INFLUENCE OF NITROGEN ON THE GROWTH OF SOME STORAGE FUNGI ON MOIST WHEAT.

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ABSTRACT

The storage fungi assayed were: Aspergillus flavus, Aspergillus chevalieri, Penicillium cyclopium, and one strain of yeast. The strains showed different growth curves in air. Growth was tested on wheat sterilized by γ-rays and inoculated by the various strains. Incubation was carried out at 30°C with the cereal at 18 - 19% moisture content, under air and ultrapure nitrogen, in order to study the possible inhibitory effect of nitrogen on mycelial growth, penetration and aflatoxin production.

Susceptibility to oxygen deficiency decreased in the order: A.chevalieri, P.cyclopium, A.flavus, yeast. The inhibiting effect of anoxia was strictly limited to the period of nitrogen application. Growth curves of A.flavus on aerated wheat following the nitrogen treatment were identical to those observed in directly incubated grain. Aflatoxin production in nitrogen was inhibited in respect to the controls in air.

Finally, unsterilized wheat was moistened to 19%, incubated first in air and consecutively in nitrogen. The inhibitory effect of anoxia was confirmed in the overall counts of fungal germs, while it induced a selection of certain mould species.

INTRODUCTION

The storage and the inalterability of foodstuffs, of seeds in

particular, represent one of the greatest problems of the worl economy and development. The fungal microflora is one of the mai causes of the food losses on a world scale as it produces discolor ation of the seeds, decrease of germinability, loss of weight, fer mentation and chemical transformation with possibly the production o mycotoxins.

Modified and controlled atmospheres are one of the most promisin methods to prevent and limit the growth of insects, fungi and th production of mycotoxins in seeds stored in silos (Diener and Davis 1969; Epstein et al., 1970; Jay and Pearman, 1973; Bailey and Banks 1974; Wilson and Jay,1975).

The research carried out on the possibility of storing moistene cereals (moisture superior to 14.5%) in nitrogen atmosphere gave goo results showing a clear inhibiting or delaying action of nitrogen o the growth of fungal microflora and maintaining moreover a goo germinability of the seeds and a low fat acidity (Shejbal and D Maggio, 1976; Di Maggio et al., 1976; Shejbal, 1978; Di Maggio an Shejbal,1979).

Not many experiments as regards the problems of storage have bee carried out to clarify the behaviour of single fungal species typica of moist stored seeds in conditions of total anoxia. We studied th behaviour of four fungal species (Aspergillus flavus, Aspergillu chevalieri, Penicillium cyclopium and Candida krusei) placed in fa vourable growth conditions both as regards the seed moisture and a regards the temperature (32°C) and in conditions of total lack o oxygen.

We have analyzed whether, in our experimental conditions, there i an inhibiting effect of nitrogen with a percentage of oxygen inferio to 0.01% on the growth of the assayed fungal species and whether ther exists a difference in the efficiency of the treatment on variou strains.

Particular attention was directed to A.flavus for its well know pathogenous action as an aflatoxin producer (Heathcote and Hibbert 1978). We have also carried out experiments concerning the duration o

the inhibiting effect of nitrogen after two different periods of exposure (30 and 60 days) of seeds infected with A.flavus and then brought back to air. At last we have examined the influence of nitrogen on the growth of A.flavus in connection with the production of aflatoxins.

MATERIALS AND METHODS

The assayed strains were: Aspergillus flavus (ATCC 22548), Aspergillus chevalieri (ATCC 24546), Penicillium cyclopium (ATCC 26162) and Candida krusei isolated from wheat.

The first 3 strains were kept on Czapek-agar culture medium (Difco) at the temperature of 25°C and Candida krusei on Malt-agar (Difco) at the temperature of 25°C. The soft wheat (Manitoba variety) utilized for experiments was sterilized by exposure to ^{90}Co for 90 min at 6926 rad min^{-1} using a Gammacell 220 (Atomic Energy Ltd of Canada) (Di Maggio et al., 1974). After sterilization seeds were moistened with sterile distilled water to a value of 18.5% moisture content measured with a thermobalance (Buhler) and were inoculated with 8×10^6 conidia of A.flavus, A.chevalieri, P.cyclopium and 10^5 of C.krusei.

