was viewed under a stereo microscope with seven fold magnification (Fig. 2-C). The submerged mycelia of the five fungal test strains were characterized by an upper layer with high hyphal density and a lower layer with low hyphal density (Fig. 2-D). The diameter and height of the layers and therefore the volume of the layers were estimated by two persons. Depending on the relative density of the lower layer, a percentage of 10, 20, 30 or 50% was added to the estimated volume of the upper layer to obtain the total volume of the submerged mycelium. The volumes estimated by the two persons, which did not differ more than 20%, were averaged. In each experiment the volume of the colony was estimated eight times during the course of time. It should be noted that in the method used the differences of density in the aerial mycelium are neglected and that the method lacks of precision in the estimation of the volume of the submerged mycelium.

Extraction and determination of mycotoxin

In the beginning, at each sampling time two cultures were taken for the analysis of mycotoxin in duplicate. Later on the two cultures were combined to make one sample for analysis of mycotoxin at each sampling time. Then the amount of mycotoxin measured was divided by 2. After taking them from the

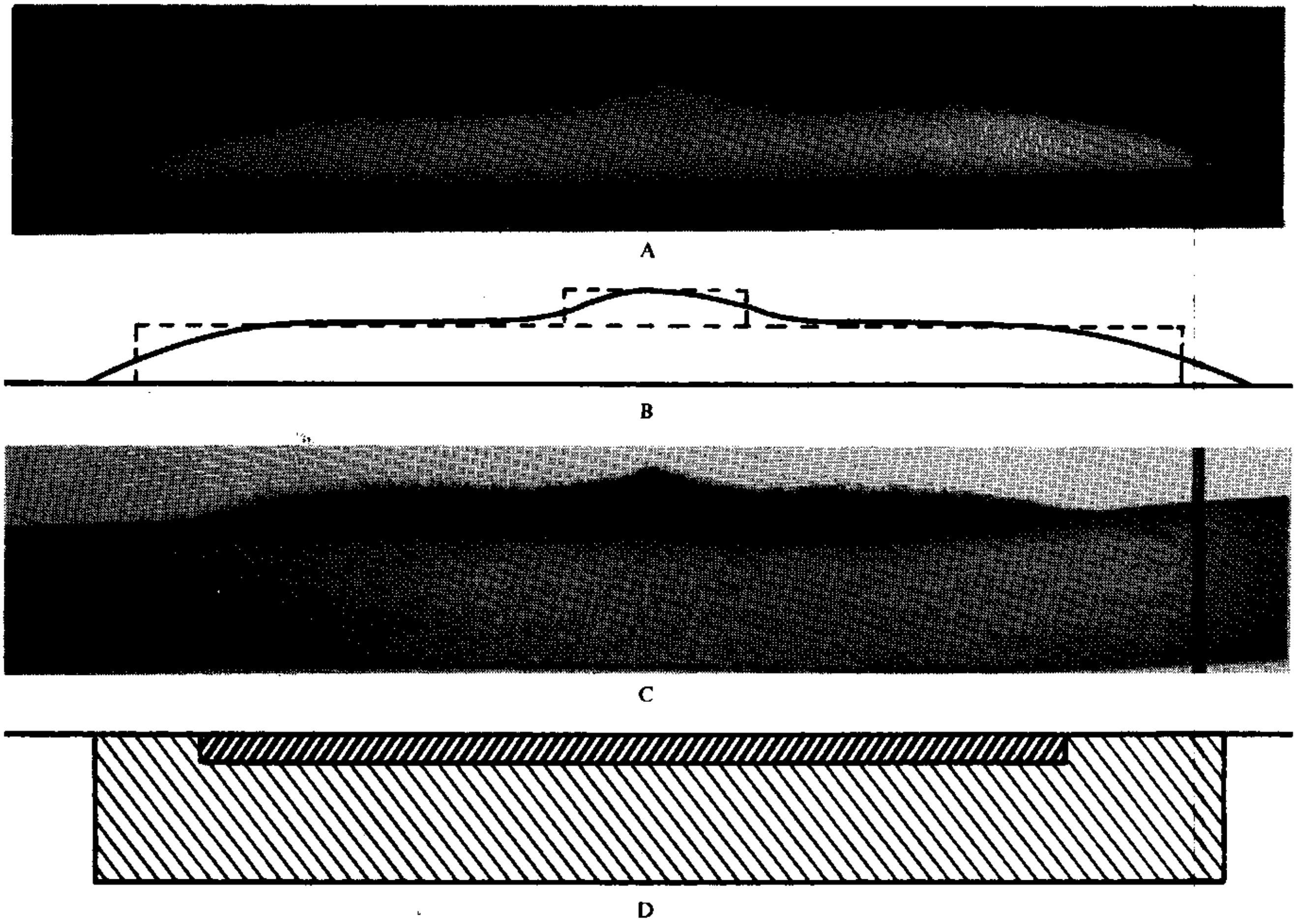

Figure 2. *Side view of a colony of* Penicillium expansum *RIV11 on malt extract sucrose agar medium of 0.99 a_w grown at 24 °C for 75 hours.*
A. Photograph of the aerial mycelium (7.2 x)
B. Drawing of two cylinder sections for estimation of the mycelium volume
C. Photograph of the submerged mycelium (6 x)
D. Drawing of the high density and low density layers for estimation of the mycelium volume.

incubator, the sampled cultures were placed at —18°C for max. 7 days. After thawing the amount of the particular mycotoxin, viz. aflatoxin B_1, patulin, penicillic acid or ochratoxin A, was determined according to the methods described in the articles 1, 3, 4 and 5, respectively.

Calculation scheme of the mycotoxin production rate (MPR)

As a consequence of the suppositions made in the introductory paragraph, the amount of mycotoxin produced is linearly related to growth time and mycelial mass, quantified as mycelial dry weight. The most important step in the calculation is the integration of mycelial dry weight over the period of growth, producing the theoretical value of the combined influence of mycelial mass and growth time. Another step, which is essential for the calculation, is the introduction of the mycotoxin production lag time (L), i.e. the period of time that elapses until the newly formed hyphae start producing mycotoxin. Hereunder the scheme of the calculation of MPR and L for one experiment including 48 cultures of a particular mold and incubated at one particular combination of a_W and temperature is given.

A part of the cultures, each consisting of 6 colonies, was used to measure the parameters amount of mycotoxin produced (x) and total surface area (a). Another part of the cultures was used to measure the parameters mycelial dry weight (w) and total surface area (a). In this way a is used to bring x and w into relation with each other. It should be noted that the parameters are expressed as a function of the growth time (t), commencing at the time of germination, and not as a function of the incubation time.

In Fig. 3 it is illustrated that the germination time can be determined by extrapolating the straight line through the averages of colony diameters to the X-axis.

The dry weight w of cultures used for the determination of mycotoxin is estimated by using the measured dry weight — surface relation and the surface — time relation, shown in Fig. 4 and 5.

Using Fig. 4 and 5, the dry weight of cultures used for the determination of mycotoxin can be plotted against growth time (Fig. 6).

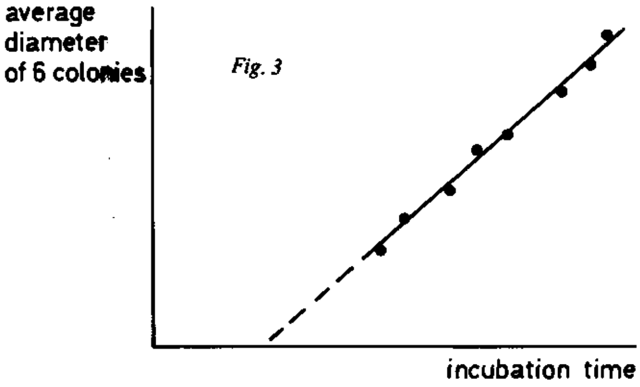

Fig. 3

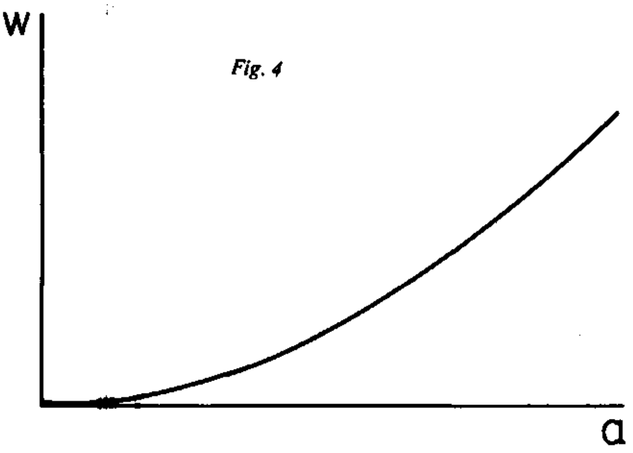

Fig. 4

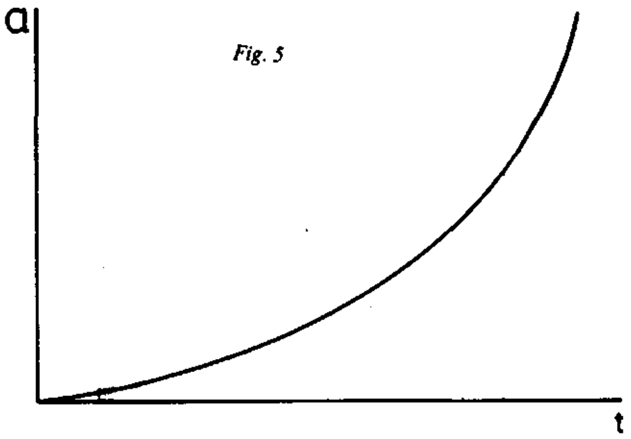

Fig. 5

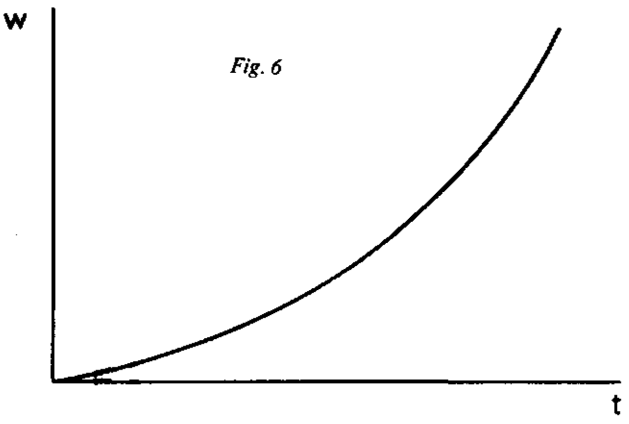

Fig. 6

At first it is assumed that the mycotoxin production lag time is zero. The increase of mycotoxin dx in a short time interval dt can be written as a function of the mycelial dry weight:

$$dx = c\,w(t)\,dt \tag{1}$$

in which c is a constant.
Then the amount of mycotoxin produced until growth time t_i is:

$$x(t_i) = c \int_0^{t_i} w(t)\,dt \tag{2}$$

Formula (2) shows how the amount of mycotoxin depends on mycelial dry weight and time. The integral in formula (2) equals the shaded area in Fig. 7.

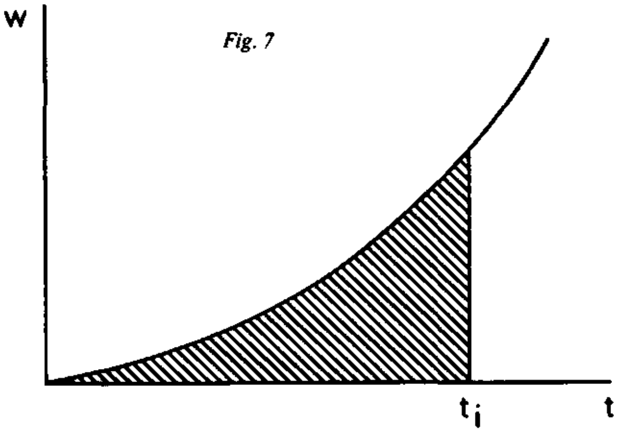

Fig. 7

If the mycotoxin production commences some time after the mycelium has been formed, the amount of mycotoxin produced until time t_i is less than that in formula (2). Assuming that the mycotoxin production lag time is L, then the mycelium formed during the last L hours does not produce mycotoxin and therefore does not contribute to the amount of mycotoxin at time t_i. Therefore the shaded area ends at $(t_i - L)$ hours (Fig. 8).

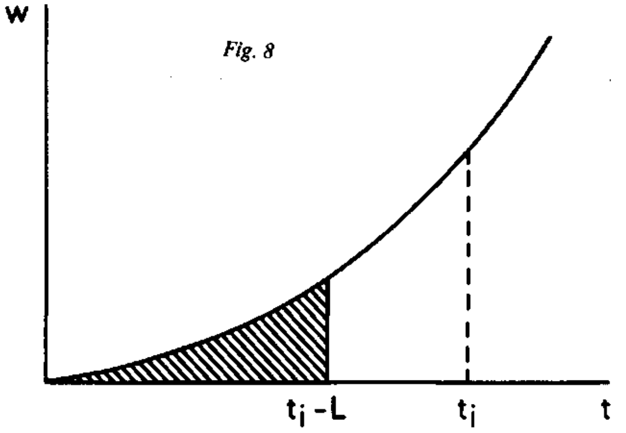

Fig. 8

The amount of mycotoxin produced until time t_i, corresponding with the shaded area in Fig. 8, can be written as:

$$x(t_i) = c \int_{0}^{t_i - L} w(t)\,dt \qquad (3)$$

Formula (3) can be rewritten as:

$$c = \frac{x(t_i)}{\int_{0}^{t_i - L} w(t)\,dt} \qquad (4)$$

The quotient in formula (4) represents the ratio of the amount of mycotoxin x at time t_i and the denominator which is the product of mycelium and production time. The ratio must be constant for the period of the experiment, and equals the mycotoxin production rate which is the production of mycotoxin per mg mycelial dry weight and per hour.

The lag time was found by trial and error. We chose some value for L and calculated the quotient c in formula (4) for a number of values of t_i. Thus a number of c values was obtained through which the linear regression line can be drawn. An example is shown in Fig. 9. A small L value may result in a negative slope.

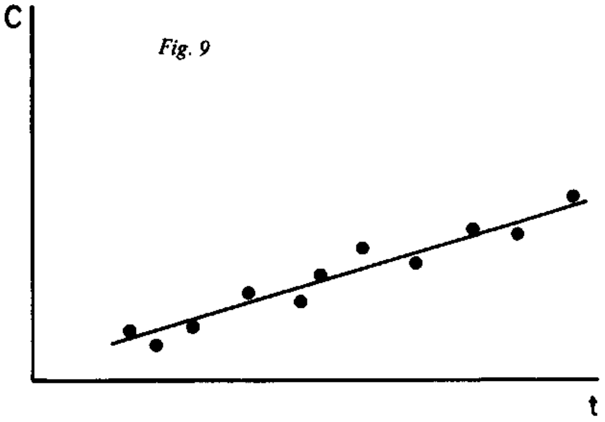

Fig. 9

If the slope of the regression line differs from zero it means that the denominator in formula (4) does not have a constant relationship with the amounts of mycotoxin measured at time t_i. Then the calculations are repeated using a different value of L until the slope of the linear regression line becomes zero. When the slope is zero, the denominator in formula (4) is the estimate of the combined influence of mycelial mass and growth time on the toxin production. Moreover, the chosen value of L that produces a zero slope can be considered as the best estimate for the lag time.

The best estimate of the lag time is used for the calculation of the quotient in formula (4), producing the best estimate for MPR. Besides the estimation of MPR it is desirable to calculate the 95 percent confidence limits of MPR. However, since the mathematical curve formulas of the mycelial dry weight-time and mycotoxin-time relationships are unknown, it is preferable to estimate the so-called range of possible values of MPR. Therefore, the 95 percent confidence limits of L and the range of possible values of $w(t_i)$ and $x(t_i)$ are estimated. The 95 percent confidence limits of L are obtained by calculating the regression line, as shown in Fig. 9, for a number of L values and testing whether or not the slope differs significantly from zero. Estimates for the minimal and maximal values of $w(t_i)$ and $x(t_i)$ are obtained by taking one half of the average deviation of the determined values from the curve. The range of possible values of MPR is estimated by taking the minimal and maximal values of L, $w(t_i)$ and $x(t_i)$.

RESULTS

Aflatoxin B$_1$ *production and growth*

Fig. 10-A and 10-B show an example of the measured mycelial dry weights and surface areas obtained with *A. flavus* RIV104. From these curves the mycelial dry weight curve in Fig. 10-C is obtained, which represents the mycelial dry weight w of the cultures used for the determination of aflatoxin B_1. The curves in Fig. 10-C were used for the calculation of the mycotoxin production lag time L and the mycotoxin production rate MPR. Similar curves obtained with *A. flavus* ATCC15517 grown under different conditions of a_W and temperature are shown in Fig. 11.

In Table 1 the estimates of L and MPR of cultures of *A. flavus* RIV104 and *A. flavus* ATCC15517 are shown. At similar conditions of a_W and temperature, *A. flavus* RIV104 needed more time to reach a surface area of colonies of 42.4 cm² and showed a significantly greater L value ($p < 0.10$) than *A. flavus* ATCC15517. The MPR value of *A. flavus* ATCC15517 was markedly higher than the MPR value of *A. flavus* RIV 104. The symbols used in Table 1 are explained in Table 2.

The results obtained with *A. flavus* ATCC15517 showed that cultures grown at 0.99 a_W and 24°C, had a much higher MPR value than those grown at the lower temperature, but there was no significant difference in L. In the experiment with cultures grown at 0.94 a_W and 24°C it was not possible to obtain a constant ratio of x and the denominator in formula (4) with any L value assumed. The small amount of aflatoxin B_1 produced at 0.94 a_W and 24°C was partly due to the small amount of mycelium which was only one third of the mycelium grown at 0.99 a_W and 24°C, and for the rest due to a low MPR.

The results of the cultures of *A. flavus* ATCC15517 grown at 0.99 a_W and 24°C, with and without aeration, showed no significant difference between the fungal growth rates and the amounts of aflatoxin B_1 produced.

In Fig. 12 the mycelial dry weight in relation to the colony volume of the *A. flavus* strains tested is shown. The intercepts of the ordinate cut by the curves are

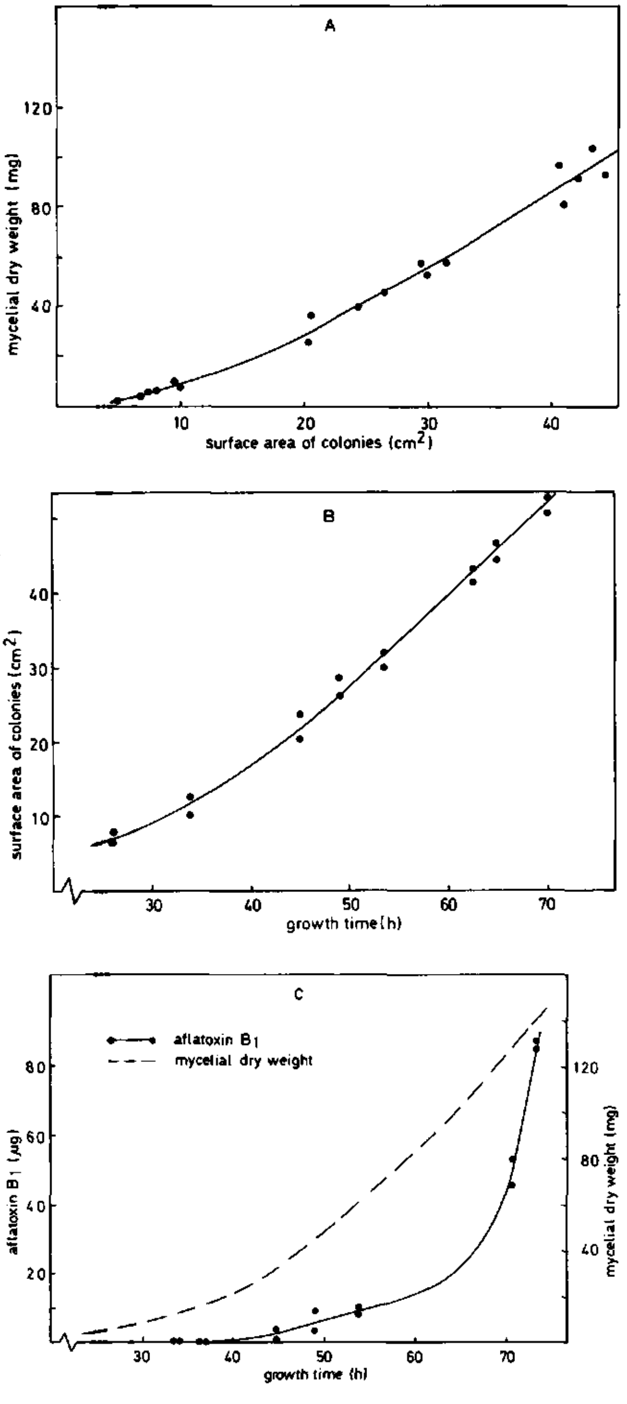

Figure 10. *Mycelial growth of and aflatoxin B_1 production by* Aspergillus flavus *RIV104 at 0.99 a_w and 24°C. A: Increase of mycelial dry weight in relation to the total surface area of colonies. B: Total surface area of colonies in relation to the growth time. C: Mycelial dry weight obtained from A and B, and aflatoxin B_1 produced at different growth times.*

small, indicating only little loss of mycelial substances during washing with hot water. Moreover the figure shows the effect of cultural condition on hyphal density.

In Table 3 the change of mycelial volume and dry weight due to a change of a_W or temperature is shown. Lowering of the a_W resulted in less submerged mycelium and a larger aerial mycelium with much lower hyphal density. Lowering of the temperature resulted in a decrease of hyphal density of the mycelium above and underneath the agar surface.

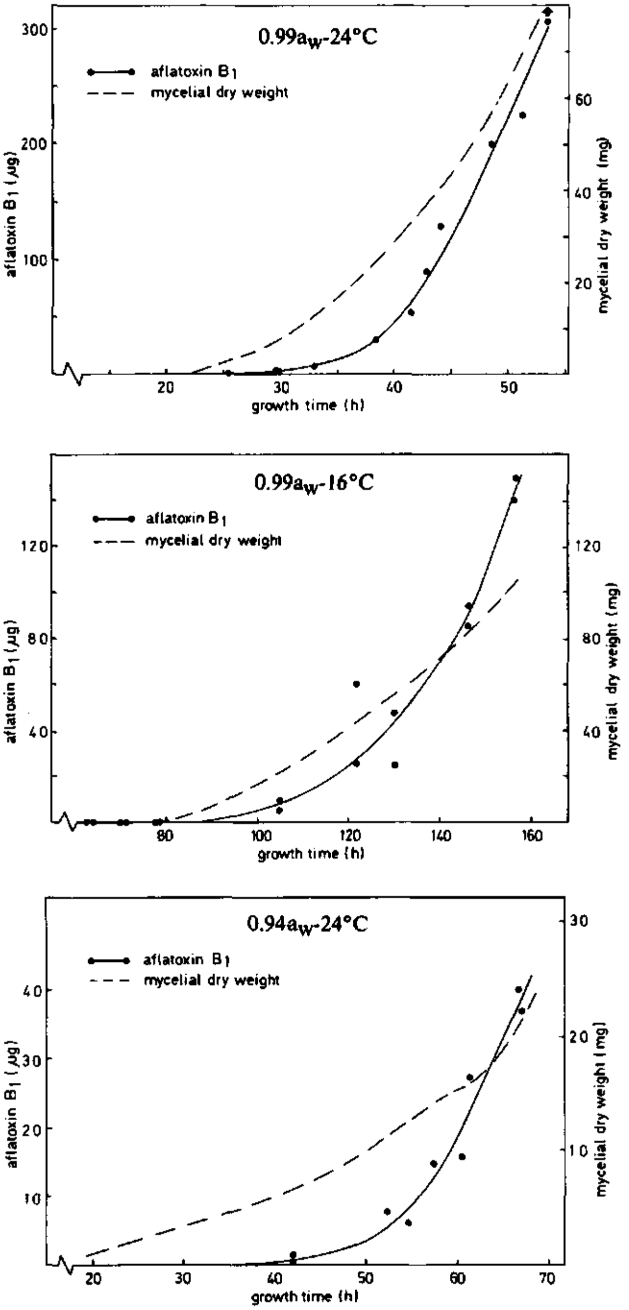

Figure 11. *Mycelial growth of and aflatoxin B_1 production by* Aspergillus flavus *ATCC15517 at three conditions of a_W and temperature.*

Patulin production and growth

In Fig. 13 the patulin production and mycelial dry weight of cultures of *P. expansum* RIV11 grown at four combinations of a_W and temperature are shown. The patulin production of the cultures grown at 0.99 a_W and 24°C, and 0.99 a_W and 16°C stopped after a growth time of 66 and 103 h, respectively, when the cultures reached a colony surface area of 31 and 36 cm², respectively. Therefore the variables L and

Table 1. *Estimates of aflatoxin B_1 production lag time (L) and aflatoxin B_1 production rate (MPR), and variables necessary for the estimation, of cultures of* Aspergillus flavus *RIV104 and* Aspergillus flavus *ATCC15517 at different conditions of a_w and temperature.*

Variable	Unit	A. flavus RIV104 Cultural condition 0.99 a_W-24°C	A. flavus ATCC15517 Cultural condition		
			0.99 a_W-24°C	0.99 a_W-16°C	0.94 a_W-24°C
t_g	h	20	23	67	36
$t_{42.4}$	h	62	51	140	69
L	h	12 (9-25)[a]	6 (5-7)[b]	4 (2-6)[b]	not estimable[c]
$x_{42.4}$	μg	17 (15-19)[d]	245 (225-265)[d]	69 (63-75)[d]	46 (42-50)[d]
$y(t_{42.4}$-L)	mg.h	515 (120-675)[d]	390 (310-470)[d]	1040 (740-1360)[d]	not estimable
MPR	10^{-2}μg.mg^{-1}.h^{-1}	3.3 (2.2-15.8)[d]	63 (48-87)[d]	6.6 (4.7-10.0)[d]	not estimable

[a] 90 percent confidence limits.

[b] 95 percent confidence limits.

[c] slope of lineair regression line shown in Fig. 9 is significantly ($p < 0.05$) larger than 0 for any value of L.

[d] range of possible values.