The seeds were then placed in 250 ml cylindrical glass jars inside which a slow flow of nitrogen (with a percentage of oxygen inferior to 0.01%) from the top to the bottom was maintained. The gas flowed from a cylinder of nitrogen through sterilizing filters, pressure regulators and micrometer valves connected to flowmeters. The gas flow was about 100 ml per day.

In a different set of jars, the nitrogen flow was replaced with an air flow.

The jars were incubated at 32°C in a thermostated incubator, itself containing a nitrogen atmosphere.

The detection of fungal growth was made by the diluition plate method: 10g of ground seeds (by Stomaker) were placed directly in 100 ml of saline solution (NaCl 0.9%). The inoculum was made by plating on Mycological agar (Difco). Incubation was carried out at 25°C for 5 days.

The fungal penetration into the seeds was studied by superficial

sterilization with sodium hypochlorite. Ten grams of whole grains were put in HClO (C.Erba, tit.min. of Cl 8% + 2%) for two minutes. Five washings were then carried out in sterile distilled water. The grains so treated were put on Mycological agar and incubated in a thermostat at 25°C for 5 days.

The samples kept in different air and nitrogen atmospheres were analyzed as regards the production of aflatoxins. The samples were prepared for extraction and quantitative analysis by High Performance Liquid Chromatography (HPLC).

Each wheat sample was homogenized by using a Waring Blendor and extracted with chloroform:methanol (2:1 v/v) for 3 hours. The extracts were filtered through phase separative paper (Whatman, 1PS) and concentrated to 1 ml on a rotatory evaporator.

To purify the aflatoxins from the lipids present in the sample, two thin layer chromatography runs were made: the first with petroleum ether: n-hexane (75:25 v/v) and the second with n-hexane: ethyl-ether: acetic acid (70:30:1.5 v/v); all were made on layers of Stratocrom SIAP, C.Erba.

The aflatoxins do not migrate and are thus totally recovered without any alterations. They were concentrated in a fixed volume of HPLC elution solvent, methanol: distilled water (50:50 v/v) and the analyses were made according to Knutti et al., (1979).

RESULTS

Fungal growth in air.

Fig.1 shows the growth curves of the 4 fungal strains on wheat in air. The growth was tested at 4, 7, 10, 14, 18, 21 and 27 days. As can be noted, P.cyclopium is the strain that grows best after a very short adaptation period. A.flavus, A.chevalieri and C.krusei follow at decreasing growth rates.

In all cases a slight decrease of the number of germs can be noted between the inoculum and the first control which is explainable by the normal adaptation of the fungus to the environmental conditions as well as by lack of full viability of the inoculated conidia.

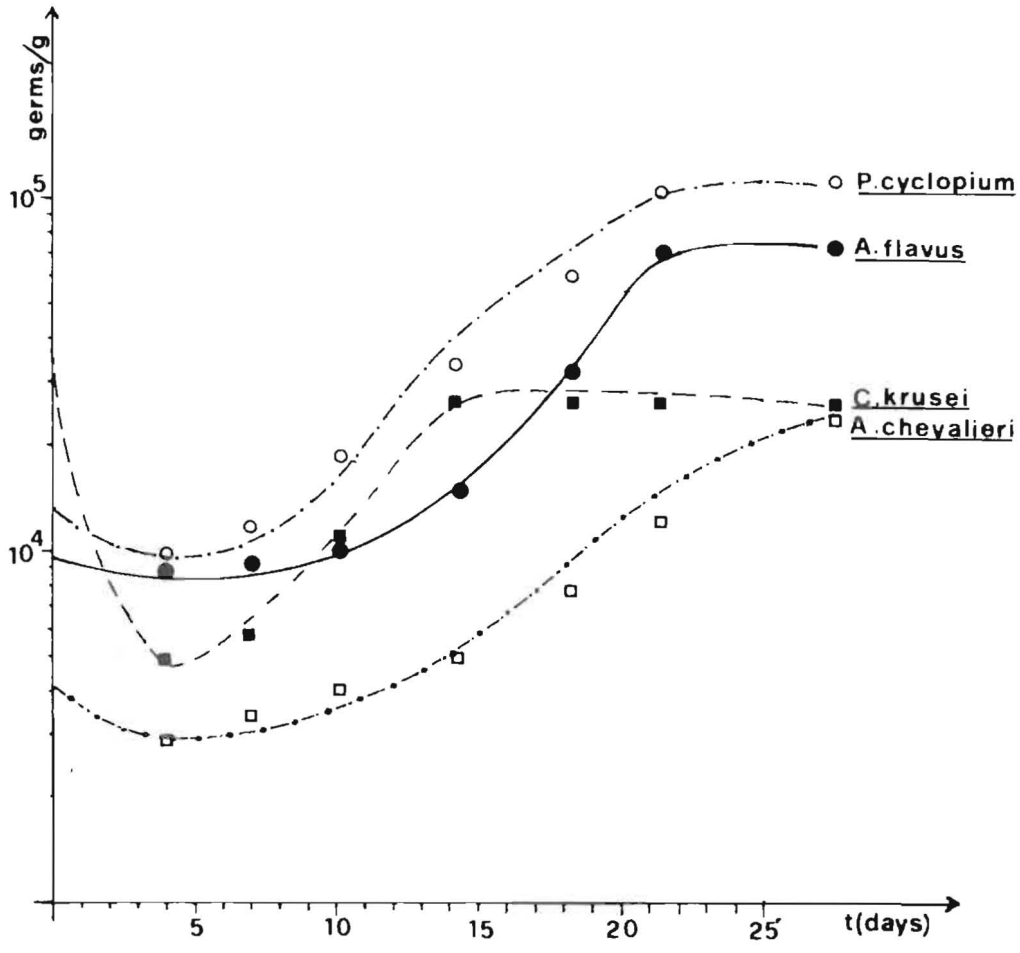


Fig.1. Growth curves on wheat seeds of 4 fungal strains in air.

Table 1 shows that the data relative to the number of germs are confirmed by those of the surface sterilization by which we have determined the fungal penetration inside the seeds.

The greater the growth of the fungus, the higher the percentage of the internally infected seeds.

The moisture content did not vary appreciably during the experiment.

Effect of nitrogen on fungal growth.

We studied the possible inhibiting effect of nitrogen on the 4 considered strains as regards fungal growth.

Fig.2 shows the inhibiting effect of nitrogen on the development of the strains after 7, 14 and 21 days. With A.flavus we carried out controls also at 60 and 70 days.

As can be seen, all the strains show a considerable decrease of the number of germs in conditions of complete anoxia, which is however different for the various strains.

P.cyclopium and A.chevalieri are the most sensitive strains to anoxic conditions, as they show at about 21 days an almost total inhibition.

Inhibition is lower for C.krusei and A.flavus. Actually, as can be seen, C.krusei is not inhibited until the 14th day, while it decreases significantly between the 21st and 27th day. This can be explained by the fact that it is possibly microaerophilic and therefore resists longer to conditions of total anoxia. Finally, though inhibited, A.flavus maintains quite high vitality up to 21 days in anoxia and only after 60 days its vitality ceases almost completely.

We wanted also to observe the effect of nitrogen at different stages of the growth curve of A.flavus in air. At 4, 7, 10, 14, 18 days we measured the effect of the exposure to nitrogen for 7 and 14 days. As shown in Fig.3, at 4, 7 and 10 days of growth (corresponding to the adaptation phase of the fungus) the inhibition produced by the nitrogen atmosphere appears only after 14 days and not yet after 7 days exposure to nitrogen.

On the contrary, in the log-growth phase (14th and 18th days of the growth curve) the effect of nitrogen appears already after 7 days of exposure, as the fungus is more sensitive to the conditions of

TABLE 1 Growth of four fungal strains on sterilized wheat in air.