Table 2. *List of symbols.*

t_g	Germination time (h)
$t_{42.4}$	Growth time (h) until the total surface area of the culture reaches a size of 42.4 cm². Similar symbols are: t_{31}, $t_{35.6}$, and t_{36}
L	Mycotoxin production lag time (h)
$x_{42.4}$	Mycotoxin (μg or mg) produced until the total surface area of the culture reaches a size of 42.4 cm². Similar symbols are: x_{31}, $x_{35.6}$, and x_{36}
$y(t_{42.4}$-L)	Integrated mycelial dry weight over the growth time until the point of time ($t_{42.4}$-L), and equalling $\int_0^{t_{42.4}-L} w(t)\,dt$ (mg.h) Similar symbols are: $y(t_{31}$-L), $y(t_{35.6}$-L), and $y(t_{36}$-L)
MPR	Mycotoxin production rate (μg.mg^{-1}.h^{-1})

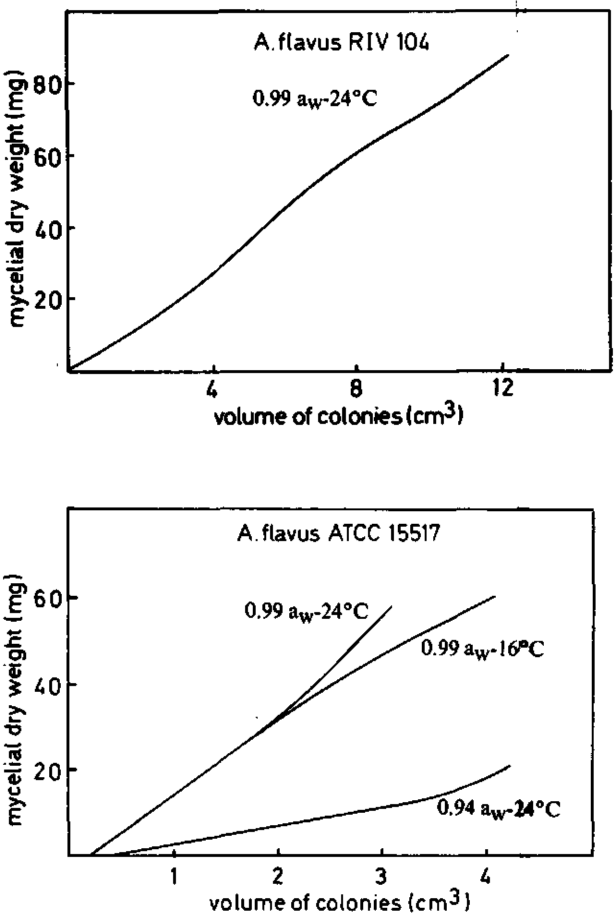

Figure 12. *Mycelial dry weight in relation to the volume of colonies of* Aspergillus flavus *RIV104 and* Aspergillus flavus *ATCC15517 grown at different conditions of a_w and temperature.*

Table 3. *Changes of volume of aerial and submerged mycelium and mycelial dry weight of* Aspergillus flavus *ATCC15517 due to a change of water activity (a_w) and temperature. All values are rounded-off.*

Change of cultural condition	Change of:		
	Mycelial volume		
	Aerial %	Submerged %	Dry weight %
0.99→0.94 a_W	+80	—50	—60
24 →16°C	+40	+40	0

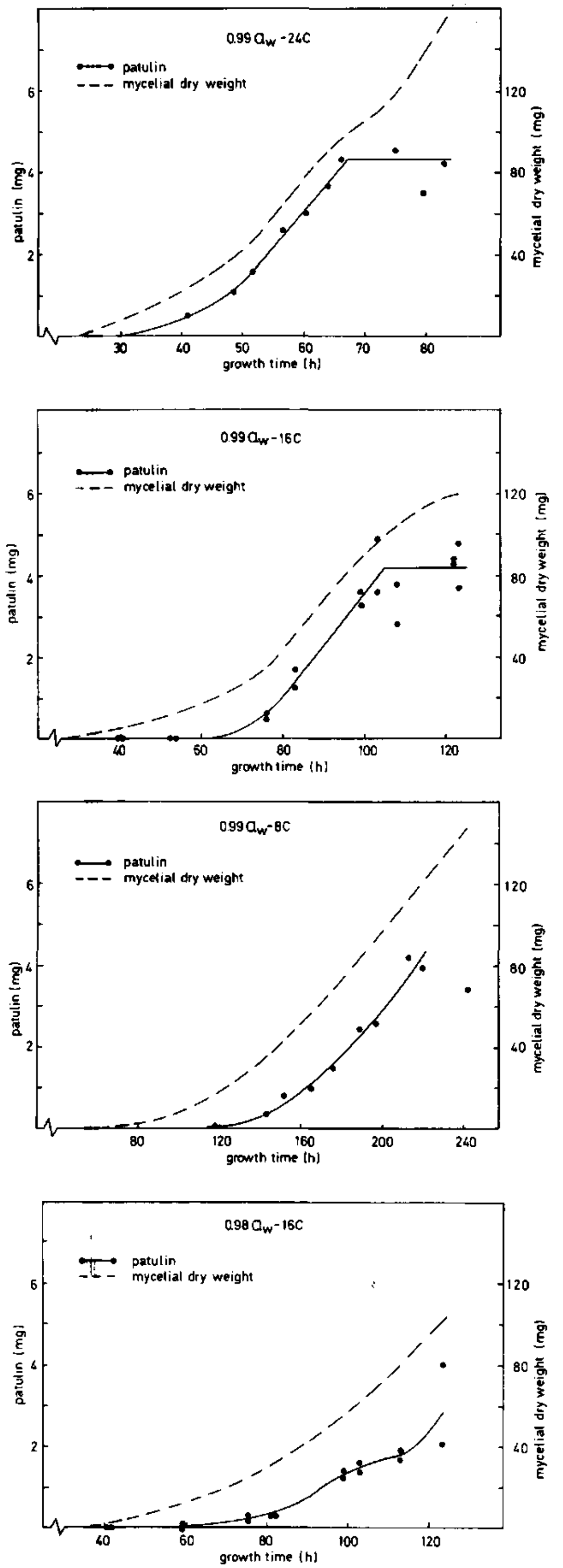

Figure 13. *Mycelial growth of and patulin production by* Penicillium expansum *RIV11 at four different conditions of a_w and temperature.*

MPR of all experiments were estimated for the period of time until the surface areas of the cultures reached 31 cm². It should be noted that the patulin production of one experiment, 0.98 a_W and 16°C, did not follow the expected pattern but increased step by step. Yet, the estimation was carried out to produce the average MPR value for the period of time mentioned.

Table 4 shows the estimates of L and MPR for cultures of *P. expansum* RIV11 grown under different conditions of a_W and temperature. At 0.99 a_W and 24°C no significant value of L could be noticed. However, at reduced a_W and temperature L amounted to a half and one day, respectively. MPR was maximal at 0.99 a_W and 16°C and was strongly dependent on the growth conditions, since at 8°C it was only a fifth of the maximal value. Also at the low a_W of 0.98 MPR was strongly reduced.

The aeration experiment revealed that the amounts of patulin produced in cultures of 31 cm² grown at 0.99 a_W and 24°C, with and without aeration, were 8.0 and 4.6 mg, respectively. The average amounts of patulin produced in cultures of 42.4 cm², with and without aeration, were 11 and 6.5 mg, respectively. Aeration had no effect on the radial growth rate.

In Fig. 14 it is shown that the intercepts of the ordinate cut by the curves are small, indicating only a slight loss of soluble substances from the mycelium during washing with hot water. Moreover the figure demonstrates that the hyphal density of *P. expansum* RIV11 grown at 0.99 a_W and 24°C is ca. twice as high as in cultures grown at lower a_W and temperature.

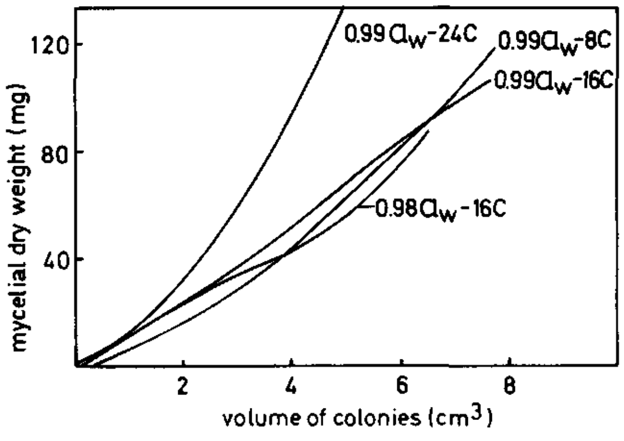

Figure 14. *Mycelial dry weight in relation to the volume of* Penicillium expansum *RIV11 grown at different conditions of a_w and temperature.*

In Table 5 the change of mycelial volume and dry weight due to a change of a_W and temperature is shown. Lowering of the a_W resulted in less submerged mycelium and a larger aerial mycelium with lower hyphal density. Lowering of the temperature resulted in a decrease of hyphal density of the aerial and submerged mycelium.

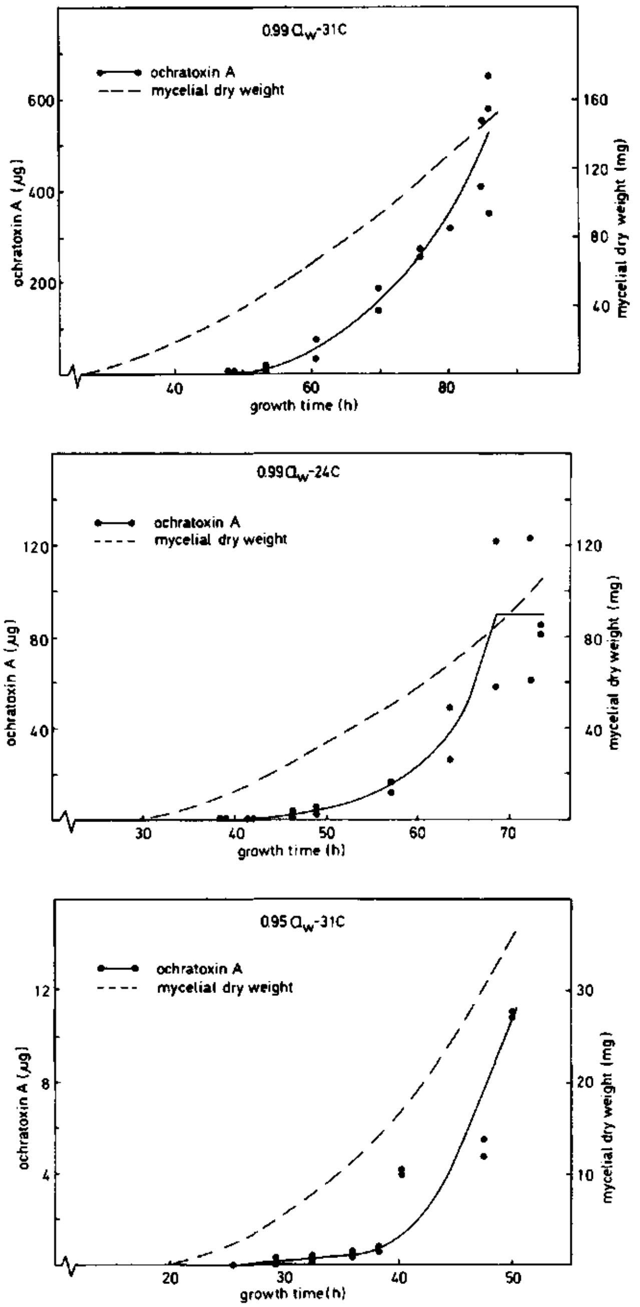

Figure 17. *Mycelial growth of and ochratoxin A production by* Aspergillus ochraceus *NRRL3519 at three different conditions of a_w and temperature.*

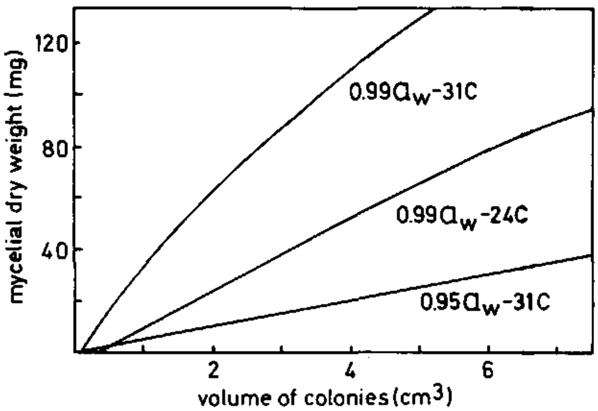

Figure 18. *Mycelial dry weight in relation to the volume of colonies of* Aspergillus ochraceus *NRRL3519 grown at different conditions of a_w and temperature.*

Table 9. *Change of volume of aerial and submerged mycelium and mycelial dry weight of* Aspergillus ochraceus *NRRL3519 due to change of water activity (a_w) and temperature. All values are rounded-off.*

	Change of:		
	Mycelial volume		
Change of cultural condition	Aerial %	Submerged %	Dry weight %
0.99→0.95 a_w	+30	—50	—80
31 →24°C	—10	+140	—40

Table 8. *Estimates of ochratoxin A production lag time (L) and ochratoxin A production rate (MPR) and variables necessary for the estimation, of cultures of* Aspergillus ochraceus *NRRL3519 at different conditions of a_w and temperature.*

		Cultural condition		
Variable	Unit	0.99 a_w-31°C	0.99 a_w-24°C	0.95 a_w-31°C
t_g	h	25	31	23
t_{36}	h	83	68	46
L	h	15 (14-16)[a]	10 (8-15)[a]	13 (12.5-13.5)[a]
x_{36}	µg	430 (410-450)[b]	90 (80-100)[b]	6 (5-7)[b]
$y(t_{36}$-L)	mg.h	1410 (1240-1610)[b]	630 (330-820)[b]	15.6 (12.1-19.1)[b]
MPR	10^{-1}µg.mg^{-1}.h^{-1}	3.0 (2.5-3.6)[b]	1.4 (1.0-3.0)[b]	3.7 (2.5-5.6)[b]

[a] 95 percent confidence limits.

[b] range of possible values.

DISCUSSION

In the articles the effect of a_W and temperature was investigated using cultures of colonies which were incubated until the colony diameter reached 3 cm. In this way the amount of mycotoxin determined at the end of the incubation period was not only dependent on the mycotoxin production rate but also on the radial growth rate and mycelial density of the colonies. In this chapter experiments are described which are carried out to estimate the effect of a_W and temperature on merely the mycotoxin production without their effects on mycelial growth.

From the results it can be concluded that the mycotoxin production rates of aflatoxin B_1 and patulin were influenced by a_W and temperature. A decrease in a_W or temperature below the optimum value decreases the production rates of these mycotoxins. Neither the penicillic acid production rate nor the ochratoxin A production rate was affected by these factors within the experimental range. The response of the mycotoxin synthetase could be different from what was suggested by the results in the articles. For instance, mycotoxin production rate of aflatoxin B_1 decreased to one tenth by a drop in temperature from 24 to 16°C, whereas the results in article 2 suggest that it decreased to one third. The reverse was true for ochratoxin A production, which appeared to decrease to one third by a drop of a_W from 0.99 to 0.95. However, the mycotoxin production rates at these values of a_W were similar.

The mycotoxin production lag time varied from 1 to 36 h depending on the type of mycotoxin and the effect of a_W and temperature. Considering only the significant effects, the results demonstrate that a decrease of a_W resulted in a shorter lag time for patulin and ochratoxin A, whereas a decrease in temperature resulted in a longer lag time for patulin and penicillic acid production.

Comparison of the data in Table 1 of two *A. flavus* strains learns that great differences of aflatoxin B_1 production rate could exist between different strains and that a more dense mycelium and a longer incubation time did not compensate for the much lower aflatoxin B_1 production rate.

For nearly all experiments with the different mycotoxins, the amount of mycotoxin produced could be correlated linearly with mycelial mass and production time. This indicates that the suppositions that all parts of the mycelium produce mycotoxin and that they do this with a constant production rate, could be true for certain conditions. However, in two experiments, *A. flavus* ATCC15517 grown at 0.94 a_W and 24°C, and *P. martensii* RIV159 grown at 0.997 and 16°C, the mycotoxin production rate increased and decreased, respectively, during the time. Since the results can not prove the validity of the suppositions further investigations are needed to solve this question.

The aeration experiments demonstrated that fungal growth and production of aflatoxin B_1 and ochratoxin A were not limited by enclosure of the cultures in polyethylene bags. Apparently the gas permeability of the bag was sufficient for growth and production of the two mycotoxins. In contrast, the patulin and penicillic acid production slowed down at conditions of a_W and temperature, where the fungal growth was fast. This phenomenon might be due to a lack of oxygen. Also, it was demonstrated that inhibition of patulin production could have occurred before the increase of the patulin curve broke off. This indicates that the production of patulin and in less extend the production of penicillic acid might require more oxygen than the production of aflatoxin B_1 and ochratoxin A. Besides, the results indicate that the production of patulin and penicillic acid requires more oxygen than mycelial growth, which did not slow down during the experiments.

The mycelial dry weight-volume curves of the different fungal species demonstrate that only little mycelial substance is lost in determinating the dry weight. Therefore the determination of dry weight is an accurate method for estimation of mycelial mass. It is concluded that the a_W and temperature had an influence on the hyphal density of the fungal colony. The hyphal density was at its highest at the highest a_W and temperature used in the experiments. For all test strains it appears that a decrease of a_W resulted in a thinner aerial mycelium and less submerged mycelium.

Our results indicate that the mycotoxins tested are produced immediately after the formation of the mycelium or a short time thereafter. The short time lags found for aflatoxin B_1 confirm the results of Reddy (10), but contrast with the results of Detroy and Hesseltine (6) who determined the toxin not until after ca. 50 h of growth time. The fact that other workers (2, 5, 6, 11) concluded that mycotoxins appear after a considerable period of time, can be explained by their method of cultures in batch fermentors. This type of cultures is characterized by an exponential growth phase, a period of decline of growth rate, and a stationary growth phase. It has been shown that the greater part of mycotoxin shows up at the end of the exponential growth phase. According to our results it can be expected that the great bulk of mycotoxin is produced immediately after the great bulk of mycelium is produced, that is in batch fermentor cultures at the end of the exponential phase. Moreover, the surface culture method should be preferred to the batch fermentor culture method because of its resemblance to fungal growth on foods where juvenile hyphae continuously arrive on fresh substrate and ageing mycelium is left behind on depleted substrate.

ACKNOWLEDGEMENTS

The author is grateful to Dr. R. A. Samson for lending the camera used in the hyphal density study.

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Erratum: Read 0.95 a_w for 0.98 a_w in Table 1 of article 3.

Discussion

COMPARISON OF CONDITIONS FOR PRODUCTION OF FOUR MYCOTOXINS

By way of comparison, the results described in the articles are summarized in Fig. 1 and Table 1. In Fig. 1 an overview of the conditions for growth of the fungal species and production of the different mycotoxins is given. It should be noted that the a_W and temperature ranges shown encompass the lowest and highest values, which may vary per strain and substrate. It is shown that aflatoxin B_1 can be produced at conditions of a_W and temperature which are close to the minimum a_W and temperature for growth. The results of Diener and Davis (11) and Sorensen et al. (31) indicate that the production of the aflatoxins B_2, G_1 and G_2 is also bound to these limits. Patulin, penicillic acid and ochratoxin A are produced within a much smaller range of a_W and temperature compared with those for growth. Moreover, the production of patulin and penicillic acid appears to be confined to high a_W only, except for *A. ochraceus* which could produce penicillic acid at low a_W on one particular substrate (poultry feed). Major differences exist between the genera *Aspergillus* and *Penicillium*. The minimum temperatures for growth and toxin production of aspergilli are higher than those of penicillia. The a_W minima for growth of penicillia differed from those of aspergilli. However, the a_W minima for mycotoxin production were species-related.

In Table 1 the influence of different fungal strains and substrates on the range and optimum condition of a_W and temperature for mycotoxin production is shown. Strains belonging to one species could have different ranges and optimum conditions of temperature. Moreover, they could have different a_W optima. The same is true for the composition of substrate, which may also affect the a_W range.

Table 1. *Conditions for mycotoxin production which are affected by fungal strain and substrate (T-temperature, a_W-water activity).*

Mycotoxin	Fungal strain	Substrate
Aflatoxin B_1	T range T optimum	a_W optimum T optimum
Patulin	T range T optimum	T optimum
Penicillic acid	T range T optimum	a_W range T range T optimum
Ochratoxin A	a_W optimum T range	T range T optimum

EFFECT OF AW ON GROWTH AND MYCOTOXIN PRODUCTION

In Table 2 the effect of a_W on two growth parameters, i.e. the mycelial dry weight and the radial growth rate, and on two parameters of mycotoxin production, i.e. the total amount of mycotoxin produced and the mycotoxin production rate is demonstrated. A decrease in a_W results in a decrease in mycelial dry weight, but has little or no effect on the radial growth rate. According to the data in Tables 3, 5 and 9 in the last experimental section, the decrease in mycelial dry weight of *A. flavus*, *P. expansum* and *A. ochraceus* is due to the decrease in submerged mycelium. Although the submerged mycelium of *P. martensii* does not seem to be decreased, this cannot be excluded because of the lack of precision in the method of determination. A decrease in a_W also results in a smaller total amount of mycotoxin and, for *A. flavus* and *P. expansum*, in a lower mycotoxin production rate. For all fungal strains the change in total amount of mycotoxin can be explained by a change in the mycotoxin production rate, mycelial dry weight and radial growth rate. For example, a decrease in a_W from 0.99 to 0.95 results in a 99% decrease in ochratoxin A which can be explained by the decrease in mycelial dry weight and the higher radial growth rate, which involves a shorter production time. In this way the effect of a_W on the total amount of mycotoxin can be satisfactorily explained by the effect of a_W on mycelial growth and the absence of an effect on the mycotoxin production rate. However, it should be emphasized that the mycotoxin production rate has been calculated from the mycelial dry weight and the growth time. This means that the mycotoxin production rate is not an independently measured variable. Moreover, the calculation of the mycotoxin production rate is based on the supposition of a linear relationship between the mycotoxin produced and the mycelial dry weight and growth time. It should also be taken into consideration that the time-course experiments are limited to only two conditions of a_W. Therefore, our explanations of the effect of a_W on the amount of mycotoxin produced should not be considered as pieces of evidence, but rather as useful wor-

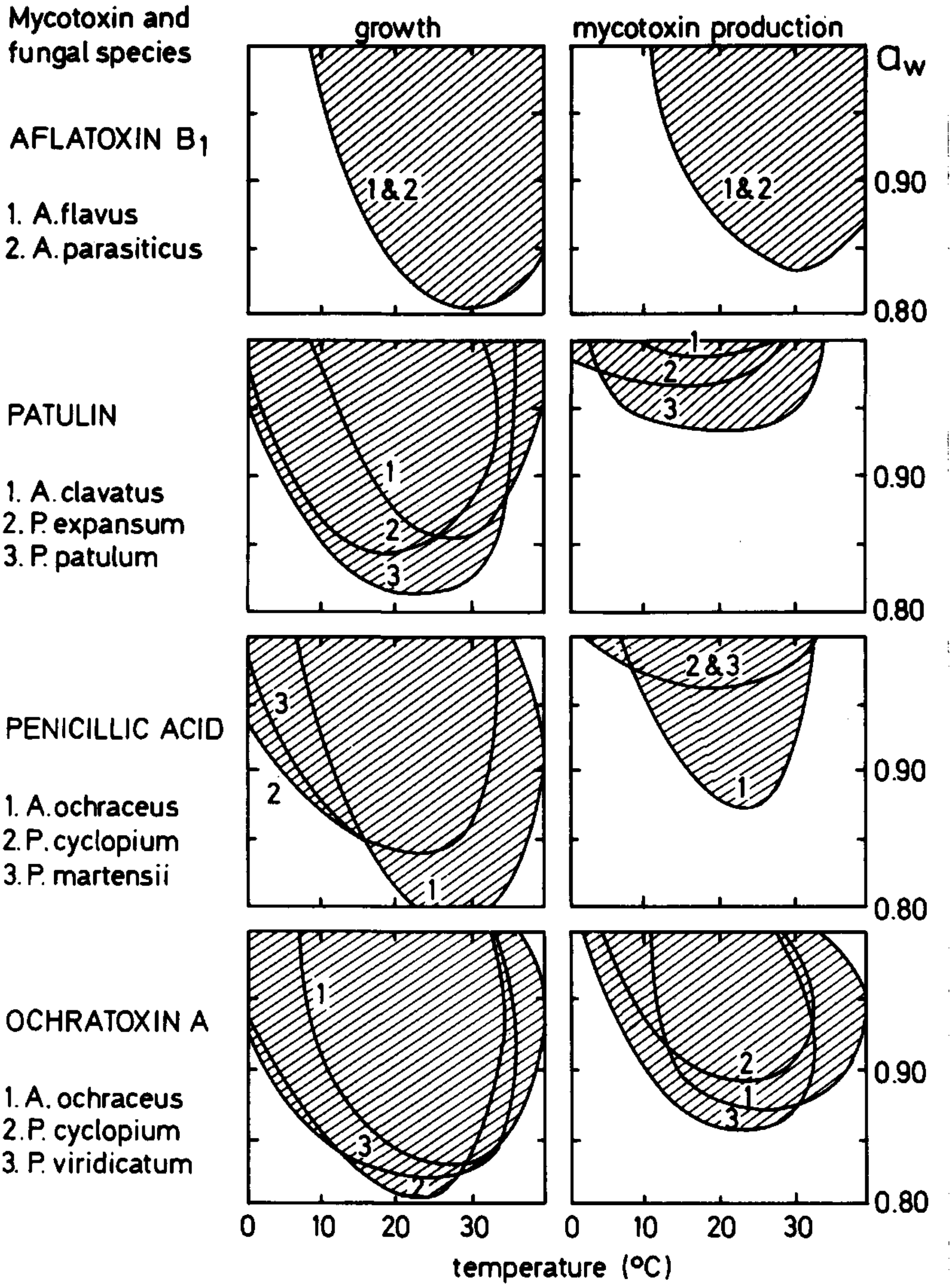

Figure 1. *Conditions of water activity (a_w) and temperature favorable (shaded area) for mycotoxin production by different fungal species. Data are obtained with different fungal strains and substrates.*

Figuur 1. *Gunstige waarden van wateractiviteit (a_w) en temperatuur (gearceerde gebieden) voor de vorming van mycotoxinen door verschillende schimmelsoorten. De resultaten zijn verkregen met verschillende schimmelstammen en voedingsbodems.*

Table 2. *Effect of water activity on growth of and mycotoxin production by four fungal strains. All values are rounded-off.*

Species, strain no. and mycotoxin	Water activity	Change of: Dry weight %	Radial growth rate %	Total amount of mycotoxin %	Mycotoxin production rate %
A. flavus ATCC 15517 aflatoxin B_1	0.99 → 0.94	—60	0	—80	decrease[a]
P. expansum RIV11 patulin	0.99 → 0.98	—20	—10	—50	—70
P. martensii RIV159 penicillic acid	0.997 → 0.992	—20	0	—30	0[a]
A. ochraceus NRRL3519 ochratoxin A	0.99 → 0.95	—80	+20	—99	0

[a] Estimated.

king hypotheses which may evoke further discussion and research.

Some other effects, connected with that of a_W, may follow when the concentration of solute is increased. First, an increase in solute may result in a nutrient effect. For sucrose and glycerol this is unlikely to occur at the concentration levels used in our investigation, since microbial growth rates show saturation kinetics at nutrient concentrations which are much lower than the solute concentrations used for the adjustment of the a_W. However, we have shown in article 2 that glycerol may have a stimulatory effect on aflatoxin B_1 production. Thus, the effect of the solute on mycotoxin production cannot be neglected. Secondly, the solute may become inhibitory at high concentrations. This effect has been found with concentrated salt solutions, but sucrose and glycerol are considered to be non-toxic (34). Thirdly, the solubility of oxygen in the substrate is inversely proportional to the concentration of the solute. A high concentration of solute may, therefore, be responsible for a relatively great inhibition of the growth of submerged mycelium, assuming that both the aerial and submerged mycelium assimilate oxygen. Indeed, the results show that the growth of submerged mycelium is inhibited at low a_W but in most instances this inhibition is no more than the inhibition of growth of the whole culture. Therefore, a possible lack of oxygen due to a high concentration could not be confirmed. Fourthly, the higher density of the low a_W agar medium may result in an inhibition of the growth of the submerged mycelium. As has been said about the oxygen in the agar medium, this observation has not been confirmed by the results. Fifthly, according to Raoult's law, the concentration of solute affects the relative humidity around the aerial mycelium. This effect may have been responsible for the enlarged volume of aerial mycelium at decreased a_W.

EFFECT OF TEMPERATURE ON GROWTH AND MYCOTOXIN PRODUCTION

In Table 3 the effect of temperature on the two growth parameters and the two parameters of mycotoxin production is summarized. A decrease in temperature has little or no effect on the mycelial dry weight of *A. flavus*, *P. expansum* and *P. martensii*, but it does slow down their radial growth rate. In cultures of *A. ochraceus* the mycelial dry weight and radial growth rate respond in the opposite manner. The effect of temperature on the total amount of aflatoxin B_1, patulin and ochratoxin A can be explained by the changes in mycelial dry weight, radial growth rate and mycotoxin production rate. For example, the decrease in the total amount of aflatoxin B_1 can be explained by the decrease in mycotoxin production rate and radial growth rate, which involves a longer production time. Likewise the decrease in the total amount of ochratoxin A can be explained as the consequence of the decrease in mycelial dry weight and the increase in radial growth rate, which involves a shorter production time. However, the decrease in the total amount of penicillic acid, due to the decrease in temperature, cannot be explained by the increase in mycelial dry weight and the decrease in the radial growth rate. Since the decrease in temperature results in less submerged mycelium (Table 7 of the last experimental section) it is suggested that the submerged mycelium of *P. martensii* may account for most of the penicillic acid produced.

As has previously been stated in the explanation of the effect of a_W on the total amount of mycotoxin, the effect of temperature on mycelial growth and the mycotoxin production rate cannot be considered as evidence but rather as a working hypothesis. Moreover, it should be noted that the results cannot be simply generalized since the time-course experiments are limited to only two temperature conditions.

Table 3. *Effect of temperature on growth of and mycotoxin production by four fungal strains. All values are rounded-off.*

Species, strain no. and mycotoxin	Change of: Temperature °C	Dry weight %	Radial growth rate %	Total amount of mycotoxin %	Mycotoxin production rate %
A. flavus ATCC15517 aflatoxin B_1	24 → 16	0	—60	—70	—90
P. expansum RIV11 patulin	16 → 8	0	—30	—30	—80
P. martensii RIV159 penicillic acid	16 → 12	+10	—20	—10	0
A. ochraceus NRRL3519 ochratoxin A	31 → 24	—40	+20	—80	0

INFLUENCE OF OTHER FACTORS

The results of the experiments have shown that the factors oxygen and composition of the substrate have influenced the outcome in some instances. In the last experimental section it has been shown that the oxygen supply of the cultures in the polyethylene bags has limited the patulin and penicillic acid production by fast-growing cultures of *P. expansum* and *P. martensii*, respectively. The results are confirmed by Orth (25) who demonstrated the inhibition of patulin production by a low oxygen concentration and the relative insensitivity of the growth of *P. expansum* to this limitation. No literature data are available on the oxygen requirements of penicillic acid production. The results of the experiments with *A. flavus* and *A. ochraceus* reveal that the oxygen concentration in these cultures was still above the level at which the production of these mycotoxins is inhibited. On the other hand, Shih and Marth (29) and Sansing et al. (27) have demonstrated that an increase of the aeration rate reduced the formation of aflatoxins and ochratoxin A. Woodhead and Walker (36) concluded from their work with *P. expansum* that a high level of aeration enhances the primary metabolism (enzyme synthesis, growth) and lessens the production of secondary metabolites (patulin and citrinin). Therefore, it is likely that different optima of oxygen supply exist for growth and mycotoxin production. Further investigations are needed to establish these optima for different molds and mycotoxins.

Although the production of the mycotoxins took place in media which enable high production of mycotoxin, there have been indications that the composition of substrate may have a profound influence on the minimum a_W for mycotoxin production. An example is the minimum a_W for penicillic acid production which was much lower on poultry feed than on malt extract agar medium.

PRACTICAL IMPLICATIONS

The practical implications of the results obtained in this investigation can be discussed using the data about the minimum a_W and temperature for fungal growth and mycotoxin production described by other investigators who used agricultural products as substrate. To explain the possible differences between their results and the results described here it is necessary to point out some factors which might have influenced the results.

First, it has been demonstrated by other investigators that microbial competition inhibits growth of and mycotoxin production by the mold tested, through which the limiting values of a_W and temperature increase (1, 3). This might happen when non-sterilized natural substrates are used in the experiments. Because this factor is the most important one in the experiments with fermented sausages, the results of these experiments are not useful for comparison.

Secondly, in experiments with grain seed it has been shown that viable seed was less readily contaminated with mycotoxin than heat-killed seed (14). Therefore, in experiments with sterilized grain seed and agar media the limiting values of a_W and temperature might be lower than those in experiments with non-sterilized grain seed.

Thirdly, depending on the composition of the substrate, the adsorption isotherm might be different from the desorption isotherm. Thus, the moisture content of the substrate which has been equilibrated to a certain a_W from the completely dry state (adsorption) is lower than that of the same substrate which has been equilibrated to the same a_W from the wet state (desorption). This pattern is found with high sugar, high protein and high starch content foods (35). Labuza et al. (18) demonstrated that the minimum a_W for fungal growth on a desorption substrate was lower than that on an adsorption substrate. In our investigations the agar media were dried to a certain extent and can be considered as desorption substrates, where-as the poultry feed and barley plates used in the experiments described in articles 4 and 5 can be considered as adsorption substrates.

Fourthly, the period of incubation determines the minimum values of a_W and temperature for growth and mycotoxin production. In experiments with a long incubation time the minimum values might be lower than in experiments with a short incubation time. By way of comparison only the literature data obtained with incubation times similar to those in our investigation are used.

In Table 4, literature data about the minimum a_W of agricultural products for fungal growth and mycotoxin production are compiled. Most available data deal with growth of *A. flavus* and aflatoxin production. A few data deal with other toxinogenic species. To simplify the comparison of the data, the small differences (< 0.05 a_W) between the literature data and those obtained in our investigation are considered to be non-significant because of possible experimental errors. An experimental error might occur when the natural substrate is not completely equilibrated to the intended a_W. It should be noted that most investigators cited equilibrated the substrate above liquids of known a_W, but without control by a_W measurement. The significant differences (≥ 0.05 a_W) between the minimum a_W values are shown in Table 5. Results of some investigations showed a minimum a_W for growth and mycotoxin production which was 0.07-0.10 a_W lower than that found in our investigation.

Table 4. *Minimum water activity (a_W) for fungal growth and mycotoxin production of different agricultural products.*

Product		Fungus		Mycotoxin	Storage temperature °C	Minimum a_W for: Growth	Mycotoxin production	Reference
	[a]		[b]				[d]	[e]
Barley	U	Aspergillus sp.	N	—[c]	20	0.80	—	32,16
	U	Penicillium sp.	N	—	20	0.87	—	32,16
	A	P. viridicatum	C	ochratoxin A	12	0.85	0.85	14
Corn	A	A. flavus	C	—	35	0.77	—	28,13
	A	A. flavus	N	—	19	0.88	—	15,13
	D	A. flavus	C	—	15	0.84	—	26,13
	D	A. flavus	N	aflatoxin B_1	24	—	0.92	33,13
	A	P. expansum	P	—	19	0.91	—	15,13
	A	A. ochraceus	P	—	19	0.76	—	15,13
	A	A. ochraceus	P	—	25	0.88	—	20,13
	A	P. viridicatum	P	—	19	0.85	—	15,13
Oats	A	—	N	—	25	0.75	—	32
Sorghum	A	—	N	—	30	0.8	—	22
Wheat	A	A. flavus	P	aflatoxins	20	—	0.80	21
	A	A. ochraceus	C	—	20	0.75	—	7
Rice	A	A. parasiticus	C	aflatoxins	30	0.70	0.80	4
	D	A. flavus	C	aflatoxins	25	0.85	0.85	5
	U	A. flavus	P	aflatoxins	30	—	0.76	19
	A	A. ochraceus	P	—	25	0.86	—	20,13
Locust beans	A	—	N	—	25	0.75	—	32
Scotch beans	A	—	N	—	25	0.75	—	32
Horse beans	A	—	N	—	30	0.9	—	22
Ground nuts	A	A. flavus	P	aflatoxins	30	—	0.86	11
Pistachio nuts	A	A. flavus	C	aflatoxins	29	0.86	0.88	10
Cashew nuts	A	—	N	—	30	0.9	—	22
Cumin	A	—	N	—	30	0.8	—	22
Coriander	A	—	N	—	30	0.8	—	22

[a] a_W obtained by adsorption of water vapour (A), by desorption (D), or unknown (U).

[b] Inoculated fungus present as pure culture (P) or with competative flora (C), fungus is part of natural flora (N).

[c] Not determined.

[d] Results obtained after 3-8 weeks of incubation.

[e] Second reference is used to convert moisture content given in first reference into terms of a_W.

Table 5. *Significant differences between the minimum water activity (a_w) found in this investigation (Fig. 1) and found by other investigators (Table 4) for fungal growth and mycotoxin production on different agricultural products.*

Product	[a]	Fungus	[b]	Mycotoxin	Differences in minimum a_w for:[d] Growth	Mycotoxin production
	D	A. flavus	N	aflatoxin B_1	—	+0.09
	A	P. expansum	P	—[c]	+0.07	—
Corn	A	A. ochraceus	P	—	−0.07	—
	A	A. ochraceus	P	—	+0.05	—
Wheat	A	A. ochraceus	C	—	−0.08	—
Rice	A	A. parasiticus	C	—	−0.10	−0.03
	U	A. flavus	P	aflatoxins	—	−0.07
Pistachio nuts	A	A. flavus	C	aflatoxins	+0.06	+0.05

[a] a_w obtained by adsorption of water vapour (A), by desorption (D), or unknown (U).

[b] Inoculated fungus present as pure culture (P) or with competative flora (C). Fungus is part of natural flora (N).

[c] Not determined.

[d] Positive value means that the minimum a_w found by other investigators is higher than that found in this investigation. Negative value: the reverse.

These differences cannot be explained by the factors mentioned above since some of these lower a_w values were obtained with adsorption substrates and non-pure cultures. The differences should probably be attributed to the composition of the substrates, which appears to play an as important role as does the a_w. Results obtained with the production of penicillic acid (Table 1) support this hypothesis. The limiting a_w values in Table 5 which were higher than those found in our investigation can be explained by the effect of the adsorption substrates and competitive fungal flora.

In Table 6 literature data about the minimum temperature for fungal growth and mycotoxin production on agricultural products are compiled. The data are similar to those obtained in our investigation. However, Ciegler and Kurtzman (8) determined a considerable production of penicillic acid by *P. cylopium* on corn, rice and sorghum at 1°C, indicating a minimum temperature for penicillic acid which could be much lower than 1°C. This difference in minimum temperature compared with that found in our investigation might be explained by the influence of fungal strain and substrate as shown in Table 1.

It can be concluded that the minimum a_w and temperature for fungal growth and mycotoxin production in some agricultural products may be lower than the values determined with mainly agar media in our investigation. It is suggested that the composition of some agricultural products could be responsible for this difference. Taking into account a safety margin of 0.10 a_w and 5°C below the minimum values of a_w and temperature, the results in this investigation can be used for recommending storage conditions for agricultural products. A somewhat larger safety margin is advisable for storage periods of several months. If the fungal flora of a stored product is unknown, the lowest value of the minima of a_w and temperature for growth of the different fungal species tested is recommended. The minima of a_w and temperature for growth rather than for mycotoxin production should be taken, since fungal growth quickly leads to mycotoxin production after an unintentional change in storage conditions. It should be noted that below the determined minimum values of a_w growth of xerophylic molds can occur. However, there is no evidence that these molds can produce mycotoxins under these circumstances. As far as is known, the minima of a_w and temperature for fungal growth determined in our investigation apply also to a number of other species of *Aspergillus* and *Penicillium,* which are known to produce other mycotoxins (2, 23, 24). Therefore, the data presented here can be used in practice in safeguarding stored agricultural products against contamination with mycotoxins.

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Table 6. *Minimum temperature for fungal growth and mycotoxin production on different agricultural products of high water activity* (≥ 0.96).

				Minimum temperature (°C)[a] for:		
Product	Fungus	[b]	Mycotoxin	Growth	Mycotoxin production	Reference
Barley	P. cyclopium	P	penicillic acid	< 1[c]	< 1[d]	8
Corn	P. cyclopium	P	penicillic acid	< 1	< 1	8
	P. martensii	P	penicillic acid	—[e]	1	17
	A. ochraceus	U	ochratoxin A	—	10	9
Wheat	A. ochraceus	U	ochratoxin A	—	10	9
	P. viridicatum	C	ochratoxin A	< 5	< 5	14
Rice	A. flavus	P	aflatoxins	—	11	31
	P. cyclopium	P	penicillic acid	< 1	< 1	8
Sorghum	P. cyclopium	P	penicillic acid	< 1	< 1	8
Ground nuts	A. flavus	P	aflatoxins	—	13	11
Stone fruits, pears	P. expansum	P	patulin	< 0	< 0	6
Apples	P. expansum	P	patulin	0	0	30
Tomatoes	P. expansum	P	patulin	5	5	12

[a] Results obtained after 3-8 weeks of incubation.

[b] Inoculated fungus present as pure culture (P) or with competative flora (C). Fungus is part of natural flora (N).

[c] Growth might be expected at lower temperature.

[d] Mycotoxin production might be expected at lower temperature.

[e] Not determined.

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Samenvatting

Mycotoxinen zijn metabolieten van schimmels die giftig zijn voor mens en dier. Ze zijn aangetoond in een groot aantal verschillende landbouwprodukten, o.a. granen, oliezaden, noten en fruit. De besmetting ontstaat door groei van bepaalde schimmelsoorten op de produkten gedurende de periode van oogst en bewaring. Door het verstrekken van besmette landbouwprodukten aan landbouwhuisdieren kan vlees en ook melk besmet worden. Omdat veel mycotoxinen nauwelijks ontleden bij de verwerking van de produkten, is preventie van de vorming van deze stoffen gewenst.

Het doel van het onderzoek was het bestuderen van de invloed van de belangrijkste omgevingsfactoren op de groei van en de mycotoxinenvorming door verschillende schimmelsoorten. De belangrijkste factoren, die in de praktijk te beïnvloeden zijn, zijn de beschikbaarheid van water, uitgedrukt als wateractiviteit (a_W) en de temperatuur. De a_W van een landbouwprodukt is gedefinieerd als de verhouding van de waterdampspanning van het landbouwprodukt en die van zuiver water. De a_W geeft derhalve de mate aan waarin de watermolekulen gebonden zijn aan de opgeloste stof, zoals zouten, eiwitten en koolhydraten.

Voor dit onderzoek werden vier mycotoxinen uitgekozen: (1) aflatoxine en (2) ochratoxine A omdat deze mycotoxinen veelvuldig in landbouwprodukten voorkomen en oorzaak zijn geweest van ziekelijke afwijkingen bij mens en dier; (3) patuline, omdat het voorkomt in appels en produkten van appels; (4) penicillinezuur omdat één der producenten van dit mycotoxine, *Penicillium cyclopium*, wijd verbreid voorkomt. De resultaten van het onderzoek zijn in een vijftal artikelen en een afsluitend experimenteel hoofdstuk beschreven.

In het eerste artikel wordt een a_W — meetopstelling op basis van een dauwpunthygrometer beschreven die voor dit onderzoek werd ontwikkeld. Dit apparaat maakte het mogelijk voedingsbodems van een gewenste a_W te maken door het toevoegen van suikers, glycerol of zouten aan een basismedium. Met behulp van de vloeistofschudcultuur- en de agarcultuurmethode werd de invloed van de a_W op de groei van en de aflatoxinevorming door *Aspergillus parasiticus* bestudeerd. Voor het verdere onderzoek werd gekozen voor de agarcultuurmethode in verband met de goede reproduceerbaarheid en de overeenkomst met praktijkomstandigheden. Onderzoekingen bij verschillende combinaties van a_W en temperatuur toonden aan dat de aflatoxinevorming bij sub-optimale a_W sterk werd geremd, terwijl de schimmelgroei juist bij een sub-optimale temperatuur sterk werd geremd. Bij 0,83 a_W werd nog wel groei maar geen aflatoxinevorming gemeten, terwijl bij 10°C geen groei en aflatoxinevorming plaatsvonden.

In het tweede artikel worden de minimale en optimale waarden van a_W en temperatuur voor de groei van en aflatoxinevorming door verschillende *Aspergillus flavus*-stammen beschreven. De optimale temperatuur voor aflatoxinevorming was verschillend voor de verschillende stammen. Bovendien was de optimale a_W bij één van de stammen afhankelijk van de temperatuur en de samenstelling van de voedingsbodem. Eén stam produceerde aflatoxine B_1 bij bijna alle combinaties van a_W en temperatuur waarbij hij kon groeien. De minimale a_W en temperatuur voor schimmelgroei en aflatoxine B_1 vorming waren 0,83 respectievelijk 12°C. De invloed van a_W en temperatuur op de schimmelgroei van aflatoxine-positieve en aflatoxine-negatieve stammen was gelijk.

In het derde artikel wordt het onderzoek naar de invloed van a_W en temperatuur op de vorming van patuline beschreven. Het onderzoek toonde aan dat patuline slechts bij hoge a_W ($\geq 0,95$) gevormd kan worden, terwijl schimmelgroei tot 0,83-0,85 a_W mogelijk was. De belangrijkste patuline-producerende schimmelsoorten *Penicillium expansum* en *Penicillium patulum* konden nog bij lage temperatuur (0-4°C) patuline vormen. Praktijkproeven met beënting van appels bewezen dat *Penicillium expansum* snelle rotting kan veroorzaken. Deze schimmel kon in Cox orange nog bij 1°C patuline produceren.

Het vierde artikel beschrijft het onderzoek met de penicillinezuur-producerende schimmelsoorten *Penicillium cyclopium*, *Penicillium martensii* en *Aspergillus ochraceus*. In agarmediumculturen was de minimale a_W voor penicillinezuurvorming door *Penicillium cyclopium* en *Aspergillus ochraceus* 0,97 en voor die door Penicillium martensii 0,99, terwijl schimmelgroei nog bij respectievelijk 0,84, 0,80 en 0,84 a_W mogelijk was. Bij optimale a_W was het temperatuurgebied voor penicillinezuurvorming 4-31°C voor de penicillia en 8-31°C voor *Aspergillus ochraceus*. Resultaten van culturen van *Penicillium cyclopium* op Tilsiter kaas met 0,96 a_W en Goudse kaas met 0,98 a_W kwamen overeen met die van de agarmediumculturen. In Tilsiter kaas kon geen penicillinezuur en in Goudse kaas kon slechts een spoor van penicillinezuur worden aangetoond. Resultaten van culturen van een *Aspergillus*

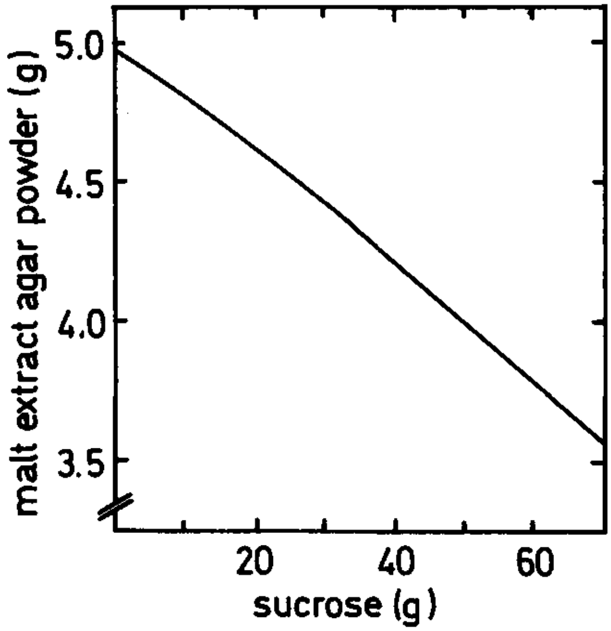

Figure 3. *Amount of malt extract agar powder required to prepare 100 g of complete medium in relation to the amount of sucrose added.*

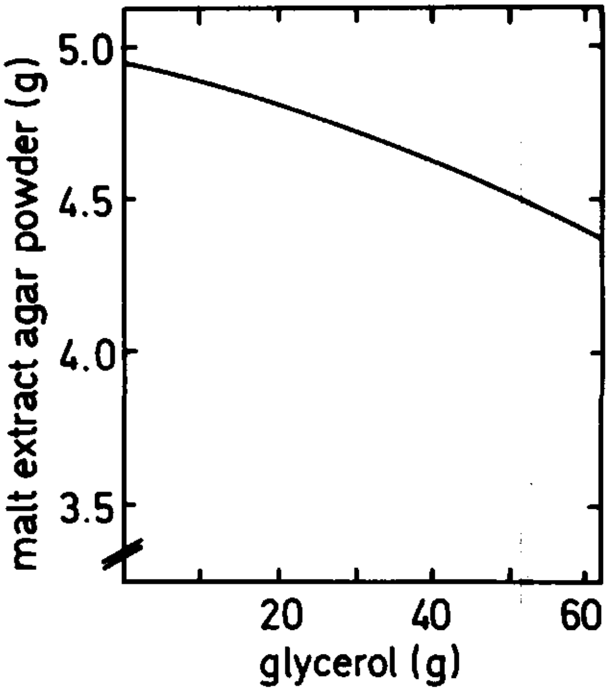

Figure 4. *Amount of malt extract agar powder required to prepare 100 g of complete medium in relation to the amount of glycerol added.*

Curriculum vitae

Martin D. Northolt werd op 8 juni 1944 geboren te Zeist. Na het behalen van het H.B.S.-B diploma in 1962 aan het Openbaar Lyceum te Zeist begon hij in hetzelfde jaar zijn studie aan de Landbouwhogeschool te Wageningen. Na het behalen van het kandidaatsexamen Levensmiddelentechnologie, chemisch-biologische richting, volgden stages aan de Keuringsdienst van Waren te Zutphen, de "Drie Hoefijzers" bierbrouwerij te Breda en de State University of Maryland, Department of Horticulture. De keuzevakken in de doctoraalstudie waren levensmiddelenchemie, biochemie, technische microbiologie en marktkunde, waarvoor een stage werd gevolgd bij het vleesbedrijf Coveco te Arnhem. Het ingenieursexamen werd in 1970 afgelegd. Voor het vervullen van de militaire dienst werd hij in 1970-1971 geplaatst bij de Inspectie Geneeskundige Dienst en part-time bij het Rijksinstituut voor de Volksgezondheid. Sinds 1971 is hij werkzaam als wetenschappelijk medewerker van het Laboratorium voor Zoönosen en Levensmiddelenmicrobiologie van het Rijksinstituut voor de Volksgezondheid. In 1974 werd hij tijdelijk uitgezonden naar het Department of National Health and Welfare, Food Protection Branch, Microbiology Division te Ottawa, en het Food Research Institute te Madison, Wisconsin. Het promotieonderzoek werd uitgevoerd onder leiding van Prof. Dr. E. H. Kampelmacher en Dr. M. van Schothorst in de periode 1974-1979. Het adres van de promovendus is Herenlaan 27 te Zeist.

Reprinted from
J. Milk Food Technol. Vol. 39, No. 3, Pages 170-174 (March, 1976)
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Effect of Water Activity and Temperature on Aflatoxin Production by *Aspergillus parasiticus*

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(Received for publication September 2, 1975)

ABSTRACT

The effect of water activity (a_w) on growth and aflatoxin production by *Aspergillus parasiticus* NRRL 2999 was determined using submerged cultures in which the a_w was adjusted by addition of glycerine, glucose, or a mixture of salts. At a sub-optimal a_w aflatoxin production was low in the glycerol and glucose media while no strong inhibition of mycelial growth occurred. A similar effect was obtained in surface cultures on agar media in which the a_w was adjusted by addition of glycerine or sucrose. The effect of a sub-optimal temperature was the reverse; compared to inhibition of mycelial growth in surface cultures, the effect on aflatoxin production was slight. No detectable quantities of aflatoxin B_1 were formed at 0.83 a_w and at 10 C nor at four combinations of higher a_w and temperature. The a_w was measured by a recently developed device using the dewpoint principle.

Fungi of the *Aspergillus flavus* group including *Aspergillus parasiticus* can produce aflatoxins under certain conditions. The most important factors determining growth and aflatoxin production by these molds are moisture and temperature. The effect of these two factors on growth of several *A. flavus* strains was studied by Ayerst (*1*), and the effect of temperature alone on growth and toxin production of two strains was investigated by Schindler et al (*5*). Little research has been done on the combined effects of these two factors on both growth and toxin production. Such information is essential as minimal, optimal, and maximal temperature permitting growth and toxin production are dependent on the moisture condition prevailing in the substrate.

The availability of water to microorganisms can be measured by the water activity (a_w), i.e. the ratio of the water vapor pressure of the substrate to the vapor pressure of pure water at the same temperature and under the same pressure (*6*). At a low a_w water is bound by salts, sugars, proteins, and other solutes. Thus growth of microorganisms cannot take place as water is not present in an available form.

The effect of a_w on growth and toxin production was studied by other workers with substrates that had been equilibrated with the water vapor above saturated salt solutions. In this investigation the a_w was adjusted by adding various amounts of glycerine, glucose, or salts to the growth medium. The a_w of the media was then measured by a dewpoint measuring device.

In the first part of the investigation the influence of a_w was examined in submerged cultures. In the second part the effect of both a_w and temperature was evaluated in surface cultures.

MATERIALS AND METHODS

Organism and spore suspension

A. parasiticus (strain NRRL 2999) was maintained at 4 C on malt extract agar. Subcultures grown for 1-2 weeks on malt extract agar were washed with 0.6% Tergitol-7 and the spore suspension obtained had about 10^6 spores/ml.

Submerged cultures

To study the effect of a_w on mycelial growth and aflatoxin production, peptone (1%)-glucose (2%) medium at different a_w values was inoculated with mycelial pellets. These were obtained by inoculating 100 ml of peptone (1%)-glucose (2%) medium in 300-ml Erlenmeyer flasks with 0.5 ml of spore suspension and then shaking the cultures at 120 rpm for 3 days at 24 C. The mycelial pellets were filtered off on sterile cheese cloth and washed first with 400 ml of distilled water and then with 300 ml of medium with the desired a_w. The pellets were transferred to graduated cylinders and the appropriate medium was added to make a total volume of 100 ml. Inoculated media were then poured into 300-ml Erlenmeyer flasks and shaken at 120 rpm for different periods at 24 C. After incubation, cultures were stored at −18 C until dry weight or aflatoxin content was determined. The dry weight of mycelium was determined to serve as a measure of growth. Mycelial pellets were rinsed with 600 ml of distilled water, dried in a ventilated oven for 24 h at 70 C and then weighed. Different a_w values were obtained by different amounts of glycerine (PGY-series), glucose (PG-series), or a mixture of NaCl, KCl and Na_2SO_4 at a molarity ratio of 5:3:2 (PGS-series). The a_w of the medium was measured after inoculation. After incubation the a_w and pH of the filtrate were determined.

Surface cultures

Various a_w values in malt extract agar (Oxoid) were achieved by adding sucrose (MES-series) or glycerine (MEG-series). Four agar plates were poured for each a_w-temperature combination and then dried for 3 days at 37 C before inoculation and the determination of a_w. Two of the four plates were inoculated with a spore suspension in three areas with an inoculation needle. The two inoculated plates, and one non-inoculated plate which served as a control on the a_w after incubation, were enclosed in a 0.3-liter polyethylene bag (gauge 0.04 mm). The fourth plate was used for measurement of the a_w at the time of inoculation. Incubation temperatures were 10, 13, 16, 24 and 32 ± 0.3 C. The growth rate of the mycelium was determined by daily measurement of two right-angled diameters of the colony which was enlarged seven times on a screen by an overhead projector. Plates were placed at − 18 C on the day that the average diameter of the three colonies reached 3 cm or on the 45th day of incubation when they did not reach this size.

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Extraction and analysis of aflatoxin

In all submerged cultures 40 ml of the filtrate was extracted with 40 ml of chloroform. In a separate series the mycelium was also extracted with 40 ml of chloroform. Extraction was then repeated twice with 25 ml of chloroform. Surface cultures were extracted by shaking the contents of the two plates in 40 ml of chloroform and by subsequent homogenization of the agar in 40 ml of chloroform with an Ultra Turrax. After centrifugation the extracts were combined. A preliminary visual determination of the aflatoxin B_1 concentration was carried out by thin-layer chromatography (TLC). The sample solution was evaporated or diluted depending on its concentration. The sample extract and 2.5 ng of B_1 standard were spotted on a silicagel G-HR chromatoplate (Machery and Nagel) and developed in chloroform: acetone (9:1 vol/vol). The intensities of fluorescence of the separated B_1 spots from the sample and the standard were measured densitometrically. The recorded intensity values for aflatoxin B_2 and G_1 were divided by 2.2 or 0.55, respectively, since the fluorescence ratio of aflatoxin B_1:B_2:G_1 is 1:2.2:0.55 under the conditions used. Aflatoxin M_1 was measured after evaporation of the extract resulting in a final concentration of about 0.1 μg M_1/ml. The fluorescence of M_1 from a sample was compared with the fluorescence of the M_1-standard after the two dimensional TLC development which employed diethyl ether: Methanol:water (94:4.5:1.5 vol/vol/vol, saturated tank) in the first direction and chloroform:aceton:methanol (90:10:2 vol/vol/vol, unsaturated tank) in the second direction.

a_w-measurement

A scheme for the a_w measurement device is presented in Fig. 1.

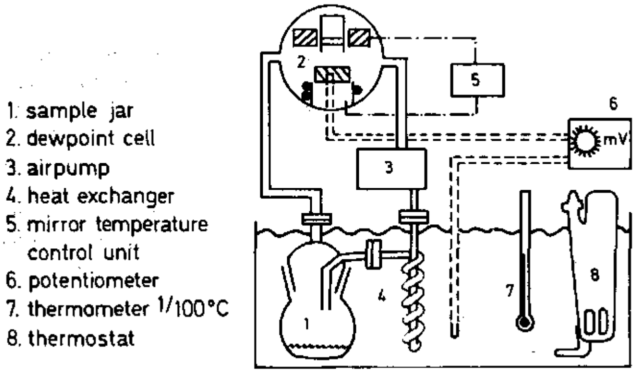

Figure 1. *Scheme of the wateractivity measuring device.*

About 20 ml of medium or culture filtrate is transferred to the 500-ml sample jar. This jar is connected via glass, polyethylene, and stainless steel tubing to a dewpoint cell (Aqmel type HCP 1 s, 50 C), a metal bellows air pump (Metal Bellows Corp.) with a flow rate of 80 liters/h, and to a glass heat exchanger. The jar and the heat exchanger are attached to a movable frame which provides room for six jars which are placed in a waterbath controlled by a thermostat. Each jar can be connected by glass valves to the dewpoint cell. The tubing and the dewpoint cell are heated to a temperature higher than that of the waterbath to prevent condensation. During equilibration of the sample with the circulating air dew droplets are formed on the gold mirror in the dewpoint cell. The mirror is constantly cooled by a Peltier element. Light reflected by the mirror is received by a photoelectric cell that electronically controls the heating of the mirror. In this way the mirror attains a temperature at which an extremely thin layer of dew droplets is maintained. This dewpoint temperature which is dependent on the relative humidity in the sample jar and the temperature of the waterbath is measured by copperconstantan thermocouples in the mirror and in the waterbath. These are connected with a potentiometer. The relative humidity is determined when the dewpoint temperature is constant, as is indicated by a mV recorder. The device is calibrated with distilled water and has a standard deviation of 0.003 a_w. All measurements were done at 25 C.

RESULTS

Submerged cultures

The a_w of the medium during incubation. The a_w of the medium increased in some instances after adding the washed mycelial pellets but it subsequently decreased slightly during incubation. On the fifth day it was at the most 0.005 a_w higher than the a_w of noninoculated medium, on the 24th day it was at the most 0.007 a_w lower. Hence it was considered sufficiently accurate to report the averaged and rounded off a_w-values on the 5th day of incubation in all experiments.

Short term cultures. The mean-value and standard error of triplicate cultures which were incubated for five days are shown in Fig. 2-4. The mycelial growth is the difference between the dry weight at the end and at the start of the experiment, the latter being the average dry weight of five batches of mycelial pellets. Growth was dependent on both the a_w and the medium. In the growth stimulating PGY-medium, the maximal production mycelium occured in the range of 0.96-0.91 a_w. An a_w of 0.87 still permitted a remarkable increase in weight. In the PGS-medium the maximum growth was

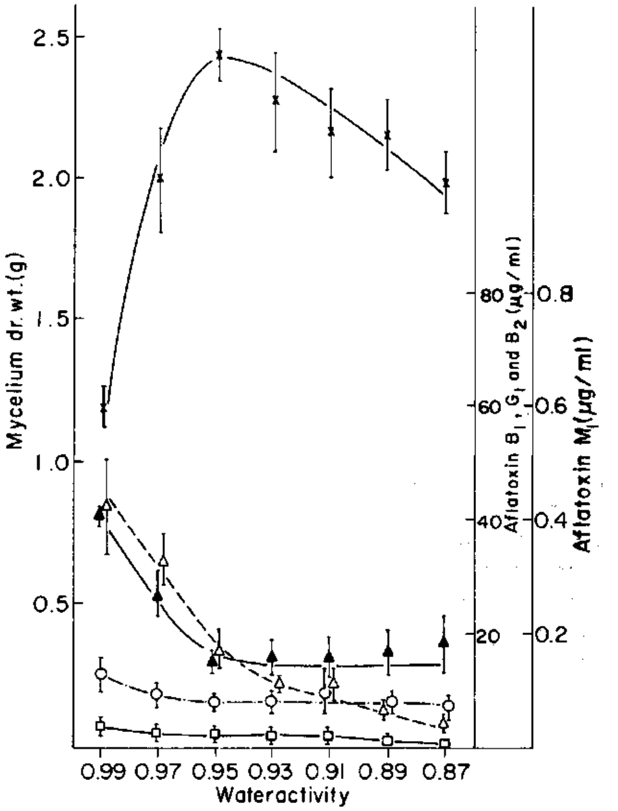

Figure 2. *Growth and aflatoxin production in peptone-glucose-glycerine medium at various water activity values after 5 days of incubation. Average initial weight of dried mycelium was 0.58 g. (Mycelium ×, aflatoxin B_1 △, G_1 ○, B_2 □, M_1 ▲)*

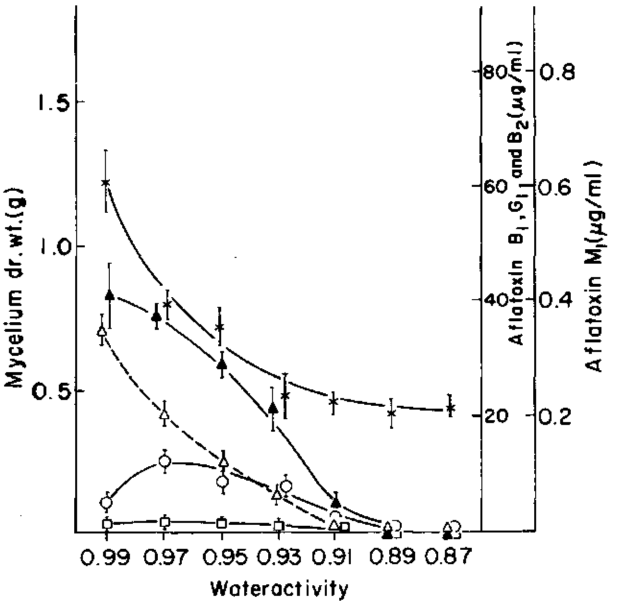

Figure 3. *Growth and aflatoxin production in peptone-glucose-salts medium at various water activity values after 5 days of incubation. Average initial weight of dried mycelium was 0.51 g. (Symbols see Fig. 2.)*

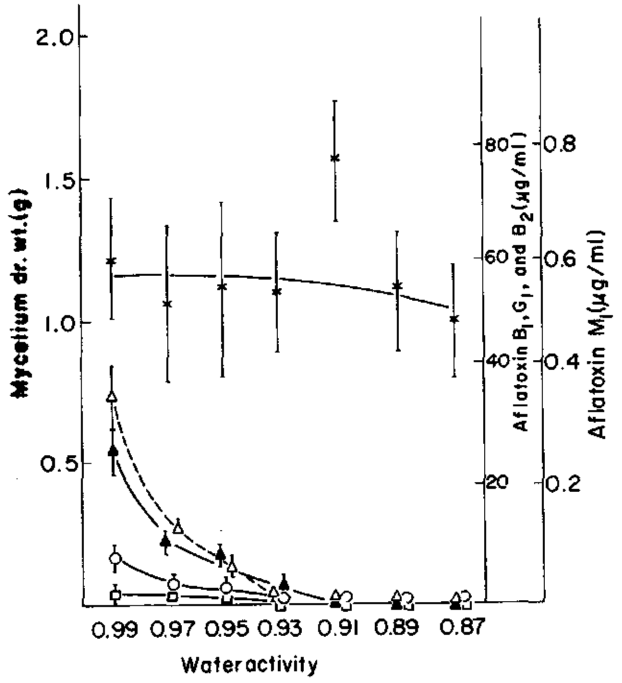

Figure 4. *Growth and aflatoxin production in peptone-glucose-medium at various water activity values after 5 days of incubation. Average initial weight of dried mycelium was 0.38 g. (Symbols see Fig. 2.)*

reached at 0.99 a_w while no growth was observed at 0.91 a_w and below. In the PG-medium growth occurred at all measured a_w-values but it varied widely.

Aflatoxin production was strongly inhibited at low a_w-values in all media. In the PGY-medium the production at 0.87 a_w was 4 µg aflatoxin B_1 per ml (7% of the maximal production). In the PGS-medium only 0.1 µg aflatoxin B_1 per ml was detected at 0.87 a_w. In the PG-medium the production at 0.87 a_w was 0.2 µg of aflatoxin B_1 per ml. The aflatoxin M_1 production followed the same pattern as aflatoxin B_1 at various a_w. However, in PGY the M_1 production was constant at a_w-values below 0.95 a_w. The same was valid for the aflatoxin B_2 and G_1,. Although the production of B_2 and G_1 was low at high a_w, the decrease in production at low a_w was considerably less than for B_1 and M_1. The washed mycelial pellets used for inoculation contained only 5 µg of aflatoxin B_1. If released into the medium this quantity would have resulted in a concentration of only 0.05 µg aflatoxin B_1 per ml of culture filtrate.

Although no growth was measured in the PGS medium at low a_w, the pH declined from 7.4 to 4.0 during incubation. This indicates that the acid producing metabolism was not affected by a low a_w.

The percentages of aflatoxins B_1 and G_1 remaining in the mycelium after separation from the culture filtrate are shown in Table 1. Depending on the medium used, 35-52% aflatoxin B_1 was retained by the mycelium at a high a_w. At a low a_w the percentage was lower. This can be explained by a higher diffusion rate of the toxin into the medium. The same pattern emerged for aflatoxin G_1. This indicates that the greater amount of toxin detected in the culture filtrate at a high a_w does indeed reflect a greater amount of toxin produced, and not merely a greater release of toxin from the mycelium into the medium.

TABLE 1. *Percentage[a] of aflatoxin B_1 and G_1 present in the mycelium after 5 days of incubation in peptone glucose media with glycerine (PGY), salts (PGS), and glucose (PG) at various water activities*

	PGY		PGS		PG	
a_w	B_1	G_1	B_1	G_1	B_1	G_1
0.99	52	27	52	40	35	25
0.97	39	29	26	15	23	13
0.95	42	27	25	11	40	25
0.93	34	19	28	10	30	18
0.91	30	15	44	26	9	6
0.89	26	15	23	10	15	12
0.87	18	11	23	6	8	1

[a]Percentage of the total amount of toxin detected in the culture filtrates and the mycelium.

Longterm cultures. In the PGS-medium the mycelial dry weight at low a_w increased slightly over 29 days (Fig. 5). Despite the low growth rate production of relatively large amounts of aflatoxin B_1 occurred at 0.93 a_w. In the PG-medium as like in the 5-day cultures, the mycelial dry weight varied widely. In both media aflatoxin B_1 production started within the first few days. While the aflatoxin B_1 concentration in PG medium decreased after 12 days, that in PGS medium kept increasing.

Surface cultures

The regression lines of colony diameter on days after inoculation were calculated for each a_w-temperature combination. The regression coefficient for all lines was at least 0.98. At low a_w or low temperature the data curves showed two stages of growth (Fig. 6). The line

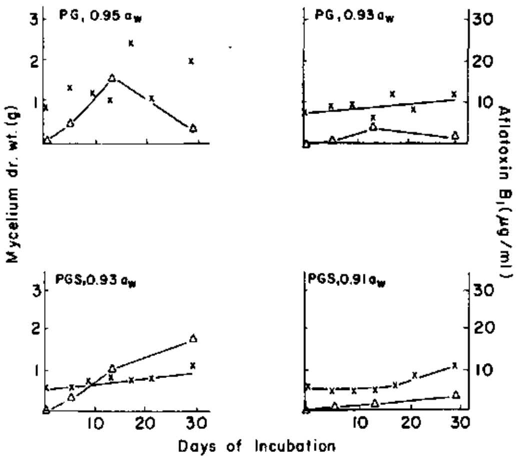

Figure 5. *Growth and aflatoxin B_1 production in peptone-glucose and peptone-glucose-salts media at various water activity values (Mycelium ×, aflatoxin B_1 △).*

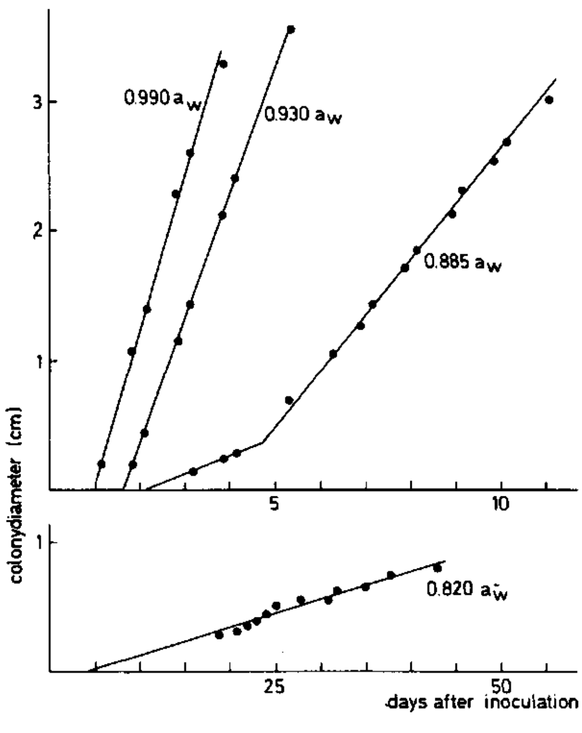

Figure 6. *Increase of colony diameter on malt extract-glycerine agar at various water activity values at 24 C.*

through the first three points was extrapolated to the x-axis to determine the germination time and the line through the remaining points was used to determine the growth rate. The average growth rate of colonies at each combination of temperature and initial a_w is shown in Fig. 7 and 8. On agar media the minimal temperature for growth was between 10 and 13 C. At high a_w and suboptimal temperature, toxin production was strikingly less affected than growth. Both figures show that the optimal temperature at 0.94 a_w was 24 C. This optimum was higher at lower a_w. Maximal growth was found at the highest a_w and incubation temperature. Growth appeared to be possible without demonstratable toxin production at 0.83 a_w on MES and 0.82 a_w on MEG. The detection level was 0.02 μg per colony.

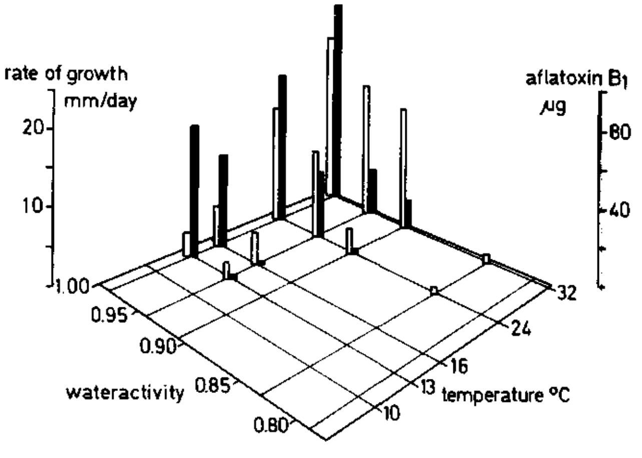

Figure 7. *Growth and aflatoxin B_1 production on malt extract-sucrose agar at various water activity values and temperatures (rate of growth-white column, average aflatoxin B_1 production of 6 colonies-black column).*

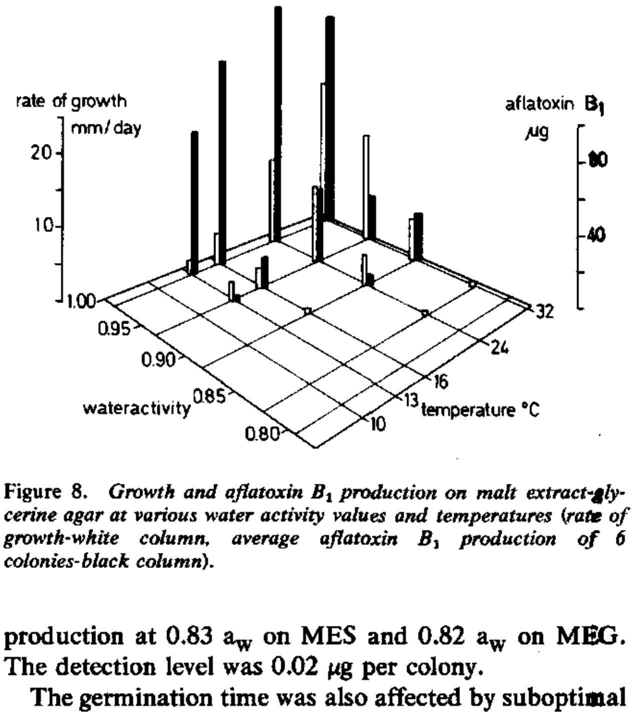

Figure 8. *Growth and aflatoxin B_1 production on malt extract-glycerine agar at various water activity values and temperatures (rate of growth-white column, average aflatoxin B_1 production of 6 colonies-black column).*

The germination time was also affected by suboptimal temperature and a_w. Under favorable conditions the growth rate exceeded 5.5 mm per day and the germination time was less than 2 days. Under unfavorable conditions it was less than 0.2 mm per day and the germination time exceeded 4 days.

The a_w of the agar media decreased only slightly during the incubation period. This was probably a result of diffusion of water vapor through the enclosing plastic bag. The decrease amounted to 0.008 a_w after 8 days and to 0.015 a_w after 40 days.

DISCUSSION

The most striking observation made in the studies with both the submerged and surface cultures was that the a_w did not have the same effect on aflatoxin production as it did on growth. With regard to growth the a_w-optimum for *A. parasiticus* in submerged cultures was dependent on the medium. The optimum lay at a lower a_W in the media containing glycerine and glucose than in the medium containing salts. This may be related to the fact that growth is influenced not only by a_W but also by composition of the substrate. However, in the surface cultures containing glycerine or sucrose the optimum for growth was close to 0.99 a_W. This was also found for *Rhizopus* and *Aspergillus niger* (*3*), and for *A. flavus* (*1*) in studies where similar methods were used. The different a_W values found for optimal growth rate suggests that a correlation does not always exist between the growth rate measured by the surface culture method and that measured by the submerged culture method. This cannot be explained simply by a depletion of oxygen in the surface culture because of the high oxygen permeability of the polyethylene bags.

In studying aflatoxin B_1 production in peanuts, Diener and Davis (*2*) found an optimum at 0.95 a_W while no significant quantity of aflatoxin was found at 0.85 a_W and lower. Hunter (*4*) found a minimal a_W of 0.84 in corn. In contrast, using the methods described here an optimum value exceeding 0.99 a_W was found in all media. A low a_W suppressed aflatoxin production in all media to about the same degree, yet at 0.87 a_W toxin production still occurred.

Besides influencing toxin production, the a_W also influenced the release of aflatoxin from the mycelium into the medium. As relatively more aflatoxin migrated into the medium at low a_W the inhibition of the aflatoxin production at low a_W is greater than may be concluded from the concentrations measured in the medium. In contrast, Shih and Marth (*7*) found that the percentage of aflatoxin in the medium increased at increasing concentrations of sodium chloride. As they used a stationary culture in yeast extract medium containing 20% sucrose and no mixture of salts other factors may have affected the outcome.

Although there was little or no mycelial growth in the medium with salts at low a_W, a slow increase in aflatoxin B_1 concentration occurred. This indicates that the aflatoxin B_1 producing enzyme system had formed before the mycelium was transferred into the medium.

The most important factor for mycelial growth was observed to be the temperature whereas for aflatoxin production it is the a_W. Slight growth is possible at low a_W without demonstrable formation of aflatoxin. Different optimal temperatures for aflatoxin production and growth was also described by Schindler et al. (*5*). The relatively high aflatoxin production at suboptimal temperature might be explained by the longer incubation period necessary to obtain the desired colony diameter. However, this long incubation period was also used for growth at a suboptimal a_W and since under these conditions relatively small amounts of aflatoxin were produced this explanation cannot be upheld. The explanation should be sought in specific influence of a_W and temperature on the aflatoxin producing mechanism.

ACKNOWLEDGMENT

The authors thank Dr. J. Harwig for his criticism and for correcting the manuscript.

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Journal of Food Protection Vol. 40, No. 11, Pages 778-781 (November, 1977)
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Differences Between *Aspergillus flavus* Strains in Growth and Aflatoxin B_1 Production in Relation to Water Activity and Temperature

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(Received for publication April 15, 1977)

ABSTRACT

The optimum and limiting conditions of water activity (a_w) and temperature for growth of and aflatoxin B_1 production by various *Aspergillus flavus* strains were determined. Agar media were used in which the a_w was adjusted by addition of sucrose or glycerine. Optimum temperatures for aflatoxin B_1 production at high a_w varied with the strain tested being 13-16, 16-24, or 31 C. Strains with a low temperature optimum for aflatoxin B_1 production showed fast growth at 37 C without aflatoxin B_1 production. A reduced a_w (0.95 and less) together with a moderate or low temperature inhibited toxin production more than growth. However, at a high temperature one strain showed stimulation of aflatoxin B_1 production on the glycerine medium at reduced a_w. No differences were noted between aflatoxin-positive and aflatoxin-negative strains with respect to growth under various conditions.

Water activity (a_w) and temperature are the most important factors controlling fungal growth. A_w and temperature optima and limits for growth of several fungi, including *Aspergillus flavus*, were studied by Ayerst (*1*). No significant differences between the *A. flavus* strains examined by this worker were shown. In a previous study, we described optima and limits for both growth and aflatoxin production by a strain of *Aspergillus parasiticus* (*5*). The optimum temperatures for aflatoxin B_1 production were 24 and 32 C, depending on the substrate. Studies by other investigators showed optima ranging from 20 to 35 C (*3,4,7,8,9*). In one such study a substrate effect on the optimum temperature for aflatoxin B_1 production was also demonstrated (*4*). Therefore, differences in results of the various investigations could be explained by differences in substrate. With respect to the optimum a_w for aflatoxin B_1 production, we found an a_w value of 0.99 (*5*), whereas Diener and Davis (*4*) found a_w values of 0.95 and 0.99 depending on the substrate.

Another explanation for the differences in optimum temperature and a_w may be sought in the use of different fungal strains. However, this has received little attention, except that in one study optimum temperatures of two strains were compared and no notable difference was found (*8*).

Since results from an investigation with one strain are too easily considered to be representative for a species, this study was undertaken with three aflatoxin-producing strains of *A. flavus* cultivated on two substrates to determine the environmental conditions necessary for aflatoxin B_1 production. Besides, two non-toxigenic strains were used to compare their growth characteristics with those of toxigenic strains.

MATERIALS AND METHODS

Three aflatoxin-producing *A. flavus* strains (RIV 103, RIV 104, ATCC 15517, further indicated by the number) and two aflatoxin-negative *A. flavus* strains (RIV 111, RIV 113, further indicated by the number) were maintained as lyophilized cultures. Cultures grown for 1 week at 24 C on malt extract agar (Oxoid) were washed with an aqueous solution of 0.6% sodium heptadecylsulphate (Tergitol-7, BDH) to prepare a spore suspension.

Various a_w conditions in malt extract agar were achieved by adding sucrose (MES-series) or glycerine (MEG-series). Preparation of agar plates, inoculation, and a_w measurement have been previously described (*5*). For each determination of rate of growth and aflatoxin B_1 production, two inoculated agar plates with three colonies each and one non-inoculated agar plate, which served as control of the a_w after incubation, were used. They were enclosed in a 0.3-liter polyethylene bag (gauge 0.04 mm). The growth rate of the mycelium was determined by daily measurement of two right-angled diameters of a colony. The regression lines of colony diameter on days after inoculation were calculated for each a_w-temperature combination. The germination time was obtained by extrapolating the regression line to the X-axis (*5*). Until extraction, the bags were placed at -18 C on the day that the average diameter of the six colonies reached 3 cm or on the 35th day of incubation when they did not reach this size. Extraction and analysis of aflatoxin B_1 have been described before (*5*). The detection limit of aflatoxin B_1 was 0.1 μg for the six colonies on the two agar plates used for each analysis.

In the first experiment the toxigenic strains 103, 104, and 15517 and the non-toxigenic strains 111 and 113 were grown at combinations of 10, 13, 16, 24, 31, 37 ± 0.3 C and five different a_w values on MES and

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Figures 1-8. *Growth of and aflatoxin* B_1 *production by three strains of* Aspergillus flavus *on MES and MEG agar under various conditions of* a_w *and temperature (rate of growth — white column; aflatoxin* B_1 *production — black column, the 1-mm black columns represent amounts of 0.1-10 µg aflatoxin* B_1*).*

fig. 1 strain 103 on MES

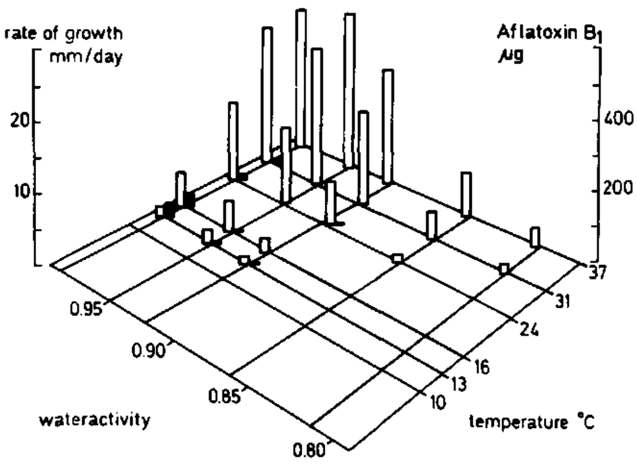


fig. 2 strain 103 on MEG

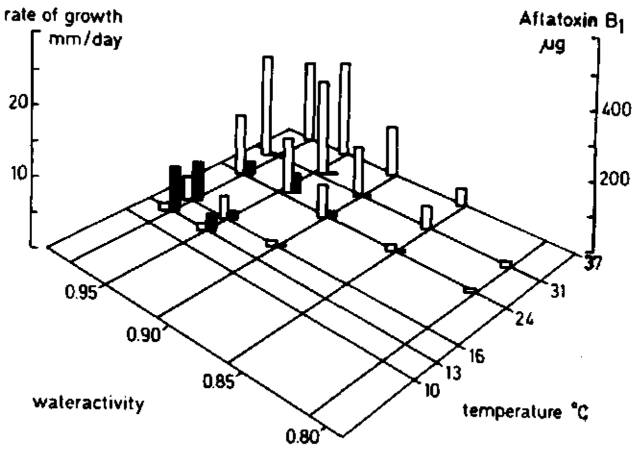


fig. 3 strain 104 on MES

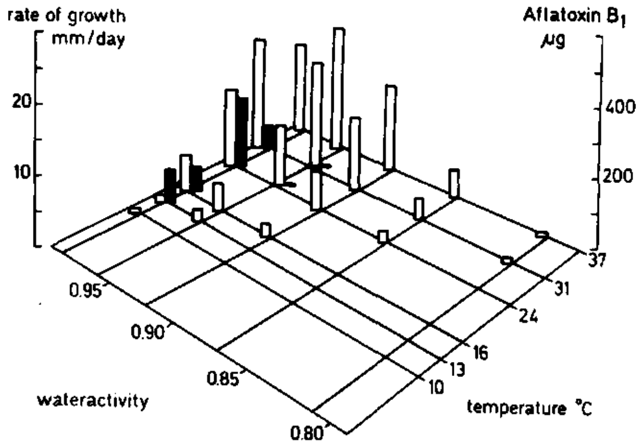


fig. 4 strain 104 on MEG

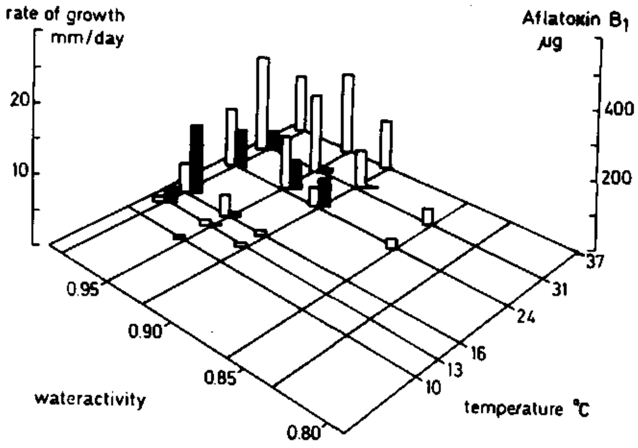


fig. 5 strain 15517 on MES (1st)

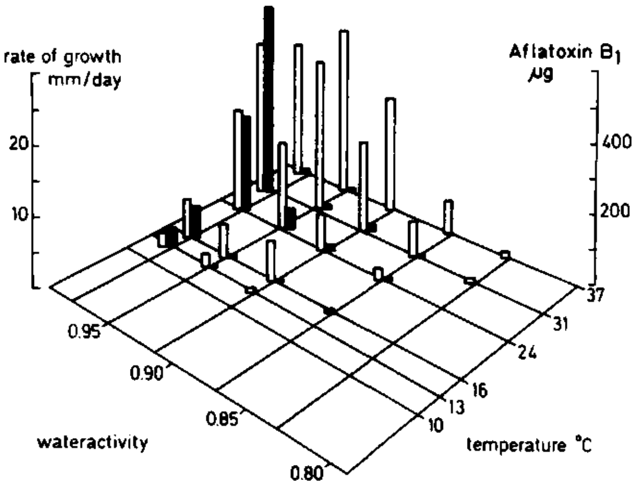


fig. 6 strain 15517 on MEG (1st)

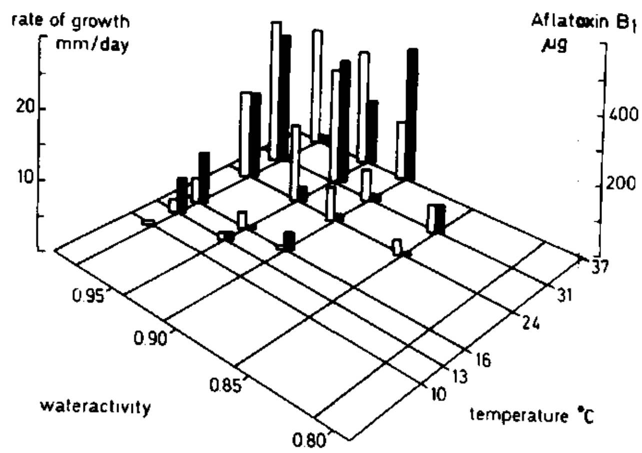


fig 7

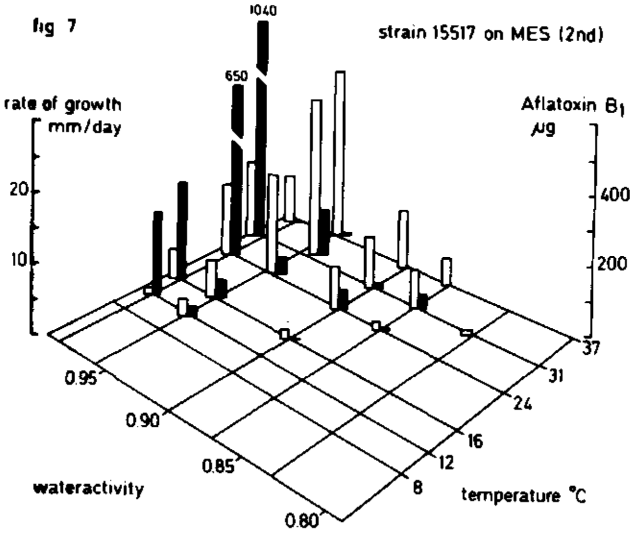
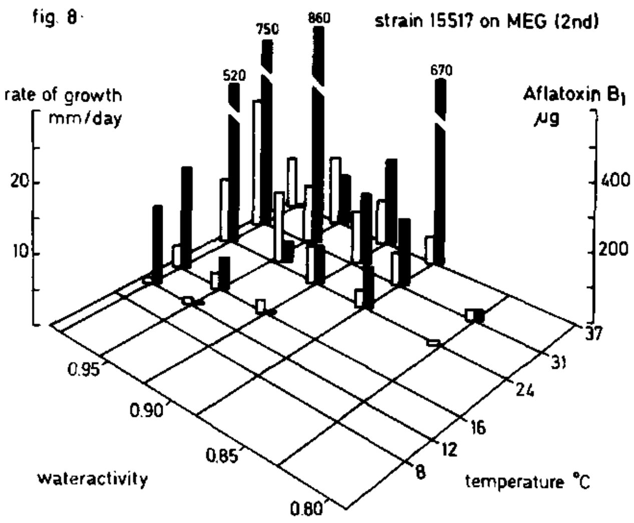

strain 15517 on MES (2nd)

fig. 8


strain 15517 on MEG (2nd)

MEG. To assess the repeatability of growth and toxin production a second experiment was carried out, in which strain 15517 was repeated at combinations of 8, 12, 16, 24, 31, 37 ± 0.3 C and five different a_w values on MES and MEG. Moreover, four bags containing MES agar of 0.99 a_w and four bags containing MEG agar of 0.99 a_w both inoculated with strain 15517, were incubated at 24 C. To reproduce the differences in optimum temperature of strains found in the first experiment, a third experiment was carried out in which strains 103, 104, and 15517 were repeated twice at 10, 13, 16, 24, 31, 37 ± 0.3 C on 0.99 a_w MES.

RESULTS

In the first experiment toxigenic and non-toxigenic strains were tested. Results of the toxigenic strains are shown in Figure 1-8. Results of the non-toxigenic strains are not shown, because their optimum and limiting conditions for growth were similar to those of the toxigenic strains. The toxigenic strains showed optima for toxin production at 13-16, 16-24, and 31 C. The optimum a_w for toxin production was 0.99 for strains 103 and 104 (Fig. 1-4). Strain 15517 had an optimum a_w on MEG which greatly depended on temperature and which varied from 0.90 to 0.99 (Fig. 6). Table 1 shows the calculated germination times in connection with the rate of growth, depending on the various conditions of a_w and temperature.

In the second experiment, results obtained with strain 15517 were repeated qualitatively (Fig. 7 and 8). The repeatability of the method was shown by comparing the aflatoxin B_1 contents of replicate cultures on 0.99 a_w MES and 0.99 a_w MEG. These differed not more than 12% from the average.

TABLE 1. *Germination time of cultures of three toxigenic Aspergillus flavus strains grown on MES or MEG agar in relation to rate of growth*

		Germination time (days)		
		Strain		
Rate of growth[1]	Agar medium	103	104	15517
Maximum	MES	0.8	0.5	0.5
	MEG	0.3	0.3	0.6
10 mm/day	MES	1	1	1-2
	MEG	0.5	0.4	1-2
1 mm/day	MES	9-10	3	6-10
	MEG	4-8	3-5	3-9

[1] *Rate of growth under various a_w-temperature conditions, as shown in Fig. 1-8*

In the third experiment, the difference in optimum temperatures for toxin production was reproduced (results are not shown). Cultures of strains 103,104, and 15517, grown at 0.99 a_w and different temperatures, contained maximal amounts of toxin at the same temperatures as in the first experiment.

DISCUSSION

This study was undertaken to investigate whether differences in fungal strains may explain the different optimum temperatures for toxin production reported by various investigators (*3,4,7,8,9*). This hypothesis has been confirmed by the results of our experiments. The other factor that determines the optimum temperature is the substrate used. The results demonstrate that a high concentration of glycerine rather than a_w did increase the optimum temperature for toxin production. This effect was most striking with strain 15517 (Fig. 6 and 8), whose optimum temperature increased from 31 to 37 C. A similar effect was also found by Diener and Davis (*4*) in a study with peanuts. They found that the optimum temperatures for aflatoxin B_1 production on damaged and sound mature peanut kernels at high a_w were 20 and 35 C, respectively.

According to the latter study, the a_w optima for aflatoxin B_1 production in damaged and sound mature kernels were 0.95 and 0.99 respectively. In our study not only substrate but also fungal strain and temperature determined the optimum a_w (Fig. 6 and 8). In a previous study (*5*) with *A. parasiticus* NRRL 2999, we concluded that a reduced a_w inhibits toxin production more than growth. This conclusion still holds true for a moderate temperature, but the present results indicate that at a higher temperature substrate and fungal strain have a greater influence.

The temperature limit for toxin production lies between 10 and 13 C, and equals that for growth. Only a few cultures grow at 10 C without toxin production. In other studies, with rice (*3*) and peanuts (*4*) the limits for toxin production were 15 and 12 C, respectively. The a_w limit for toxin production lies between 0.83 and ca. 0.87, whereas in other studies the a_w limits were 0.70 - 0.75 with rice (*2*) and 0.85 with peanuts (*4*). However, it is difficult to compare the results because of the different and wide intervals of temperatures and a_w values used in this and the other studies.

The data in this study indicate that foodstuffs contaminated with *A. flavus* spores may contain large amounts of aflatoxin after a short period, because our results demonstrate that six colonies of a potent *A. flavus* strain could accumulate 500 μg aflatoxin B_1 in one and a half days.

Summarized, it can be stated that differences between *A. flavus* strains exist not only with regard to maximal amount of aflatoxin produced, but also with regard to the optimum conditions for aflatoxin production.

ACKNOWLEDGMENT

The authors thank Dr. J. Harwig for his criticism and suggestions.

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Reprinted from:
Journal of Food Protection Vol. 41 No. 11, Pages 885-890 (November 1978)
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Patulin Production by Some Fungal Species in Relation to Water Activity and Temperature

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(Received for publication February 17, 1978)

ABSTRACT

The combined effects of water activity (a_w) and temperature on growth and patulin production by strains of *Penicillium expansum, Penicillium patulum*, and *Aspergillus clavatus* were determined. Malt agar media were used, in which the a_w was adjusted by addition of sucrose or glycerine. The minimum a_w values for patulin production by *P. expansum, P. patulum*, and *A. clavatus* were 0.99, 0.95, 0.99, respectively. The temperature ranges for patulin production by *P. expansum, P. patulum*, and *A. clavatus* were 0-24, 4-31, and 12-24 C, respectively. The optimum temperatures for patulin production by *P. expansum* and *A. clavatus* were low compared with those for growth. Optimum temperatures for patulin production at high a_w by *P. patulum* varied with the strain tested and were 8 or 31 C. The temperature range for patulin production in apples by *P. expansum* was determined. The minimum temperatures for rotting and patulin production were 1 C in Cox Orange cv. and 4 C in Golden Delicious cv. The amount of patulin accumulating in rotten tissue of six apple varieties differed greatly. The invasiveness of and patulin production by various strains of four patulin-producing fungal species were tested. All *P. expansum* strains tested caused rot containing patulin. The increase of rot and patulin production by *P. crustosum* and *A. clavatus* depended on the strains tested. None of the *P. patulum* strains was able to invade Golden Delicious apples.

Patulin is a metabolite of various *Penicillium, Aspergillus*, and *Byssochlamys* species. The toxicity of patulin to microorganisms, plants and animals, and its carcinogenicity to mice have been reviewed by Stott and Bullerman (*23*). Patulin has been found frequently in apples decayed by *Penicillium expansum* (*2, 7*). Results of Harwig et al. (*7*) indicate that where apples processed for juice include unsound fruit, patulin may be introduced into the end product. This has been confirmed by the demonstration of patulin in apple juice in Canada (*17*), the United States (*21, 25*) and Sweden (*8*). Patulin has also been detected in fruit other than apples (*1,5,17*). *P. patulum*, synonymous with *P. urticae* and *P. griseofulvum* (*16*), was frequently isolated by Graves and Hesseltine (*6*) from flour and refrigerated dough products and it could have been the dominant mold of spontaneously molded bread containing patulin (*13*). Bullerman and Olivigni (*3*) isolated molds from commercial Cheddar cheese. Some of these molds produced patulin in laboratory media; one of the isolates was identified as *P. patulum* (*22*). Another patulin producing fungal species is *Aspergillus clavatus*. This species was involved in intoxication of calves fed moldy barley (*11*). *P. crustosum*, which occurs only rarely on food products, is another patulin producer. *P. verrucosum var. cyclopium* is proposed as its correct name (*16*).

The most important factors determining growth and, therefore, production of patulin are temperature and moisture. The effect of temperature on growth and production of patulin has been determined by various investigators (*5,15,20*). The temperature range for production of patulin by *P. expansum* has been determined for tomatoes and bread (*5, 15*), but not for apples. The moisture requirement for germination of spores of *P. expansum* and *P. patulum* isolated from stored corn was determined by Mislivec and Tuite (*10*); however, the effect of moisture on growth and production of patulin received little attention.

The range of temperature over which microorganisms can grow is influenced by the water activity (a_w) (*19*). Therefore, in this investigation the effects of temperature on fungal growth and patulin production were studied at different levels of a_w. In addition, the temperature limits of patulin production in apples were determined.

MATERIALS AND METHODS

Organisms and spore suspensions

Fungal strains were maintained as lyophilized cultures. *P. expansum* strains RIV 50 and RIV 287, and *P. crustosum* strain RIV 58 had been isolated from a meat product, a naturally rotten apple, and a bakery product, respectively. *A. clavatus* strains RIV 612 and RIV 672 had been isolated from compost. Other strains of *P. expansum, P. patulum, P. crustosum*, and *A. clavatus* had been received from Dr. R. A. Samson, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands. Cultures grown for 1 week at 24 C on malt extract agar (Oxoid) were washed with an aqueous solution of 0.6% sodiumheptadecyl-sulfate (Tergitol-7, BDH) to prepare spore suspensions.

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Cultures on agar media

In a preliminary study patulin production by each of the strains was checked on two malt extract agar plates (Oxoid) of 1.00 a_w incubated at 24 C. Each plate was inoculated at three different spots with an inoculation needle dipped in a spore suspension of ca. 10^6 spores per ml. As soon as the colonies reached an average diameter of 3 cm, agar plates were stored at -18 C until extraction for patulin. A high patulin producing strain of each fungal species, i.e. *P. expansum* RIV 11, *P. patulum* RIV 56, and *A. clavatus* CBS 114.48, was used to determine the environmental conditions for patulin production. Various a_w conditions in malt extract agar were achieved by adding sucrose (MES-series) or glycerine (MEG-series). In a previous paper (*12*) preparation of agar plates, inoculation and a_w measurement have been described. The a_w measurement device consisted of a sample jar submerged in a temperature-controlled waterbath which facilitated equilibration of a vapor pressure around the sample, and connected with a closed circuit with a dewpoint meter. The accuracy of the device was 0.005 a_w unit. For each determination of rate of growth and patulin production, two inoculated agar plates with three colonies each and one non-inoculated agar plate, which served as control of the a_w after incubation, were used. They were enclosed in a 0.3-liter polyethylene bag (gauge 0.04 mm). Cultures were grown at combinations of 0, 4, 8, 12, 16, 24, 31, and 37 ± 0.3 C and six different a_w values on MES and MEG. The growth rate of mycelium was determined by daily measurement of two right-angled diameters of a colony. The regression lines of colony diameter on days after inoculation were calculated for each a_w-temperature combination. The germination time was obtained by extrapolating the regression line to the X-axis (*12*). Until extracted for patulin, cultures were stored at -18 C on the day the six colonies reached an average diameter of 3 cm or on the 35th day of incubation when they did not reach this size.

To assess whether or not the strains tested were representative of the mold species, the limiting a_w and temperature for growth and patulin production were determined for three other strains of *P. expansum* and *P. patulum*, which were grown on MES at the same time as the test strains.

Four replicate MES cultures of *P. expansum* RIV 11, grown at 0.99 a_w and 16 C, were analyzed for patulin to determine the repeatability of the method.

Cultures in apples

The pathogenicity to apple of the various strains was determined by stabbing Golden Delicious apples to a depth of ca. 1 mm with a needle covered with spores. Twenty apples were used for each strain. The apples were incubated at 20 ± 0.3 C in open polyethylene bags. Apples inoculated with strains of *P. expansum* were incubated for 11 days and apples inoculated with strains of the other fungal species for 15 days. On the last day of incubation, diameters of rotten areas were measured, and the rotten tissue was removed from the fruit and stored at -18 C until extracted for patulin. The effect of temperature on increase of rot and production of patulin was determined by inoculating Golden Delicious and Cox Orange apples with *P. expansum* RIV 11. Twelve apples were incubated at each of the following temperatures: 1, 4, 8, 12 16, 20, and 24 ± 0.3 C. Each day diameters of rot were measured. Incubation of the individual apples was continued until the diameter of rot reached 1.5 cm. To determine the influence of apple variety on increase of rot and patulin production, apples of the following varieties were inoculated with *P. expansum* RIV 287: Golden Delicious, Cox Orange, Ingrid Marie, Goudreinette, Notaris, and Jonathan. Twelve apples of each variety were incubated at 8 C. Incubation of individual apples was continued until the diameter of rot reached 4 cm. Apples were taken from the wholesale market shortly after harvest. Experiments were done with apples of equal maturity and size.

Extraction and analysis of patulin from the agar media

After thawing, the cultures were inactivated by adding 20 ml of chloroform. After evaporation of chloroform, the two agar plates with the cultures were blended in 75 ml of H_2O by means of a homogenizer (Ultra Turrax). After centrifugation, 5 ml of the supernatant fluid was extracted two times with 50 ml of chloroform. The chloroform extract was evaporated to dryness and the residue was dissolved in 1 ml of chloroform. After preliminary visual determination of patulin concentration by thin layer chromatography, the sample solution was diluted, depending on its concentration. Sample extract and patulin standard were spotted on a silica gel thin layer plate (Merck 60) with a thickness of 0.25 mm, and developed in toluene-ethyl acetate-formic acid (6:3:1 vol/vol/vol). The amounts of patulin from sample and standard were determined densitometrically by reflection measurement at 276 nm. After measurement, the identity of patulin was confirmed by spraying the plate with MBTH solution (0.5% 3-methyl-2-benzothiazolinone hydrazone hydrochloride in distilled water), patulin spots turned yellow (*14*). The limit of detection was ca. 0.1 mg of patulin per two agar plates.

Extraction and analysis of patulin from rotten tissue of apples

Rotten tissue was blended in ethyl acetate with an Ultra Turrax homogenizer. The ratio of ethyl acetate (in ml) to rotten tissue (in g) was 1:1. After homogenization, the ethyl acetate fraction was dried over anhydrous Na_2SO_4, evaporated to dryness and the residue taken up in 1 ml of chloroform. After preliminary visual determination of patulin concentration by thin layer chromatography the sample solution was diluted depending on its concentration. The extract was then analyzed by thin layer chromatography and densitometric measurement as described above for the quantitative analysis of agar plates. The limit of detection was 1-2 μg of patulin per gram of rotten tissue.

RESULTS

Cultures on malt extract agar

Results of the preliminary study showed that the strains of *P. expansum* and *P. patulum* produced patulin on malt extract agar plates. This was also true for *A. clavatus* strains RIV 672 and CBS 114.48. However, neither the strains of *P. crustosum* nor *A. clavatus* RIV 612 produced patulin on this agar medium.

Figure 1 shows an example of the data used for calculation of mold growth. The regression coefficients for all regression lines of colony diameter were at least 0.98. The average growth rate of colonies and patulin production on MES and MEG at each combination of temperature and initial a_w are shown in Fig. 2-8. The optimum temperatures for patulin production by *P. expansum* and *A. clavatus*, both 16 C, were lower than those for growth, 24 and 31 C, respectively. Optimum temperatures for patulin production at high a_w by *P. patulum* varied with the strain tested: strain RIV 56 has an optimum temperature at 8 C, whereas the optimum temperature of strain CBS 315.63 was 31 C. Temperature ranges for patulin production by *P. expansum*, *P.*

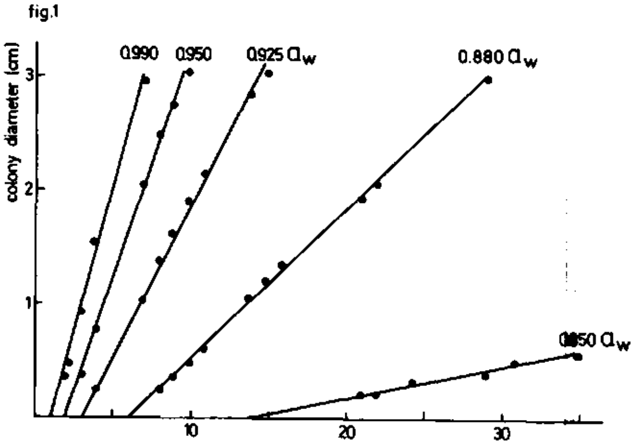

Fig. 1. *Growth of* Penicillium expansum *RIV 11 on malt extract glycerine agar at various a_w levels and 16 C.*

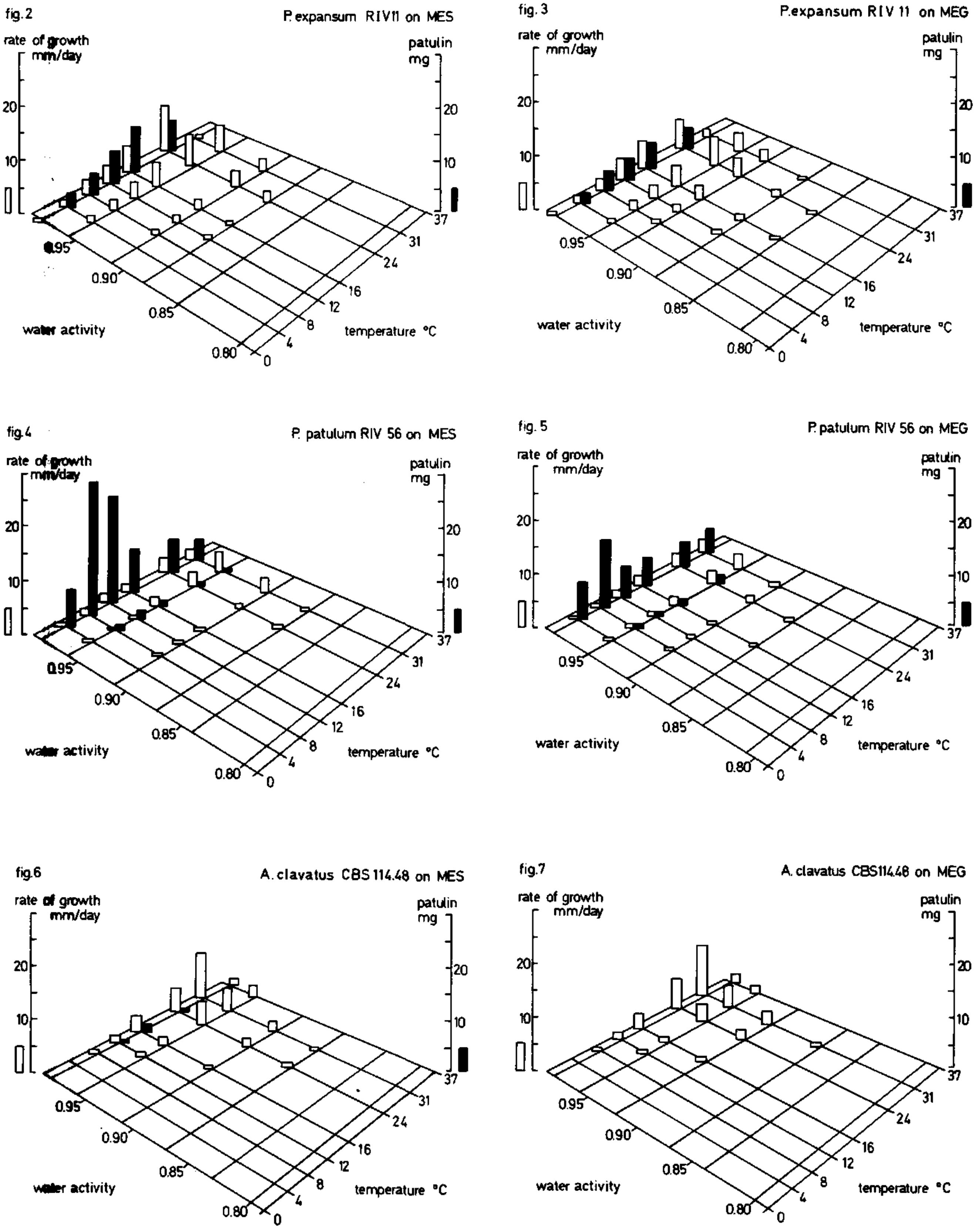

Fig. 2-7. *Growth of and patulin production by three fungal species on malt extract sucrose agar [MES] and malt extract glycerine agar (MEG) under various conditions of a_w and temperature (rate of growth - white column; patulin production - black column, the 1 mm black columns represent amounts of 0.1-1.5 mg patulin).*

patulum and *A. clavatus* were 0-24, 4-31, and 12-24 C, respectively.

Water activity had a profound effect on patulin production. *P. expansum* and *A. clavatus* showed patulin production at 0.99 a_w only, whereas *P. patulum* produced relatively little patulin at 0.95 a_w and large amounts at 0.99 a_w. No patulin could be determined in cultures of *P. crustosum* RIV 58 on MES and MEG. The optimum conditions for growth of this strain were 0.96 a_w and 24 C, whereas limiting conditions for growth were 0 and 31 C and 0.83 a_w. Germination times of strains of the four species were 5-10 h under favourable conditions and they increased to 12-20 days under unfavourable conditions.

fig. 8

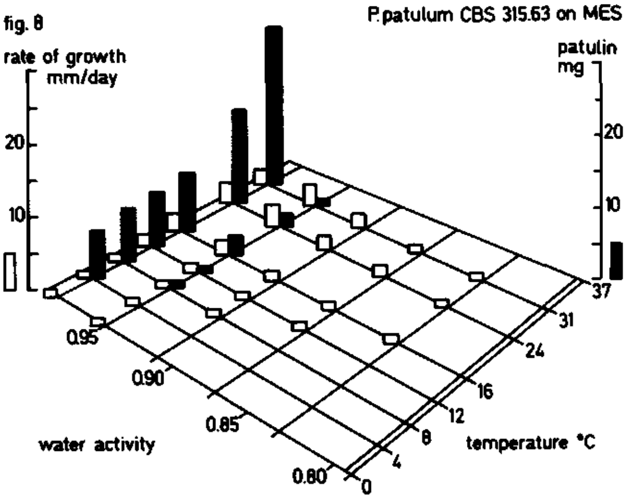

Fig. 8. *Growth of and patulin production by* Penicillium patulum *CBS 315.63 on malt extract sucrose agar [MES] under various conditions of a_w and temperature [rate of growth - white column; patulin production - black column, the 1 mm black columns represent amounts of 0.1 - 1.5 mg patulin].*

In Table 1 it is demonstrated that other strains of *P. expansum* and *P. patulum* had limiting conditions for growth and patulin production similar to strains of which the results are given in Fig. 2-5 and 8. A minor difference was found: at 0 C, *P. expansum* RIV 9 showed patulin production; its growth was faster than that of the other *P. expansum* strains and it therefore reached a greater colony diameter within 35 days.

Table 1. *Growth of patulin-producing penicillia and patulin production on malt extract sucrose agar at unfavorable conditions of a_w and temperature.*

Species and strain no	0.99 a_w			0.98 a_w	0.91 a_w
	0 C	31 C	37 C	16 C	12 C
P. expansum					
RIV 9	G +[1]	NG[3]	—[4]	G-	—
RIV 11	G-[2]	G-	—	G-	—
RIV 51	G-	NG	—	G-	—
RIV 52	G-	G-	—	G-	—
P. patulum					
RIV 14	G-	—	NG	—	G-
RIV 56	NG	—	NG	—	G-
CBS 315.63	G-	—	NG	—	G-
CBS 384.48	G-	—	NG	—	G-

[1] Growth and patulin detected.
[2] Growth and no patulin detected.
[3] No growth.
[4] Not tested.

The patulin contents of the four replicate cultures of *P. expansum* RIV 11 differed by not more than 8% from the average, when grown on MES at 0.99 a_w and 16 C.

Patulin production in apples

Pathogenicity of various strains of the four fungal species and patulin production are shown in Table 2. Fast rotting and patulin production were observed in Golden Delicious inoculated with *P. expansum* strains, *P.* crustosum RIV 58, and *A clavatus* RIV 612. Neither the *P. patulum* strains nor two strain of *A. clavatus* were able to produce rot. In Golden Delicious (0.984 a_w) the optimum temperature for patulin production of *P.*

TABLE 2. *Pathogenicity of four fungal species to and patulin production in Golden Delicious apples stored at 20 C.*

Species and strain no.	Incubation period (days)	Average diameter of rot spots (cm)	Patulin concentration in rotten tissue (µg/g)[1]
P. expansum			
RIV 9	11	5	1
RIV 11	11	5	18
RIV 12	11	5	4
RIV 50	11	4	15
RIV 287	11	3	ca. 16
P. patulum			
RIV 14	15	0	NT[2]
RIV 56	15	0	NT
CBS 315.63	15	0	NT
CBS 384.48	15	0	NT
CBS 746.70	15	0	NT
P. crustosum			
RIV 14	15	4	4
NRRL 1983	15	1	5
IMI 52736	15	2	3
IMI 91920	15	1	<1
IMI 143338	15	2	<1
A. clavatus			
RIV 612	15	4	4
RIV 672	15	0	NT
CBS 114.48	15	0	NT

[1] Lower detection limit ca. 1 µg/g rotten tissue.
[2] Not tested.

fig. 9

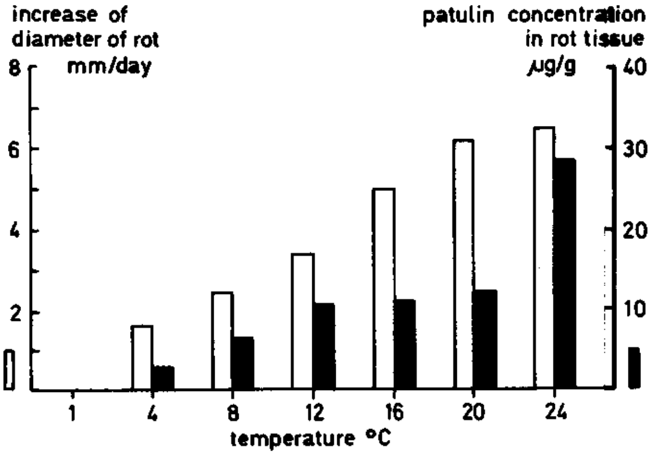

Fig. 9. *Increase of rot and patulin production by* Penicillium expansum *RIV 11 in Golden Delicious of 0.984 a_w in relation to incubation temperature (increase of rot - white column, patulin concentrations - black column).*

expansum RIV 11 was at least 24 C (Fig. 9), which is higher than that on MES or MEG. However, in Cox Orange apples incubated at different temperatures, the patulin concentrations produced in rotten tissue were about the same. Rot and patulin were detected in apples stored at 1 C.

Apples of various varieties inoculated with *P. expansum* and incubated at 8 C contained different concentrations of patulin in their rotten tissue. Compared with the patulin concentration in Golden Delicious, levels in Notaris and Ingrid Marie were at least two times higher, and those in Goudreinette and Jonathan at least five times higher. The patulin concentration in Cox Orange equaled that in Golden Delicious.

DISCUSSION

The results demonstrate that a_w is a very important factor for production of patulin as growth was observed over a relatively wide a_w range but patulin production only over a narrow range. The lowest a_w permitting production of patulin was 0.95, which was the a_w limit for production by *P. patulum*. On the other hand, the temperature range for production of patulin was wide and almost equalled that for growth. The narrow a_w range for production of patulin may explain the small amounts detected by Reiss (*15*) in wheat bread inoculated with *P. expansum*; our measurements of the a_w of wheat bread showed values of ca. 0.96. Therefore substantial amounts of patulin may only be encountered in foodstuffs of high a_w, such as fresh fruit. Decreasing the a_w of fruit by adding sugar to manufacture jams and marmalade is an effective means of preventing production of patulin on these products, as confirmed by Frank et al. (*5*). The small amounts of patulin detected in inoculated Cheddar cheese should not be explained only by the effect of the substrate (*24*), which is low in total carbohydrate, or by instability of patulin in this product (*22*) but also by the sub-optimal a_w for patulin production; Cheddar cheese has an a_w of 0.95 according to our measurements.

The limiting a_w values for growth of *P. patulum* and *P. expansum* lay at 0.83-0.85, a little higher than those determined by Mislivec and Tuite (*10*) for germination of spores. Further, our results with the *Penicillium* strains (Fig. 2-5, Table 1) suggest that, at a high a_w, patulin may be produced at the whole temperature range permitting growth. It is possible that even at the upper and lower temperature limits for growth, patulin had been formed, however in non-detectable quantities, due to the long germination time, leaving only a short period for growth.

Our results obtained with *P. expansum* RIV 11 confirm those of Sommer et al. (*20*). However, they contrast with those of Reiss (*15*), who found a rather low optimum temperature of 10 C for growth on wheat bread. This might be due to the use of different strains or substrates, although we found no difference in results obtained with apple and malt extract media. On the other hand we noticed a profound influence of strain as well as substrate on the optimum temperature for patulin production.

It has been known that *P. expansum* is a common storage rot organism and it is the most important *Penicillium* species causing rot of apple (*2, 7*). Also in this investigation *P expansum* caused rotting of apples, whereas the strains of the other fungal species tested caused slow rot or did not invade apples at all. However, other investigators observed low invasiveness of *P. patulum*, some strains of which produced patulin in the rotten tissue (*4, 9*). The relatively great invasiveness of *P. expansum* may explain the high frequency of this fungal species in apple rot. Our results indicate that in the manufacturing of apple juice, varieties such as Goudreinette and Jonathan should be sorted carefully as they favour greater production of patulin in rotten tissue than other varieties.

ACKNOWLEDGMENTS

The authors are grateful to Dr. J. Harwig for correcting the manuscript.

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Bacon, Richard B. Russell Agricultural Research Center, Athens, Georgia and was originally labeled with the number 107. A *P. cyclopium* isolate from a shelf in a cheese warehouse had been supplied by Mr. E. de Boer, Gist-Brocades, Delft, The Netherlands. Other strains of *Penicillium chrysogenum, Penicillium crustosum, P. cyclopium, P. martensii, P. notatum, Penicillium palitans, P. roqueforti,* and *Penicillium viridicatum* had been received from Dr. R. A. Samson, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands. *P. cyclopium* isolates from cheese were determined according to Stolk and Samson (*28*) and Samson et al. (*26*). The identity of other *P. cyclopium* strains and other *Penicillium* species were checked according to Raper and Thom (*24*).

Cultures grown for 7-10 days at 24 C on malt extract agar (Oxoid) were washed with an aqueous solution of 6 g of sodium heptadecylsulphate (Tergitol-7, BDH)/l to prepare spore suspensions of ca. 10^6 spores per ml.

After the desired periods of incubation, cultures of these organisms on the substrate described below were extracted for PA analysis either immediately or after storage at -18 C for a maximum of 7 days.

Cultures on agar media

In a preliminary study, mold strains were tested for PA production at 24 C on two agar plates of each of the following media: malt extract agar medium (Oxoid) (ca. 1.00 a_w, pH 5.4, ME), malt extract agar medium (Oxoid) supplemented with 20 g of yeast extract/l (Oxoid) and 100 g of sucrose/l (0.99 a_w, pH 6.0, MEYS), 10 g of peptone/l (Oxoid) - 20 g of dextrose/l - agar medium (ca. 1.00 a_w, pH 6,2, PD), and minerals-tartrate-dextrose agar medium according to Bentley and Keil (*2*) (ca. 1.00 a_w, pH 3.9, MTD). Each plate was inoculated at three different spots with an inoculation needle dipped in a spore suspension. Incubation was terminated as soon as the colonies reached an average diameter of 30 mm.

High PA-producing strains of each fungal species, i.e. *P. cyclopium* CBS 434.73, *P. cyclopium* RIV 129, *P. martensii* RIV 159, *A. ochraceus* NRRL 5175, and *A. ochraceus* RIV 1215, were used to determine the environmental conditions for PA production. Various a_w conditions in malt extract agar were achieved by adding sucrose (MES-series) or glycerol (MEG-series). Preparations of agar plates, inoculation and a_w measurement have been described in a previous paper (*20*). The a_w measurement device consisted of a sample jar that was submerged in a temperature controlled water bath, thus facilitating equilibration of the vapor pressure around the sample, and was connected to a dew-point meter through a closed circuit. The accuracy of the device was 0.005 a_w. All measurements were done at 24 C. For each determination of rate of growth and PA production, two inoculated agar plates with three colonies each and one non-inoculated agar plate, which served as control for determination of the a_w after incubation, were used. They were enclosed in a 0.3-liter polyethylene bag (gauge, 0.04 mm). Cultures were grown at combinations of eight temperatures and six a_w values on MES or MEG. The temperature variation was 0.3 C. The growth rate of the mycelium was determined by daily measurement of two right-angled diameters of the colonies. The regression lines of colony diameter on days after inoculation were calculated for each a_w-temperature combination. The germination time was obtained by extrapolating the regression line to the X-axis (*20*). Where rates of growth were decreasing during incubation, initial rates of growth obtained by drawing the tangent are given. Incubation was terminated on the day the six colonies reached an average diameter of 30 mm or on the 35 days of incubation when they did not reach this size.

To assess whether or not the strains tested were representative of the mold species, the limiting a_w and temperature for growth and PA production were determined for some other strains of *P. cyclopium* and *A. ochraceus* which were grown on MES at the same time as the test strains.

To investigate whether a decrease of rate of growth might be due to lack of oxygen, two series of cultures of *P. cyclopium* RIV 129 were grown at combinations of different temperatures and water activities. In one series, cultures were enclosed in polyethylene bags until the colonies reached 30 mm in diameter according to the method described above, whereas in the other series the cultures were aerated daily by opening the bag, replacing the air in the bag with fresh air, and closing it again.

Four replicate MES cultures of *P. cyclopium* CBS 434.73, grown at 0.99 a_w and 24 C, were analyzed for PA to determine the repeatability of the method.

To determine the effect of substrate on growth and PA production, MTD agar plates supplemented with dextrose yielding 0.99 a_w were inoculated with spores of *P. cyclopium* CBS 434.73. Incubation, storage, and extraction were as for MES or MEG cultures.

Screening P. cyclopium *isolates from cheese for toxin production*

Spore suspensions of *P. cyclopium* strains isolated from cheese and of a PA-producing strain of *P. cyclopium* isolated by de Boer from a warehouse shelf (*13*) were used to inoculate MTD agar plates. Two plates of agar medium were inoculated at three different spots with a needle covered with spores and then were incubated at 24 C for 14 days. The strains from warehouses were also screened for PA production by the method of de Boer (*13*). One hundred ml of a medium with 20 g of yeast extract (Oxoid)/l and 150 g of sucrose/l in 500-ml flasks were inoculated with 1 ml of a spore suspension and incubated as stationary cultures at 24 C for 9 days.

P. cyclopium *cultures on cheese*

In the first experiment, growth rate of and PA production by *P. cyclopium* CBS 434.73 was measured on Gouda cheese of 0.98 a_w and on Tilsiter cheese of 0.96 a_w. The 8-day-old Gouda cheese of 5.2 kg was obtained from a warehouse, whereas the Tilsiter cheese of 1.4 kg of unknown age was obtained from a shop. The cheeses were aseptically cut in slices of 40 × 40 × 4 mm. To obtain slices of similar a_w value, the central part and the outer layer of the cylindrical Gouda cheese and the outer layer of the brick-shaped Tilsiter cheese were discarded. The cultural method was similar to that of the agar cultures; for each determination of rate of growth and PA production, two inoculated slices of cheese with three colonies each and one non-inoculated control slice of cheese, were used. They were placed in three petri dishes, which were enclosed in a polyethylene bag. Cultures were incubated at six different temperatures until the six colonies reached an average diameter of 30 mm or until the 42nd day when they did not reach this size. Other incubated slices of Gouda cheese were incubated at 16 C for 20, 27, 34, and 42 days and of Tilsiter cheese fo 30, 37, and 44 days; these colonies grew beyond 30 mm diameter.

A second experiment was carried out with potential PA-producing *P. cyclopium* strains RIV 212, CBS 161.42, NRRL 1888, as well as CBS 434.73. Seven-day-old Gouda cheese was obtained from a warehouse, stored at 4 C for 7 days, cut into slices and treated as described for the first cheese experiment. The a_w of the slices was 0.97. Inoculated slices were incubated at 16 C for 14, 28 and 42 days.

A. ochraceus *cultures on poultry feed*

Complete feed meal for laying hens was blended with water to a water content of 200, 260, 320, 380, 440, 500, and 620 g/kg (wet weight basis), giving after autoclaving water activities of 0.88, 0.93, 0.96, 0.97, 0.975, 0.98 and 0.99, respectively. The feed was autoclaved at 121 C for 1 h, thoroughly mixed, stored for 5 days and pressed into petri dishes to a thickness of 5 mm. Each plate was inoculated at three different spots with 1 μl of a spore suspension of *A. ochraceus* RIV 1215. Feed plates of 0.99 a_w were incubated at eight different temperatures, whereas feed plates of 0.88, 0.93, 0.96, 0.97, 0.975, and 0.98 were incubated only at 24 C. In another experiment, feed plates of 0.95, 0.97, and 0.99 were inoculated with *P. cyclopium* CBS 434.73 and incubated at 24 C. For each a_w-temperature combination two inoculated plates and one non-inoculated control plate were enclosed in a polyethylene bag.

The method for determination of growth rate and germination time of mycelium was the same as that for cultures on agar. When the average diameter of the colonies reached 11, 17, 22, and 26 mm respectively, the bag was opened a short time to aerate the cultures. Incubation was terminated as soon as the colonies reached an average diameter of 30 mm. Total protein and amino acid content of the feed were determined by Mr. K. Terpstra, Spelderholt Institute for Poultry Research, Beekbergen, The Netherlands.

Extraction and determination of PA from agar and liquid media

After thawing, cultures were inactivated by adding 20 ml of chloroform to each pair of plates. The chloroform was allowed to stand in contact with the culture for at least 2 h at room temperature. For qualitative analyses, 20 μl of the chloroform solution was spotted on a thin layer chromatography (TLC) plate next to a PA standard. The plate was developed in toluene-ethyl acetate-formic acid (6:3:1 vol/vol/vol) in an unsaturated tank, dried, exposed to ammonia vapor for 10 min and then held at 100 C for 2 min. The presence of PA was indicated by a blue fluorescent spot on the plate when exposed to long wave UV light at 365 nm (9).

For quantitative analysis, the chloroform was evaporated and the contents of the two inoculated agar plates were blended in 75 ml of H_2O by means of an homogenizer (Ultra Turrax). After centrifugation, 5 ml of 0.5 mol H_2SO_4/l was added to 5 ml of the supernatant fluid and the mixture was extracted twice with 50 ml of chloroform. The collected chloroform extracts were dried over Na_2SO_4, evaporated to dryness and the residue dissolved in 1 ml of chloroform. After semi-quantitative determination of the PA concentration by TLC, the sample solution was diluted to the desired concentration. Sample extract and PA standard were spotted next to each other on a silica gel TLC plate (Merck 60) with a thickness of 0.25 mm and developed in toluene-ethyl acetate-formic acid (6:3:1 vol/vol/vol) in an unsaturated tank. After development, the plate was exposed to ammonia vapor for 10 min and held at 100 C for 2 min.

The resulting blue fluorescent spot of PA was measured densitometrically by fluorescence measurement (excitation wavelength 365 nm, emission wave-length 430 nm). The limit of detection was ca. 0.1 mg of penicillic acid per two agar plates.

Extraction and determination of PA from cheese

Quantitative analysis was preceded by qualitative analysis of mycelium scrapings. Mycelium of six colonies together with the ca. 0.5-mm upper layer of cheese was suspended in 2 ml of chloroform. After at least 2 h at room temperature, 20 μl of the chloroform extract were spotted on a TLC plate and the same procedure was carried out as for the qualitative check for PA on agar plates. If PA was detected in the mycelium scrapings, these were recombined with the scraped cheese slices and the extraction procedure was carried out as follows: 10 g of cheese were blended in 50 ml of acetonitrile and 1.5 ml of concentrated formic acid (5) by means of an Ultra-Turrax. After filtration through glass wool, the mixture was shaken twice with 50 ml of n-hexane in a separatory funnel. The n-hexane phase was discarded. Then 25 ml of water were added to the remaining solution and the water layer was extracted with 50 and 25 ml of chloroform, successively. The chloroform extracts were collected and extracted with 50 ml of a 30 g $NaHCO_3$/l solution in water. The $NaHCO_3$ solution was shaken with 10 ml of chloroform, which was discarded. The water phase was acidified to ca. pH 1 with 2 mol H_2SO_4/l and then extracted three times with 50 ml of chloroform. The collected chloroform extracts were dried over Na_2SO_4, evaporated to dryness and the residue was dissolved in 1 ml of chloroform. After preliminary visual determination of the PA concentration by one-dimensional TLC, the sample solution was diluted to the desired concentration and 5 μl of the extract was spotted on a 66 × 66-mm Merck 60 TLC plate (thickness 0.25 mm), and developed two-dimensionally in diethyl ether (first direction) and toluene-ethyl acetate-formic acid (6:3:1 vol/vol/vol, second direction), both times in an unsaturated tank. Ammonia fumigation, heat treatment and densitometric measurement were carried out as for quantitative analysis of agar cultures. The limit of detection was ca. 2.5 mg/kg of cheese.

Extraction and determination of PA from poultry feed

The amounts of reagents used indicated below are for 50 g of dry weight of feed. They were proportionally increased or decreased when the amount of dry feed to be analyzed varied.

After thawing, 250 ml of acetonitrile and 25 ml of 0.1 mol H_3PO_4/l were added to the two inoculated feed plates. The mixture was blended by means of an Ultra-Turrax, and this was followed by filtration. Fifty ml of filtrate were shaken in a separatory funnel with 50 ml of acetonitrile, together with 50 ml of a solution of 12.5 g of $NaHCO_3$/l and 30 g of NaCl/l in water and 50 ml of iso-octane. After mixing, three layers were formed of which the lower water phase was drained and shaken with 50 ml of chloroform. The chloroform phase was discarded and the water layer was acidified with 5 ml of 2 mol H_2SO_4/l and extracted three times with 50 ml of chloroform. The collected chloroform fractions were dried over Na_2SO_4, evaporated to dryness and the residue was dissolved into 0.5 ml of chloroform. TLC determination of PA was carried out as described for the analysis of agar cultures. The detection limit was 2.5 mg/kg of poultry feed.

To assess the effect of water content on the recovery of PA from feed, 3.8 mg of PA were added to four batches of 150 g moistened feed containing 380, 440, 500, and 620 g of water/kg which corresponded to 0.97, 0.975, 0.98, and 0.99 a_W. Extraction and analysis of each batch were done in duplicate.

RESULTS

Cultures on laboratory media

The results of the preliminary study on different agar media are shown in Table 1. PA-producing strains were found in the species of *P. cyclopium, P. martensii. P. viridicatum,* and *A. ochraceus,* but not in the species of *P. chrysogenum, P. crustosum, P. notatum, P. palitans* and *P. roqueforti.* On MTD agar medium, *P. cyclopium* produced more PA than on ME or PD. Enrichment of ME agar medium by addition of protein-rich yeast extract and sucrose (MEYS) did increase PA production by *P. martensii,* but did not influence PA production by *A. ochraceus.*

Results of the examination for PA production of *P. cyclopium* strains isolated from cheese are shown in Table 2. None of these strains produced PA on MTD agar medium or in YES liquid medium. However, the strain isolated from a cheese shelf produced PA on MTD agar medium but not in YES liquid medium.

Figure 1 shows examples of colony growth curves of cultures of *P. cyclopium, P. martensii,* and *A. ochraceus* on agar medium. Under certain conditions of a_W and temperature, the growth rate of *P. cyclopium* and *P. martensii* decreased after a period of time. This phenomenon could not be correlated with a_W or temperature and it occurred very rarely with cultures of *A. ochraceus.*

The average growth rate of colonies and PA production on MES and MEG agar medium at each combination of temperature and initial a_W are shown in Fig. 2-10. For cultures with decreasing growth rate the average initial growth rate is given in the figures. Optimum temperatures for PA production by *P. martensii* were 16-24 C, whereas *A. ochraceus* produced maximum quantities at 24-31 C. Optimum temperatures for PA production by *P. cyclopium* varied with the strain tested: strain CBS 434.73 had an optimum temperature of 24 C,whereas the optimum temperature of strain RIV 129 was 31 C (Fig. 11). Temperature ranges for PA production by *P. cyclopium, P. martensii,* and *A. ochraceus* were 4-31, 4-31, and 12-31 C, respectively.

Water activity had a profound effect on PA production. In the Fig. 2-11 all species showed PA production at 0.99 a_W only. However, additional cultures grown on MES at intermediate a_W values (0.96-0.98) showed that *A.*

ochraceus and *P. cyclopium* had a minimum a_W for PA production of 0.97. To demonstrate whether or not PA could have been produced on MES of 0.95 a_W, the possible disappearance of PA was checked by mixing 2 mg of toxin with a MES plate of 0.99 a_W and a MES plate of 0.95 a_W and incubating them for 60 h at 24 C. The recovery of toxin was 84 and 78%, respectively. *A. ochraceus* NRRL 5175, a low producer of PA compared to strain RIV 1215, showed no detectable PA production on MEG agar medium. Germination times of the three species were 0.5-2 days under favorable conditions for growth and they increased to 7-20 days under unfavorable conditions.

In Table 3 it is demonstrated that two other strains of *P. cyclopium* had limiting conditions for growth and PA production similar to strains of which the results are given in Fig. 2, 3, and 10. In contrast to *A. ochraceus* RIV 1215, other strains of *A. ochraceus* showed growth at 8 C and of which some produced PA. Therefore, the temperature range for PA production by *A. ochraceus* should be regarded as 8-31 C.

Fig. 10 and 11 show that there is no effect of aeration on growth; the time of decreased growth rate, noticed under some conditions of a_W and temperature, was quite comparable with those of the non-aerated cultures. However, an effect on PA production was demonstrated; aerated cultures at 4-31 C showed an increased PA production compared with that of non-aerated cultures.

TABLE 2. *Penicillic acid production on different media by* Penicillium cyclopium *strains isolated from cheese in shops and from cheese and shelf in warehouses.*

Isolates		Medium[1]	
Origin	Number	MTD-agar	YES-liquid
Cheese in shops	34	0/34[2]	NT[3]
Cheese in warehouses	48	0/48	0/8
Shelf in warehouse	1	1/1	0/1

[1] Incubated at 24 C.

[2] Number of toxin producing isolates and isolates examined.

[3] Not tested.

The PA contents of the four replicate cultures of *P. cyclopium* CBS 434.73 differed by not more than 7% from the average when grown on MES of 0.99 a_W and at 24 C.

In Fig. 12, growth of and PA production by *P. cyclopium* CBS 434.73 on MTD, a mineral agar medium without protein, is shown. Temperature range and optimum temperature equalled those determined for cultures on MES agar medium; however, PA production on MTD was higher than that on MES.

Cultures on natural substrates

Figure 13 shows colony growth rate curves of *P. cyclopium* CBS 434.73 on two types of cheese. Initial rate of growth and PA production are summarized in Fig. 14. The temperature range for growth on Gouda cheese was 0-24 C, whereas on Tilsiter cheese, it was 4-16 C. Neither

TABLE 1. *Penicillic acid production (mg per six colonies) by various strains of different fungal species on various agar media incubated at 24 C.*

	Agar medium[1]			
Species and strain no.	ME	MEYS	PD	MTD
P. cyclopium				
RIV 127	4.6	–[2]	0.4	8.0
RIV 156	< 0.1[3]	–	< 0.1	< 0.1
RIV 157	< 0.1	–	< 0.1	< 0.1
RIV 212	0.1	–	< 0.1	6.6
RIV 232	< 0.1	–	< 0.1	< 0.1
CBS 434.73	4.6	–	1.1	27
NRRL 1888	< 0.1	–	< 0.1	37
P. martensii				
RIV 158	0.5	1.3	0.1	–
RIV 159	1.7	11	< 0.1	–
RIV 160	< 0.1	3.4	0.8	–
RIV 161	< 0.1	< 0.1	0.2	–
P. viridicatum				
RIV 80	< 0.1	< 0.1	–	< 0.1
RIV 188	< 0.1	–	–	0.3
RIV 189	< 0.1	–	–	< 0.1
A. ochraceus				
RIV 45	< 0.1	< 0.1	–	–
RIV 86	8.9	9.0	–	–
CBS 263.67	5.8	5.3	–	–
CBS 588.69	< 0.1	< 0.1	–	–
CBS 589.68	19	15	–	–
NRRL 3174	23	29	–	–
NRRL 3519	< 0.1	< 0.1	–	–
NRRL 5175	16	13	–	–
ATCC 18641	32	15	–	–
P. chrysogenum	< 0.1 /2[4]	–	< 0.1 /2	–
P. crustosum	< 0.1 /4	–	–	< 0.1 /4
P. notatum	< 0.1 /2	–	< 0.1 /2	–
P. palitans	< 0.1 /8	–	< 0.1 /8	–
P. roqueforti	< 0.1 /4	–	< 0.1 /4	–

[1] ME: malt extract agar, MEYS: malt extract yeast extract sucrose agar, PD: pepton dextrose agar, MTD minerals tartrate dextrose agar.

[2] Not tested.

[3] Detection level 0.1 mg per 6 colonies.

[4] Number of strains tested.

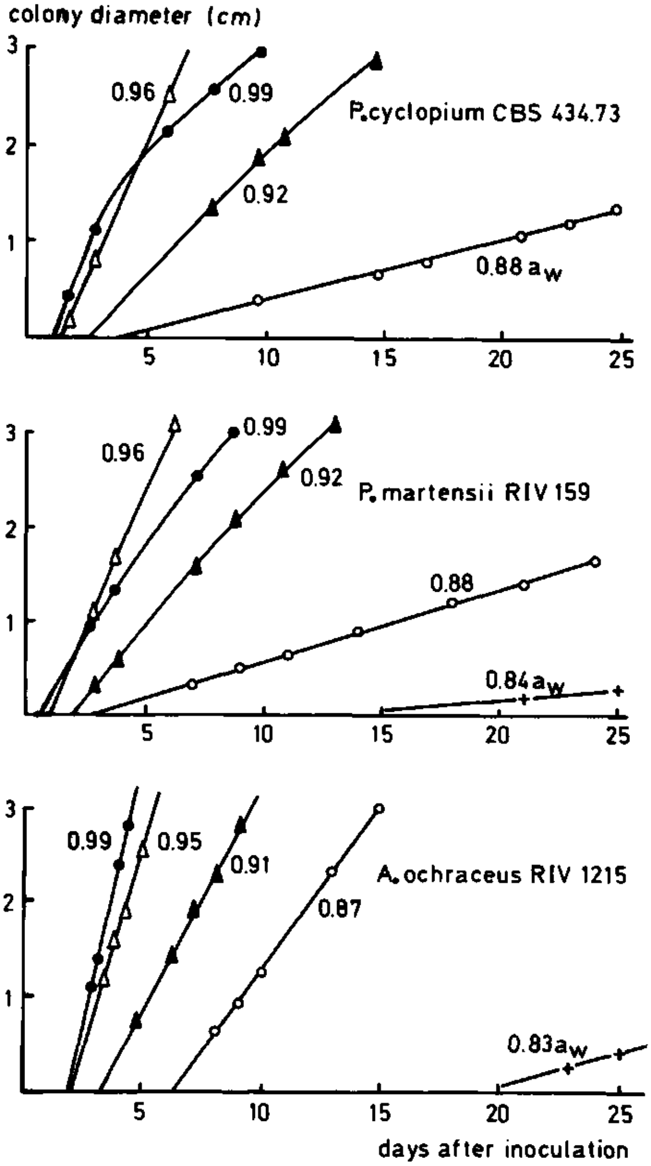


Figure 1. *Growth of strains of three different fungal species on malt extract sucrose agar at various water activity levels and 24 C.*

cultures on Gouda cheese nor cultures on Tilsiter cheese contained PA when extracted after reaching an average colony diameter of 30 mm. Cultures on Tilsiter cheese which were incubated at 16 C for longer periods up to 44 days, also did not contain PA. However, a culture of six colonies on Gouda cheese incubated for 42 days contained 4 μg of PA. Results of cultures on Gouda cheese of 0.97 a_w of four potential PA-producing strains of *P. cyclopium* revealed that these strains did not produce PA on Gouda cheese at 16 C after 14, 28 and 42 days of incubation (Table 4).

Growth rate of and PA production by *A. ochraceus* on
are shown in Fig. 15. At 0.99 a_w the
nge for PA production was 12-24 C.
ivity was adjusted to lower values, PA
ed more than growth. PA could be
tivity as low as 0.88. The PA
w optima; one optimum at 0.99
.975 a_w. This phenomenon was repeatable and was not caused by the small variation in recovery of PA due to differences in moisture content of feed, because the average recovery of PA added to the batches of feed of 0.97, 0.975, 0.98 and 0.99 a_w was 82, 87, 89, and 92% respectively. The wide a_w range of toxin production could only be demonstrated in *A. ochraceus* RIV 1215 cultures on poultry feed; in another experiment, *P. cyclopium* CBS 434.73 produced toxin in poultry feed at 0.99 and 0.97, but not at 0.95 a_w. Because of the high protein content of poultry feed, reaction of toxin with several amino acids could be expected to result in only little detectable toxin. It was determined that the protein content of feed amounted to 200 g/kg of the dry weight (d.w.), and the total amount of reactive amino acids (*11*), i.e. cysteine, lysine, histidine, and arginine, was 28 g/kg of d.w. To explain the high amount of toxin found in poultry feed, leaching of toxin from mycelium and reactivity of toxin with poultry feed were examined. In one experiment, it was shown that mycelial pellets obtained from a liquid MES 0.99 a_w shaken culture contained only 0.5% of the initial total amount of toxin after draining the culture medium and washing the pellets with water. In another experiment, 2 mg of toxin was mixed with a plate of poultry feed of 0.99 a_w and a plate of 0.95 a_w and incubated for 60 h at 24 C. The recovery of toxin was 43 and 38% respectively.

DISCUSSION

The temperature range for PA production almost equalled that for growth of the fungi tested. *P. cyclopium* and *P. martensii* produced PA over a wide temperature range starting at 4 C, whereas *A. ochraceus* produced PA starting at 8 C. The optimum temperature for PA production by *P. cyclopium* and *A. ochraceus* varied with the strain tested. This property was strain-related rather than species-related and had also been demonstrated with aflatoxin-producing strains of *Aspergillus flavus* and patulin-producing strains of *Penicillium patulum* in previous investigations (*21,22*). Aeration of *P. cyclopium* cultures stimulated PA production, but it did not influence the optimum temperature for PA production, nor the a_w range of PA production. The decrease in growth rate at the end of the incubation period was not abolished by extra supply of air, which indicated that there was no shortage of oxygen in the cultures.

The substrate played an important role in the a_w range for PA production. On MES and MEG, *A. ochraceus* had a narrow a_w range for PA production, similar to those of *P. cyclopium* and *P. martensii*, but it had a wide a_w range on poultry feed, as was also found by Bacon et al. (*1*). The recovery studies with PA in MES and poultry feed demonstrate that the low amounts of PA found at low a_w could not be explained by an increase of instability of PA, but merely by a depression of the production of PA. The absence of PA in dry sausages ripened by penicillia capable of producing PA (*10*) should be explained by the impossibility of PA production by penicillia at 0.95 a_w, fig.2 P.cyclopium CBS 434.73 on MES

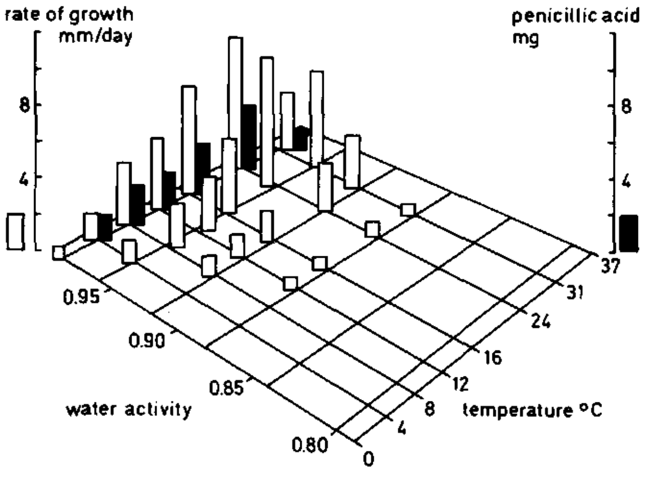


fig.3 P.cyclopium CBS 434.73 on MEG

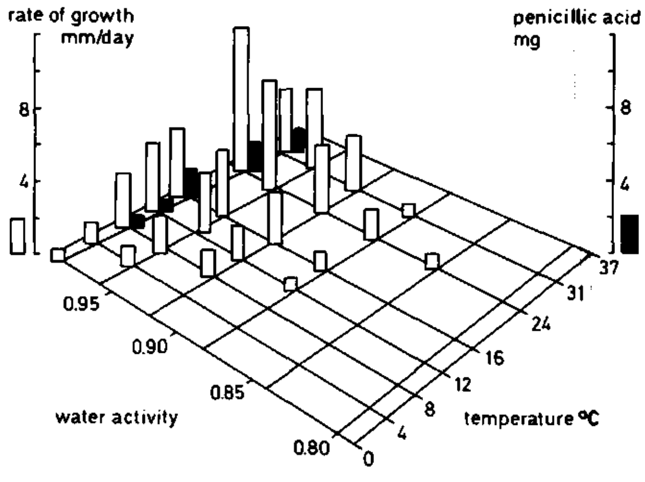


fig.4 P.martensii RIV159 on MES

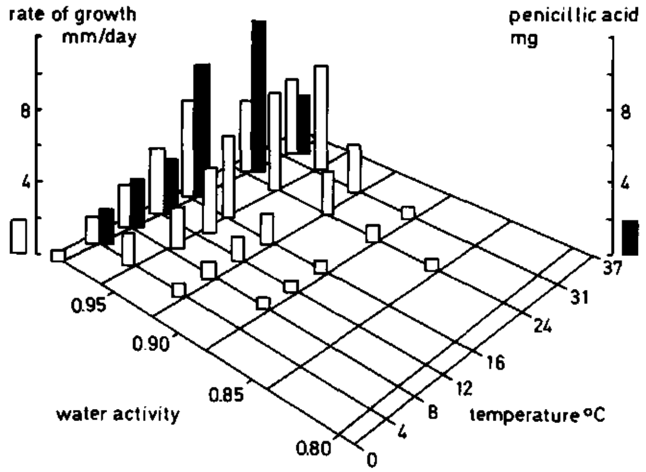


fig.5 P.martensii RIV159 on MEG

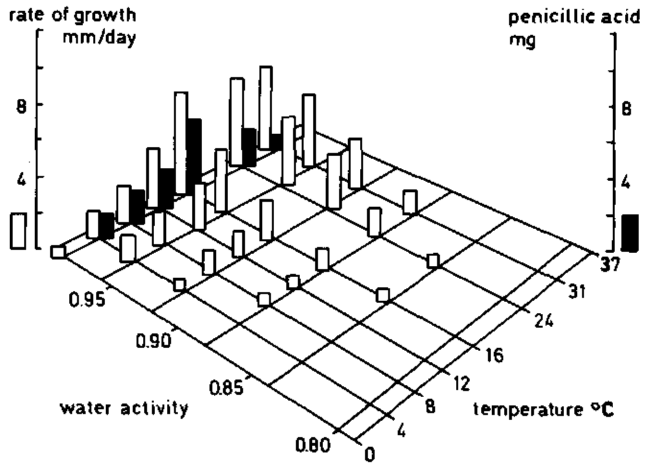


fig.6 A.ochraceus NRRL 5175 on MES

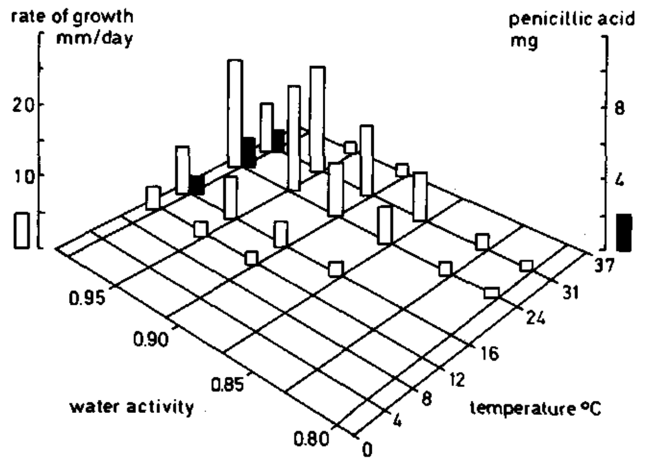
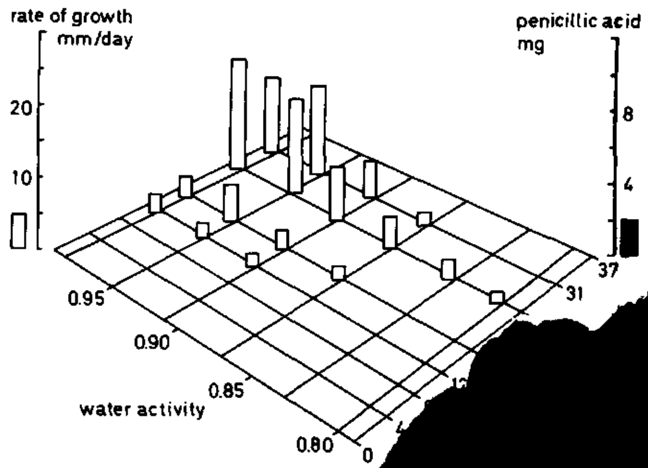


fig.7 A.ochraceus NRRL 5175 on MEG



Figures 2-7. *Growth of and penicillic acid production by three fungal species on malt extract sucrose agar (MES)* *(MEG) under various conditions of water activity and temperature (the 1 mm black columns represent amounts of 0.1-*

fig.8

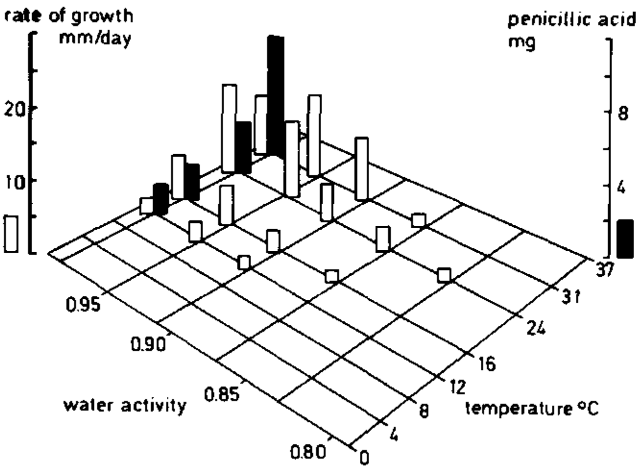
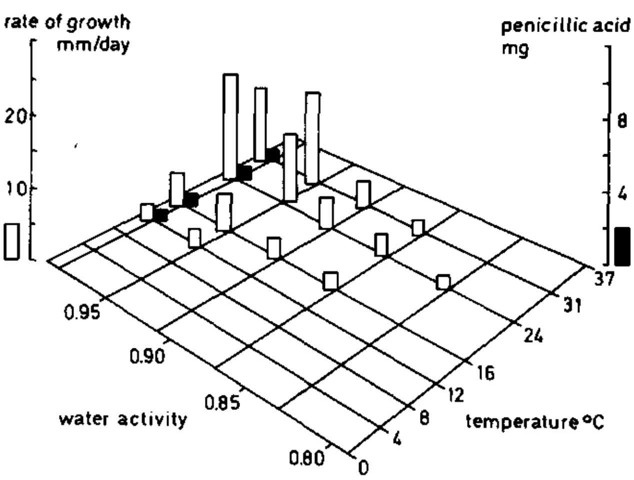

A. ochraceus RIV 1215 on MES

fig.9


A. ochraceus RIV 1215 on MEG

Figures 8 and 9. *Growth of and penicillic acid production by* Aspergillus ochraceus *RIV 1215 on malt extract sucrose agar (MES) and malt extract glycerol agar (MEG) under various conditions of water activity and temperature (the 2 mm black columns represent amounts of 0.1-0.4 mg of penicillic acid).*

fig.10

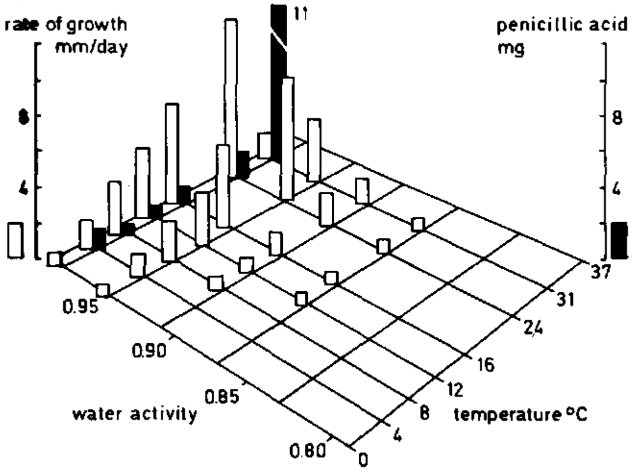
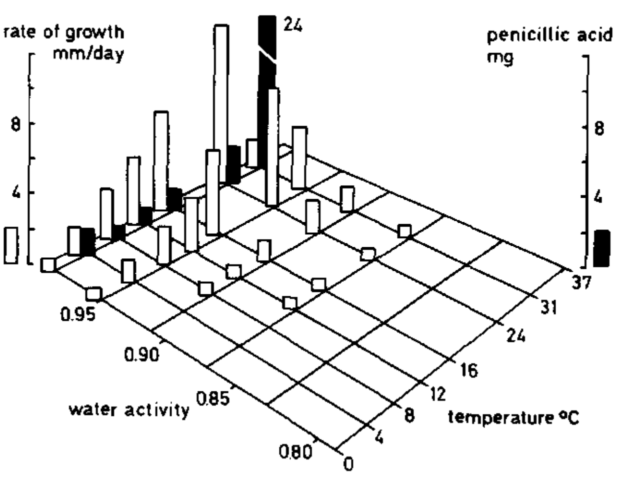

P. cyclopium RIV 129 on MES without aeration

fig.11


P. cyclopium RIV 129 on MES with aeration

Figures 10-11. *Growth of and penicillic acid production by* Penicillium cyclopium *RIV 129 on malt extract sucrose agar (MES) and malt extract glycerol agar (MEG) under various conditions of water activity and temperature, with and without regular opening of culture bags (the 1 mm black columns represent amounts of 0.1-0.4 mg of penicillic acid).*

TABLE 3. *Growth of penicillic acid-producing molds and penicillic acid production on malt extract sucrose at unfavourable conditions of a_w and temperature.*

	0.99 a_w						
Species and strain no.	0	4	8	12	24	37(C)	0.95 a_w 24 (C)
P. cyclopium							
RIV 127	G –[1]	NT[2]	NT	NT	G +[3]	NG[4]	G –
RIV 129	G –	NT	NT	NT	G +	NG	G –
RIV 196	G –	NT	NT	NT	G +	NG	G –
CBS 434.73	G –	NT	NT	NT	G +	NG	G –
A. ochraceus							
RIV 86	NT	NG	G +	G +	G +	NG	G –
RIV 1215	NT	NG	NG	G +	G +	NG	G –
CBS 589.68	NT	NG	G +	G +	G +	NG	G –
NRRL 3174	NT	NG	G –	G +	G +	NG	G –
ATCC 18641	NT	NG	G –	G +	G +	NG	G –

rowth and no penicillic acid detected (< 0.1 mg per 6 colonies).

t tested.

owth and penicillic acid detected.

growth.

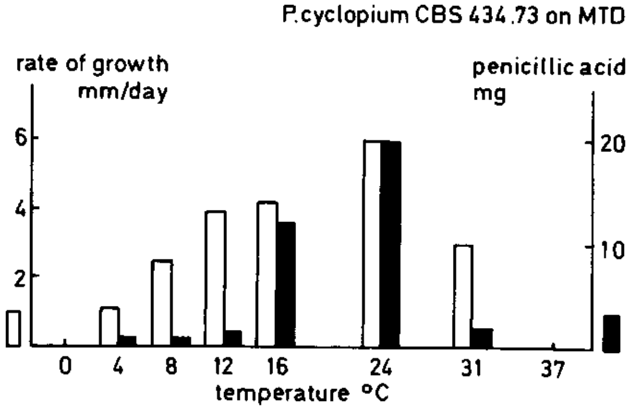


Figure 12. *Growth of and penicillic acid production by* Penicillium cyclopium *CBS 434.73 on minerals tartrate dextrose agar (MTD) at different temperatures (the 1 mm black columns represent amounts of 0.1-0.7 mg of penicillic acid).*

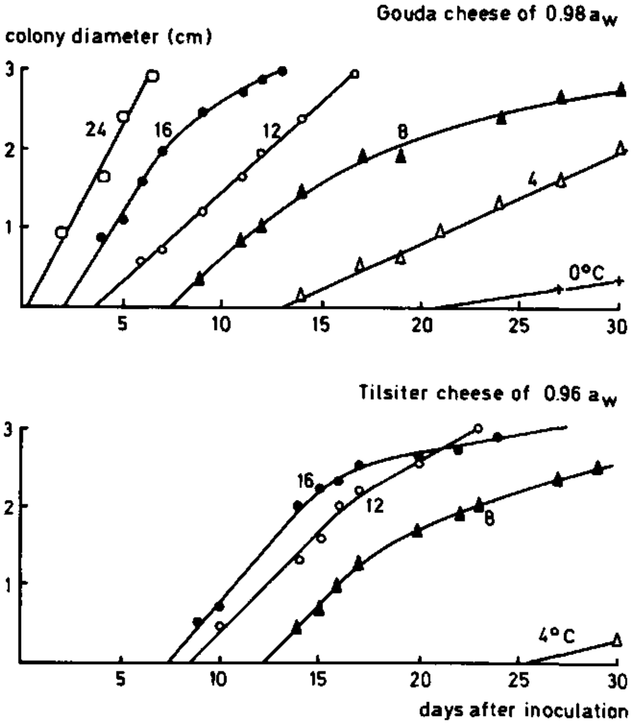


Figure 13. *Growth of* Penicillium cyclopium *CBS 434.73 on slices of Gouda and Tilsiter cheese at different temperatures.*

rather than by the instability of PA in the product. Although we used the same strain of *A. ochraceus* as Bacon (*1*) did, our results showed two a_w optima for PA production, e.g. 0.99 and 0.975 a_w, instead of an a_w optimum of 0.90, as observed by Bacon. This might be explained by a difference in the poultry feed used.

The PA production by *A. ochraceus* demonstrated at low a_w in poultry feed may be related to the composition of this substrate. Poultry feed is high in protein compared to MES and MEG, and this might suggest that protein stimulates PA production by this mold. However, this explanation is not supported by our results, where no increase of PA production was noticed in the yeast extract-enriched malt extract medium, MEYS, compared to malt extract medium, ME.

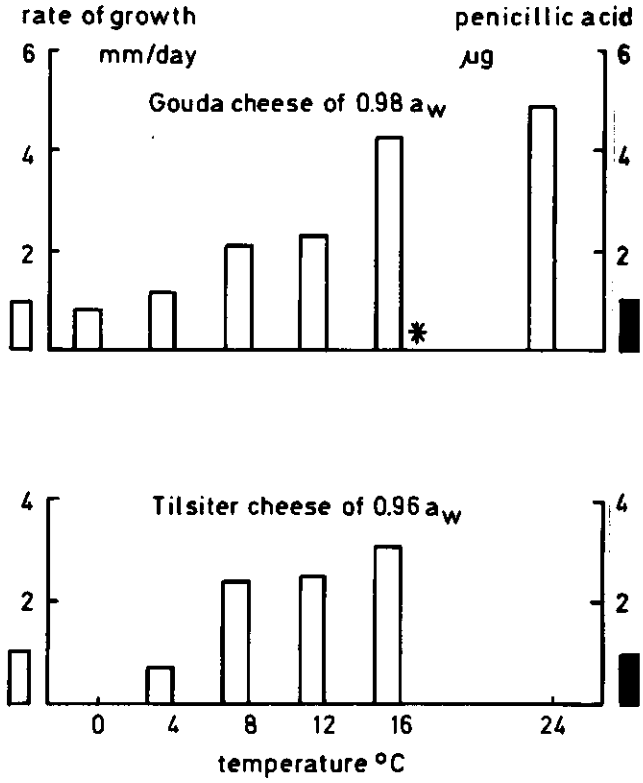


Figure 14. *Growth of and penicillic acid production by* Penicillium cyclopium *CBS 434.73 on slices of Gouda and Tilsiter cheese at different temperatures (* after 6 weeks of incubation 4 µg of penicillic acid per 6 colonies were determined).*

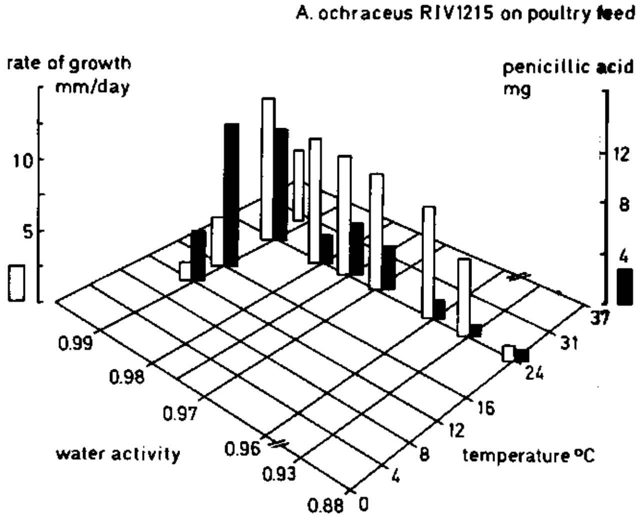


Figure 15. *Growth of and penicillic acid production by* Aspergillus ochraceus *RIV 1215 on poultry feed under various conditions of water activity and temperature (the 1 mm black columns represent amounts of 0.1-0.6 mg of penicillic acid).*

TABLE 4. *Penicillic acid production by penicillic acid-producing strains of* Penicillium cyclopium *on 1 week old Gouda cheese of 0.97 a_w at 16 C.*

Strain of	Incubation period (days)		
P. cyclopium	14	28	42
RIV 212	—[1]	—	—
CBS 161.42	—	—	—
CBS 434.73	—	—	—
NRRL 1888	—	—	—

[1] No penicillic acid detected.

Therefore, an explanation for the substrate effect should be sought in other unknown factors in poultry feed.

In the survey of cheese, *P. cyclopium* was isolated most frequently from naturally moldy Gouda cheese ripening in warehouses or on display in shops (*23*). All strains except the one that was isolated from a cheese shelf failed to produce PA in media favorable for PA production. In several experiments with cheese inoculated with *P. cyclopium* strains of known ability to produce PA, PA could be detected in only one sample of cheese after a long incubation period. The absence of PA-producing *P. cyclopium* strains on cheese and the low production of PA in inoculated cheese suggest that there is little chance to detect PA in Gouda cheese molded by *P. cyclopium*. The small amount of PA in cheese can be explained by a depression of PA production in substrates low in carbohydrates (*16*) and the sub-optimal a_w of cheese for PA production. Besides, PA is unstable in cheese, possibly reacting with sulfhydryl compounds and becoming chemically undetectable (*16*). However, a large amount of toxin was found in cultures on protein- and carbohydrate-rich poultry feed containing reactive amino acids. It was demonstrated that the mycelium readily released the toxin, and yet the toxin was rather stable in poultry feed, as shown by a 43% recovery rate of PA after 60 h at 24 C. This indicates that the main reason for the small amount of PA in cheese is low production rather than instability of PA in the cheese.

It is concluded that the temperature and water activity as well as the nature of the substrate play an important role in production of PA. With regard to reactive mycotoxins such as penicillic acid one should keep in mind that the final amount of detectable toxin depends on how much is produced and how much is lost as a result of the toxin with substrate components.

ACKNOWLEDGMENTS

The authors thank Mr. K. Terpstra for the analysis of poultry feed and Dr. C. W. Bacon, Mr. E. de Boer, Dr. J. Harwig, and Dr. R. A. Samson for supplying fungal strains. Moreover, they are grateful to Dr. J. Harwig for correcting the manuscript.

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Reprinted from
Journal of Food Protection Vol. 42, No. 6, Pages 485-490 (June, 1979)
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Ochratoxin A Production by Some Fungal Species in Relation to Water Activity and Temperature

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(Received for publication January 3, 1979)

ABSTRACT

The effects of water activity (a_w) and temperature on growth of and ochratoxin A (OA) production by strains of *Aspergillus ochraceus, Penicillium cyclopium*, and *Penicillium viridicatum* were investigated. On agar media in which the a_w had been adjusted by addition of sucrose or glycerol, the minimum a_w values for OA production by *A. ochraceus, P. cyclopium* and *P. viridicatum* lay between 0.83-0.87, 0.87-0.90, and 0.83-0.86, respectively. At 24 C, optimum a_w values for OA production by *A. ochraceus* and *P. cyclopium* were 0.99 and 0.95-0.99, respectively, whereas that of *P. viridicatum* varied and was 0.95 and 0.99 for the two strains tested. At optimum a_w, the temperature range for OA production by *A. ochraceus* was 12-37 C, whereas that of *P. cyclopium* and *P. viridicatum* was 4-31 C. Optimum temperature for OA production by *A. ochraceus* was 31 C, whereas that of *P. cyclopium* and *P. viridicatum* was 24 C. On Edam cheese of 0.95 a_w, the minimum temperature for OA production by *P. cyclopium* was 20 C. On barley meal, *P. viridicatum* produced maximal quantities of OA at 0.97 a_w and could produce OA at temperatures as low as 12 C.

Ochratoxins are a group of structurally related secondary metabolites produced by molds belonging to several species of the genera *Aspergillus* and *Penicillium* (*33*). The most extensively studied compound of this group, ochratoxin A (OA), is toxic to many test animals, as has been reviewed by Chu (*5*). The main pathological changes after fatal dosing of animals, such as rats and chicks, with OA were confined primarily to damage to renal tubules and necrosis of kidneys and periportal liver cells. OA was also found to be teratogenic (*3*). According to Krogh et al. (*15*), OA may be a disease determinant of Balkan endemic nephropathy, a chronic kidney disease affecting rural populations in some areas of the Balkans.

The presence of OA has been demonstrated in a number of agricultural products. In the United States, corn (*31*), barley (*9*), and wheat (*32*) have been found to be contaminated with OA. In Sweden, OA-contaminated barley and oat products have been obtained from shops (*13*). In some districts of Denmark with a high incidence of swine nephropathy, OA was demonstrated in cereal grains, especially barley, used as feed (*14*). Residues of OA were found at slaughter in kidneys of pigs (*16*) and poultry (*7*) showing nephropathy changes.

OA-producing fungi have been characterized as storage fungi. *Penicillium viridicatum* has been frequently isolated from stored corn (*4,20*), dried beans (*25*), fermented sausages (*19*), cured hams (*17*) and hard cheese (*23*). The evidence points to *P. viridicatum* as the main cause of OA contamination of grains (*29*), but *Aspergillus ochraceus* has also been associated with the presence of OA in corn (*10*) and green coffee beans (*18*). The fungus was one of the predominant species isolated from dried beans (*21*) and black pepper (*4*). Other OA-producing fungi are *Penicillium cyclopium* and *Penicillium palitans. P. cyclopium* was the chief species isolated from stored corn (*20*), hard cheese (*23*), and fermented and cured meat products (*11*). *P. palitans* has been isolated from fermented and cured meat products (*11*) and mixed feed containing OA (*29*).

Although much work has been done on the toxicity of OA and the examination of foodstuffs for OA, little is known about the environmental conditions for growth of and OA production by molds. The most important factors in safeguarding stored foodstuffs are water activity (a_w) and temperature. The first factor, defined as the equilibrium relative water vapor pressure of a substrate, is of great value as a measure for the amount of water available to microorganisms (*30*).

In the present study, cultures of various fungal species were grown on semi-synthetic media at different combinations of a_w and temperature to determine the limits and optimum conditions for mycelial growth and production of OA. Moreover, natural substrates were inoculated with OA-producing molds to estimate the possible influence of substrate.

MATERIALS AND METHODS

Organisms and spore suspensions

Fungal strains were maintained as lyophilized cultures. Strains of *A. ochraceus* and *P. cyclopium* had been received from the late D. I. Fennell, Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois. Strains of *A. ochraceus* and *P. viridicatum* had been supplied by J. Harwig, Health and Welfare Canada, Health Protection Branch, Ottawa, of which strains RIV 28, 29, 80, 126 and 712 were originally labeled with the numbers 129, 136, 183, 182 and 583, respectively. Strains of *A. ochraceus, P. cyclopium, P. palitans*, and *P. viridicatum* had been received from R. A. Samson, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands, of which the *P. cyclopium, P. palitans* and *P. viridicatum* strains were isolated from meat products by R. Hadlok, Giessen, G. F. R. Moreover, the laboratory's own strains, belonging to the four species and isolated from different kinds of foods, were used. Aspergilli were classified according to Raper and Fennell (*27*), penicillia according to Raper and Thom (*26*). It should be noted that in several European countries *P.*

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cyclopium and *P. viridicatum* are now classified as *P. verrucosum* var. *cyclopium* and *P. verrucosum* var. *verrucosum*, respectively, whereas *P. palitans* is classified as either *P. verrucosum* var. *cyclopium or* P. *verrucosum* var. *melanochlorum*, depending on the color of the colonies (*28*).

Cultures grown for 7-10 days at 24 C on malt extract agar (Oxoid) were washed with an aqueous solution of 6 g of sodium heptadecylsulfate (Tergitol-7, BDH)/l to prepare spore suspensions of ca. 10^6 spores per ml.

After the desired periods of incubation, cultures were stored at - 18 C for 10-30 days until extracted for OA.

Cultures on agar media

In a preliminary study, mold strains were tested for OA production at 24 C on two agar plates of malt extract agar medium (Oxoid) (ca. 1.00 a_w, ME) and Czapek maize extract agar medium (ca. 1.00 a_w, CM). CM agar medium was prepared by mixing a sterilized solution of 70 g of Czapek Dox broth powder (Difco)/l and 30 g of agar no. 1 (Oxoid)/l with an equal volume of sterilized maize extract. The components were separately sterilized at 121 C for 20 min. The maize extract was prepared by boiling 200 g of crushed maize in 1 liter of water for 30 min, followed by centrifugation. Each plate was inoculated at three different spots with an inoculation needle dipped in a spore suspension. Incubation was terminated as soon as the colonies reached an average diameter of 30 mm.

A. ochraceus NRRL 3519, *P. cyclopium* NRRL 6065, *P. viridicatum* RIV 28 and *P. viridicatum* RIV 712 were used to determine the environmental conditions for mold growth and OA production. Agar media favoring high production of OA were chosen: *A. ochraceus* NRRL 3519 was cultured on malt extract agar supplemented with sucrose (MES-series) or glycerol (MEG-series) to obtain the desired a_w conditions, whereas the *Penicillium* species were cultured on Czapek maize extract agar supplemented with sucrose (CMS-series) or glycerol (CMG-series) to obtain the desired a_w conditions. The two agar series were used to demonstrate the effect of a_w on toxin production rather than the effect of concentration of the supplemented compound. To determine the effect of substrate on mold growth and OA production, *A. ochraceus* NRRL 3519 was also cultured on CMS at different conditions of a_w and temperature. Preparation of agar plates, inoculation, and a_w measurement have been described in a previous paper (22). The a_w measuring device consisted of a sample jar that was submerged in a temperature-controlled water bath, thus facilitating the equilibration of the vapor pressure around the sample, and was connected to a dewpoint hygrometer through a closed circuit. The accuracy of the device was 0.004 a_w. All measurements were done at 24 C. For each determination of rate of growth and OA production, two inoculated agar plates with three colonies each and one non-inoculated agar plate, which served as control for the a_w after incubation, were used. They were enclosed in a 0.3-liter polyethylene bag (gauge 0.04 mm). Cultures were grown at combinations of eight temperatures and six a_w values on MES and MEG or CMS and CMG. The variation of temperature was 0.3 C. The growth rate of the mycelium was determined by daily measurement of two right-angled diameters of the colonies. The regression lines of colony diameter on days after inoculation were calculated for each a_w-temperature combination. The germination time was obtained by extrapolating the regression line to the X-axis (22). Where rates of growth decreased during incubation, initial rates of growth obtained by drawing the tangent are given. Incubation of strains *A. ochraceus* and *P. viridicatum* was terminated on the day the six colonies reached an average diameter of 30 mm or on the 35th day of incubation when they did not reach this size. The incubation of *P. cyclopium* NRRL 6065 lasted until the average colony diameter reached a size of 20 mm, due to the slow growth rate of the strain.

To assess whether or not the strains tested were representative of the mold species, the limiting a_w and temperature for mold growth and OA production were determined for some other strains of *P. viridicatum*, which were grown on CMS at the same time as the test strain, *P. viridicatum* RIV 28.

Four replicate CMS cultures of *P. viridicatum* RIV 28, grown at 0.95 a_w and 24 C, were analyzed for OA to determine the repeatability of the method.

P. cyclopium *cultures on cheese*

Growth rate of and OA production by *P. cyclopium* NRRL 6065 was measured on Edam cheese of 0.95 a_w. The ca. 5-week-old brick-shaped cheese of 2.6 kg was obtained from a shop and was aseptically cut in slices of 40 × 40 × 4 mm. To obtain slices of similar a_w value, the outer layer of the cheese was discarded. The culture method was similar to that used for the agar cultures; for each determination of rate of growth and OA production, two inoculated slices of cheese with three colonies each and one non-inoculated control slice of cheese were used. They were placed in three petri dishes, which were enclosed in a polyethylene bag. Cultures were incubated at seven different temperatures until the six colonies reached an average diameter of 20 mm or until the 35th day when they did not reach this size.

P. viridicatum *cultures on barley*

Barley meal was blended with water to a moisture content of 144, 179, 236, 289, 341, 495 and 649 g/kg (wet weight basis), giving water activities of 0.80, 0.86, 0.91, 0.93, 0.95, 0.97 and 0.98, respectively, after autoclaving. The barley was autoclaved at 121 C for 1 h, thoroughly mixed, pressed into petri dishes to thickness of 5 mm and stored for 5 days. Each plate was inoculated at three different spots with 1 µl of spore suspension of *P. viridicatum* RIV 28. Barley plates of 0.95 a_w were incubated at eight different temperatures, whereas barley plates of 0.80, 0.86, 0.91, 0.93, 0.97 and 0.98 were incubated only at 24 C. For each a_w-temperature combination two inoculated plates and one non-inoculated control plate were enclosed in a polyethylene bag. The method for determination of growth rate and germination time of mycelium was the same as that for cultures on agar. Incubation was terminated as soon as the colonies reached an average diameter of 30 mm or after 30 days when they did not reach this size.

Extraction and determination of OA from agar media

After thawing, cultures were inactivated by adding 20 ml of chloroform to each pair of plates. After shaking, the chloroform was allowed to stand in contact with the culture for at least 2 h at room temperature. For qualitative analysis, 20 µl of the chloroform solution was spotted on a thin layer chromatography (TLC) plate next to an OA standard. The plate was developed in toluene-ethyl acetate-formic acid (6:3:1 vol/vol/vol/) in an unsaturated tank, dried and exposed to ammonia vapor for 10 min. The presence of OA was indicated by a blue fluorescent spot on the plate when exposed to longwave UV light at 365 nm.

For quantitative analysis, the chloroform was decanted, the agar plates were washed twice with 10 ml of chloroform and the chloroform fractions were combined. The agar plates were blended with 50 ml of H_2O, 50 ml of chloroform and 5 ml of H_2SO_4 (0.5 mol/l) by means of a homogenizer (Ultra Turrax). After centrifugation, the mixture was transferred into a separatory funnel, and the chloroform layer was combined with the chloroform fractions collected before. The chloroform extracts were dried over anhydrous Na_2SO_4, evaporated to dryness and the residue was dissolved in 1 ml of chloroform. After preliminary visual determination of OA concentration by TLC, the sample solution was diluted to a measureable concentration. Twenty µl of sample solution and OA standard were spotted next to each other on a silica gel TLC plate, Merck 60, with a thickness of 0.25 mm and developed in toluene-ethyl acetate-formic acid (6:3:1 vol/vol/vol) in an unsaturated tank. After development, the plate was exposed to ammonia vapor for 10 min and covered with a glass plate. The resulting blue fluorescent spot of OA was measured densitometrically by fluorescence measurement (excitation wavelength 365 nm, emission wavelength 430 nm). The limit of detection was ca. 0.1 µg of OA per two agar plates.

Extraction and determination of ochratoxin A from cheese

Quantities of chemicals for extraction of OA indicated below are related to the weight of the cheese; they were proportionally increased or decreased when the amount of cheese to be analyzed varied.

Twenty-five g of cheese was blended in 125 ml of chloroform and 12.5 ml of H_3PO_4 (0.1 mol/l) for 90 sec by means of an Ultra Turrax (2). Ten g of diatomaceous earth (Celite) was added and the moisture was filtered. Two g of diatomaceous earth was mixed with 1 ml of $NaHCO_3$ (0.15 mol/l) in water and brought into a 700 × 17-mm chromatographic column, fitted with a plug of glass wool (24). After tamping the column, a mixture of 50 ml of cheese extract and 40 ml of hexane was brought onto the column. The column was eluted with 75 ml of chloroform and the eluate was discarded. Ochratoxin A was eluted with 75 ml of chloroform-formic acid (99:1 vol/vol). Immediately thereafter the eluate was evaporated almost to dryness and the residue was dissolved in 1 ml of chloroform. Concentration of OA was determined as described for the quantitative analysis of agar plates. However, instead of one-dimensional TLC, two-dimensional TLC was applied with chloroform-methanol (8:2 vol/vol) and toluene-ethyl acetate-formic acid (6:3:1 vol/vol/vol) as first and second developing solvent, respectively. The limit of detection was ca. 0.05 μg per culture.

Extraction and analysis of ochratoxin A from barley

The quantities of chemicals indicated below are related to the dry weight of barley; they were proportionally increased or decreased when the amount of barley to be analyzed varied.

After thawing, barley from the pair of plates were blended with 250 ml of acetonitrile and 25 ml of H_3PO_4 (0.1 mol/l) by means of an Ultra Turrax, followed by filtration. Fifty ml of filtrate was shaken in a separatory funnel with 50 ml of acetonitrile, 50 ml of iso-octane and 50 ml of $NaHCO_3$ (0.15 mol/l) and NaCl (0.5 mol/l) solution in water. After shaking, three layers were formed of which the lower phase was the water phase. The water phase was drained off, acidified with 5 ml of H_2SO_4 (2 mol/l), and extracted three times with 50 ml of chloroform. The combined chloroform fractions were dried with anhydrous Na_2SO_4, evaporated to dryness and the residue was dissolved in 0.5 ml of chloroform. TLC determination of OA was carried out as described for the analysis of agar cultures. The detection limit was ca. 1 μg per culture.

RESULTS

Cultures on agar media

Results of the preliminary study of ME and CM agar medium are given in Table 1. One strain each of *A. ochraceus* and *P. cyclopium* and several strains of *P. viridicatum* produced OA, but none of the *P. palitans* strains tested produced OA. On ME, *A. ochraceus* produced more OA than on CM, whereas *P. cyclopium* and *P. viridicatum* produced more OA on CM than on ME.

TABLE 1. *Production of ochratoxin A (in μg per 6 colonies) by various strains of different fungal species on malt extract agar (ME) and Czapek maize extract agar (CM) incubated at 24 C.*

Species and strain no.	Agar medium ME	Agar medium CM
A. ochraceus		
NRRL 3519	410	8
13 other strains	< 0.1	—[a]
P. cyclopium		
NRRL 6065	24	47
5 other strains	< 0.1	—
P. palitans		
13 strains	< 0.1	—
P. viridicatum		
RIV 28	3.8	230
RIV 29	0.7	—
RIV 80	0.1	—
RIV 126	1.0	—
RIV 188	2.6	—
RIV 712	0.8	220
4 other strains	< 0.1	—

[a] Not tested.

Figure 1 shows examples of colony growth curves of cultures of *P. viridicatum* RIV 28 on CMS agar medium. Under certain conditions of a_w and temperature, growth rate decreased after a period of time. This phenomenon could not be correlated with a_w or temperature and it occurred rarely.

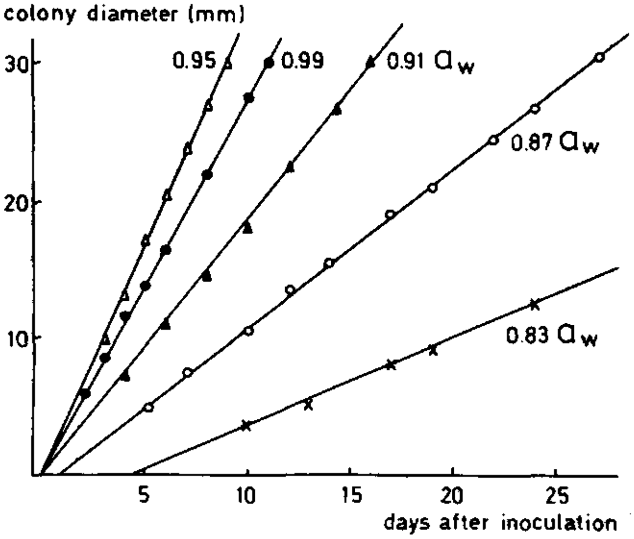


Figure 1. *Growth of* Penicillium viridicatum *RIV 28 on Czapek maize extract sucrose agar at various water activity levels and 24 C.*

Average growth rate of colonies and OA production on agar media at each combination of temperature and initial a_w are shown in Fig. 2-9. For cultures with decreasing growth rate, the average initial growth rate of colonies is given in the figures. Optimum a_w value for OA production by *A. ochraceus, P. cyclopium* and *P. viridicatum* RIV 712 was 0.99. However, that of *P. viridicatum* RIV 28 was 0.95. The minimum a_w value for OA production by *A. ochraceus* and *P. viridicatum* RIV 28 lay between 0.83 and 0.87, that of *P. viridicatum* RIV 712 between 0.83 and 0.86 and that of *P. cyclopium* between 0.87 and 0.90. The minimum a_w value for mold growth lay between 0.79 and 0.83 and did not differ significantly for the different fungal species. Optimum temperatures for OA production by *A. ochraceus* were 31 or 37 C, depending on a_w, whereas *P. cyclopium* produced maximum quantities at 24 C. *P. viridicatum* RIV 28 produced more OA on CMS at 24 C than at 16 C, but no difference was noticed on CMG. Temperature ranges for OA production by *A. ochraceus, P. cyclopium* and *P. viridicatum* were 12-37, 4-31 and 4-31 C, respectively. The temperature range for mold growth by *A. ochraceus*, 8-37 C, was higher than that of *P. cyclopium* and *P. viridicatum*, which was 0-31 C.

Germination times of *A. ochraceus* and *P. viridicatum* were 0.5-2 days under optimum conditions for mold growth and they increased to 12-14 days under unfavorable conditions. Those for *P. cyclopium* were 1-2 days and 21 days, respectively.

Data in Table 2 demonstrated that two other strains of *P. viridicatum* had limiting conditions for mold growth and OA production similar to those for strain RIV 28.

fig. 2

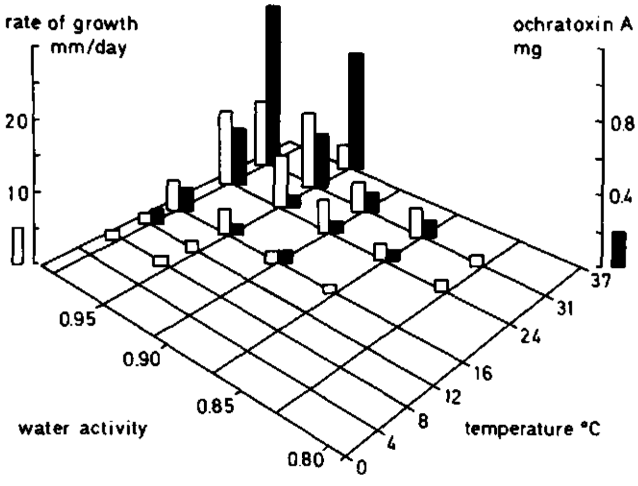
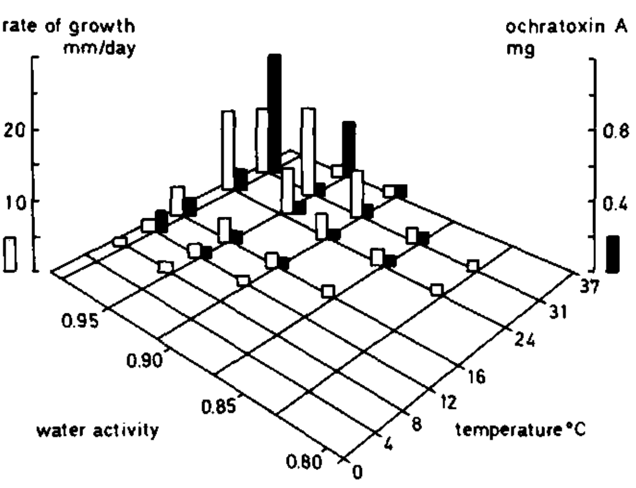

A. ochraceus NRRL 3519 on MES

fig. 3


A. ochraceus NRRL 3519 on MEG

Figures 2-3. *Growth of and ochratoxin A production by* Aspergillus ochraceus *NRRL 3519 on malt extract sucrose agar (MES) and malt extract glycerol agar (MEG) under various conditions of water activity and temperature (the 1 mm black columns represent amounts of 0.1-40 µg of ochratoxin A).*

However, two strains failed to produce toxin at 4 C and did not grow at 31 C.

The OA contents of the four replicate cultures of *P. viridicatum* RIV 28 differed by not more than 15% from the average when grown on CMS of 0.95 a_W at 24 C. The difference between initial and final a_W of substrates could amount to 0.006.

Cultures on natural substrates

Figure 10 shows average growth rate of and OA production by *P. cyclopium* NRRL 6065 on Edam cheese of 0.95 a_W. The temperature range for OA production was 20-24 C, whereas that for mold growth was 0-24 C.

Growth rate of and OA production by *P. viridicatum* on moistened barley meal are shown in Fig. 11. At 0.95 a_W the temperature range for OA production was 12-24, whereas that for mold growth was 0-31. Optimum a_W for OA production as well as for mold growth lay at 0.97. OA could be detected at 0.91 a_W, but not at 0.86.

DISCUSSION

The a_W range for OA production was similar for the three fungal species tested. In general, 0.99 a_W was optimal for OA production. The difference between the optimum a_W for OA production by *P. viridicatum* RIV 28 and *P. viridicatum* RIV 712 indicate that this feature may be strain-related. The effect of a_W on OA production was independent of substrate, since results obtained with cultures of *P. viridicatum* RIV 28 on Czapek maize extract agar and on barley meal were similar. This was also true for results obtained with cultures of *P. cyclopium* NRRL 6065 on Czapek maize extract agar and cheese, and those of *A. ochraceus* NRRL 3519 on MES, MEG and CMS.

Data in figures show that the penicillia tested can grow and produce OA at lower temperatures than *A. ochraceus*. Therefore penicillia may thrive in moderate climates, whereas *A. ochraceus* may be found especially in tropical areas. This has been indicated by the association of *P. viridicatum* with the presence of OA in Canadian (*29*) and U.S. (*34*) wheat and Danish barley (*34*), and the predominance of *A. ochraceus* in black pepper (*4*) and green coffee beans containing OA (*18*).

Our results demonstrate a minimum a_W of 0.86 - 0.90 for OA production and confirm the data of other investigators using wheat and barley (*12*) or poultry feed (*1*) as substrate. However, they contrast with results reported by Escher et al. (*8*), who inoculated *A. ochraceus* on cured ham and found that even more toxin was produced in ham of a moisture in equilibrium with 70-75% ERH than in ham with more moisture. Although unlikely, this might be due to a difference of strains. The minimum temperature of 12 C for OA production by *A. ochraceus* almost equalled that determined in corn and wheat by Ciegler (*6*) and in cured ham by Escher et al. (*8*). The temperature range for OA production by *P. viridicatum* seems to be dependent on the type of substrate; we found minimum temperatures for OA production of 4 and 12 C on Czapek maize extract agar and barley, respectively, whereas other workers (*12*) reported a minimum temperature of 5 C on wheat.

Besides environmental conditions, production of OA is influenced by fungal species and strain. Species may have different requirements for production of OA since *A. ochraceus* produced more OA on malt extract agar than on Czapek maize extract agar. This was reversed for *P. viridicatum*. Production of OA by *P. cyclopium* on cheese was low compared with that on Czapek maize extract agar and might be related to type and age of cheese. As far as is known, this is the first report of production of OA in cheese. It indicates a potential risk of contamination of commercial cheese with OA as *P. cyclopium* was found to be the predominant fungal species on hard types of cheese in warehouses, shops and households showing mold growth (*23*).

Summarizing, it can be concluded that only one strain of *A. ochraceus* and *P. cyclopium* could produce OA, whereas several OA-producing strains of *P. viridicatum* fig. 4

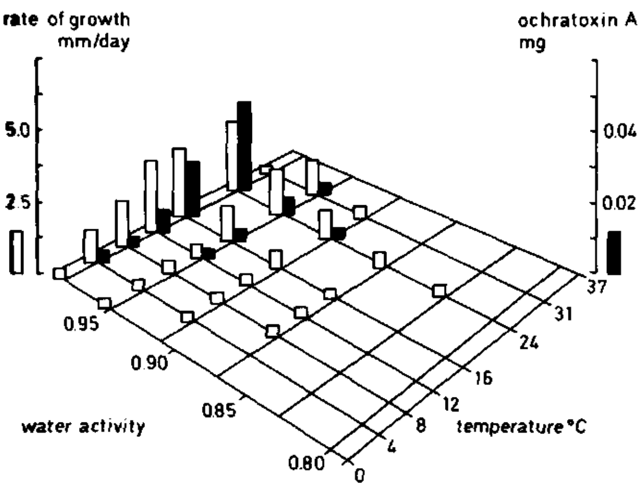

P.cyclopium NRRL 6065 on CMS

fig. 5

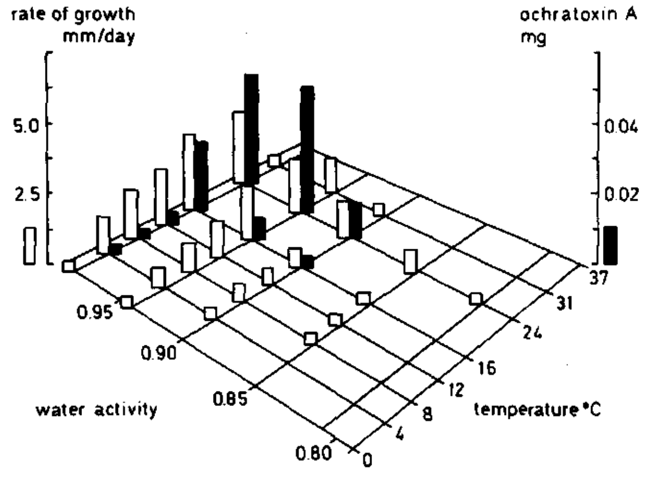

P.cyclopium NRRL 6065 on CMG

fig. 6

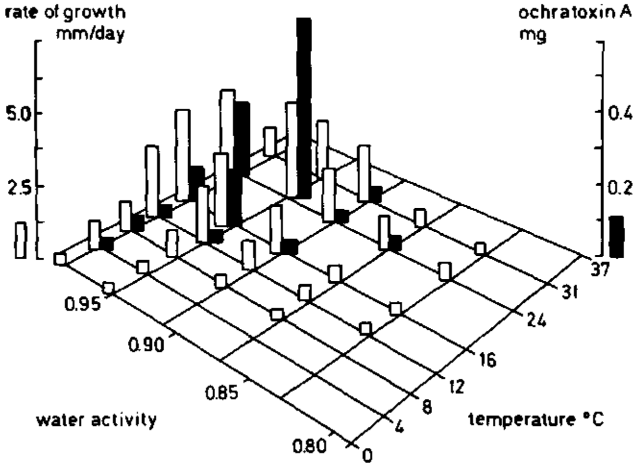

P.viridicatum RIV 28 on CMS

fig. 7

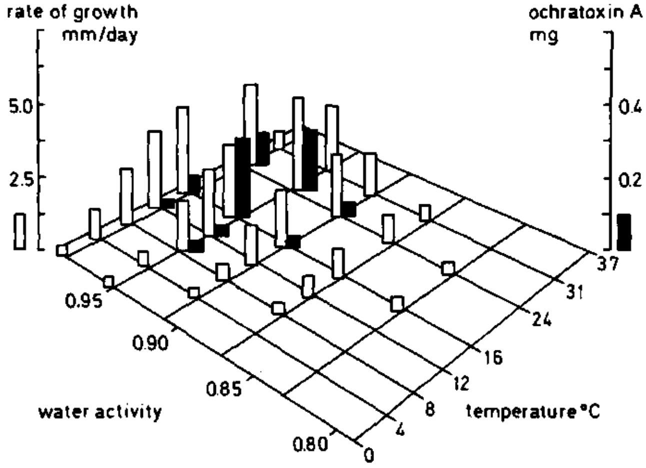

P. viridicatum RIV28 on CMG

fig. 8

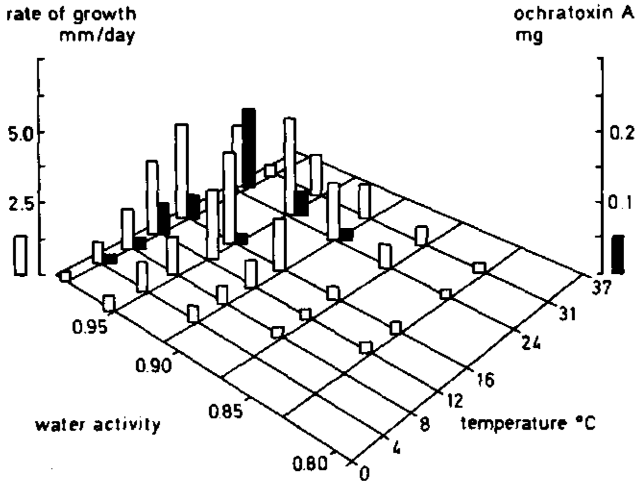
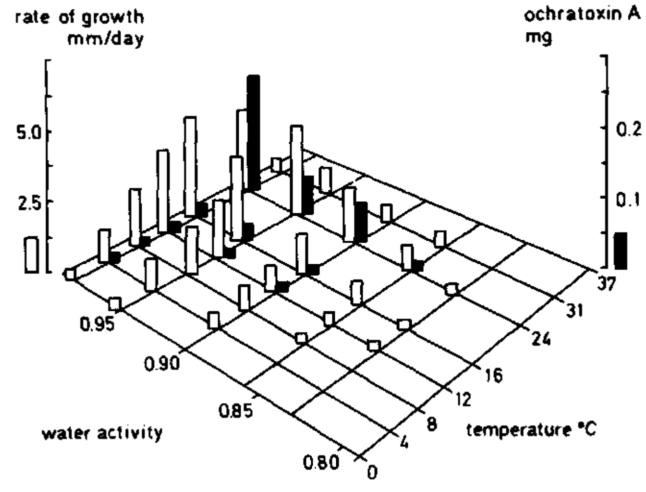

P. viridicatum RIV712 on CMS

fig. 9


P. viridicatum RIV712 on CMG

Figures 4-9. *Growth of and ochratoxin A production by one strain of* Penicillium cyclopium *and two strains of* Penicillium viridicatum *on Czapek maize extract sucrose agar (CMS) and Czapek maize extract glycerol agar (CMG) under various conditions of water activity and temperature (N.B. the scales of toxin axis are different: the 1 mm black columns represent amounts of 0.1-2, 0.1-20, and 0.1-10 μ g of ochratoxin A for* P. cyclopium, P. viridicatum *RIV 28, and* P. viridicatum *RIV 712, respectively).*

were found. Large quantities of OA can be produced at intermediate and high temperatures and high a_w by species belonging to the genera *Penicillium* and *Aspergillus*, whereas at low temperatures the toxin can be produced by penicillia only.

TABLE 2. *Growth of and ochratoxin A production by various strains of* Penicillium viridicatum *on Czapek maize extract sucrose agar at unfavorable conditions of a_w and temperature.*

	0.99 a_w				0.83 a_w
Strain no.	0 C	4 C	24 C	31 C	(24 C)
RIV 28	G –[a]	G +[b]	G +	G –	NG[c]
RIV 29	G –	G +	G +	G –	NG
RIV 44	G –	G –	G +	NG	NG
RIV 126	G –	G +	G +	G –	NG
RIV 188	G –	G –	G +	NG	NG

[a] Growth and no ochratoxin A detected (< 0.1 μg per 6 colonies).
[b] Growth and ochratoxin A detected.
[c] No growth.

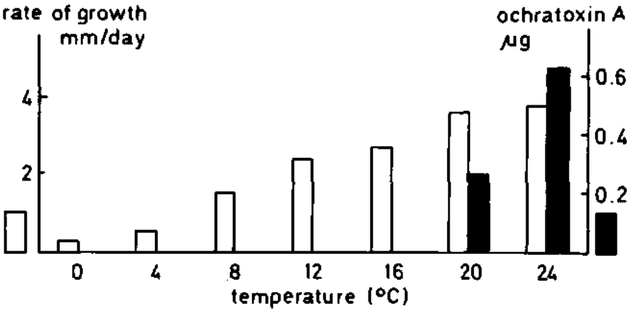


Figure 10. *Growth of and ochratoxin A production by* Penicillium cyclopium *NRRL6065 on Edam cheese of 0.95 a_w at different temperatures.*

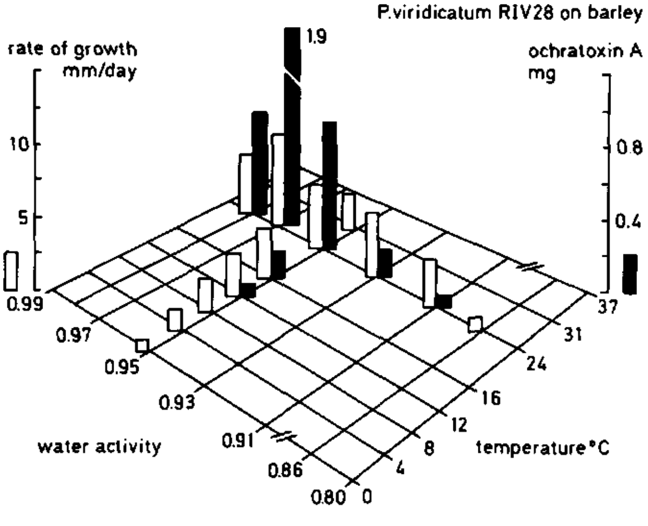


Figure 11. *Growth of and ochratoxin A production by* Penicillium viridicatum *RIV 28 on barley meal under various conditions of water activity and temperature (the 1 mm black columns represent amounts of 1-40 μg of ochratoxin A).*

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