Time (days)	A. flavus germs/g	A. flavus infected seeds %	P. cyclopium germs/g	P. cyclopium infected seeds %	A. chevalieri germs/g	A. chevalieri infected seeds %	C. krusei germs/g
0	10×10^3	0	14×10^3	0	43×10^2	0	40×10^3
4	9×10^3	20	10×10^3	7	30×10^2	7	5×10^3
7	9.5×10^3	20	12×10^3	25	35×10^2	16	7×10^3
10	10.5×10^3	30	19×10^3	30	42×10^2	20	10×10^3
14	15×10^3	60	34×10^3	44	52×10^2	34	27×10^3
18	30×10^3	100	60×10^3	88	80×10^2	78	26.7×10^3
21	70×10^3	100	102×10^3	100	124×10^2	100	26.4×10^3
27	72×10^3	100	114×10^3	100	240×10^2	100	26×10^3

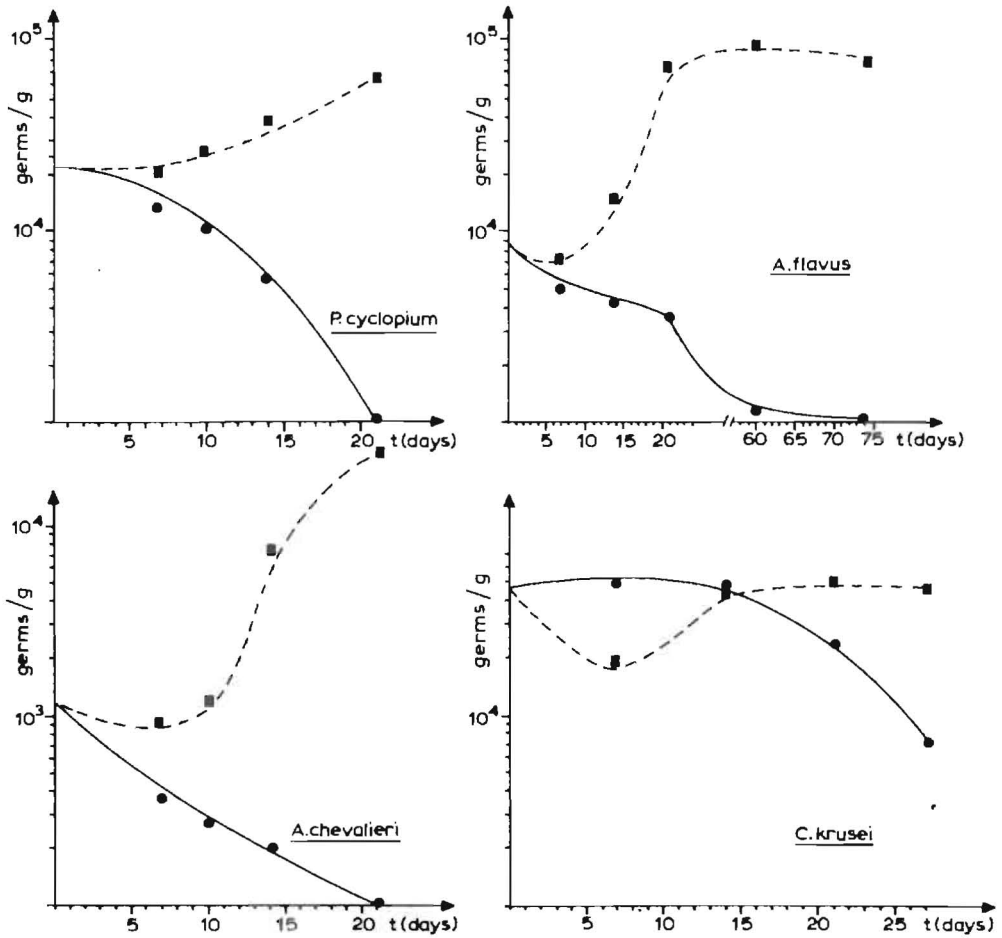


Fig.2. Growth curves on wheat of 4 fungal strains in air and nitrogen.
(dotted line: growth curve in air, full line: growth curve in nitrogen)

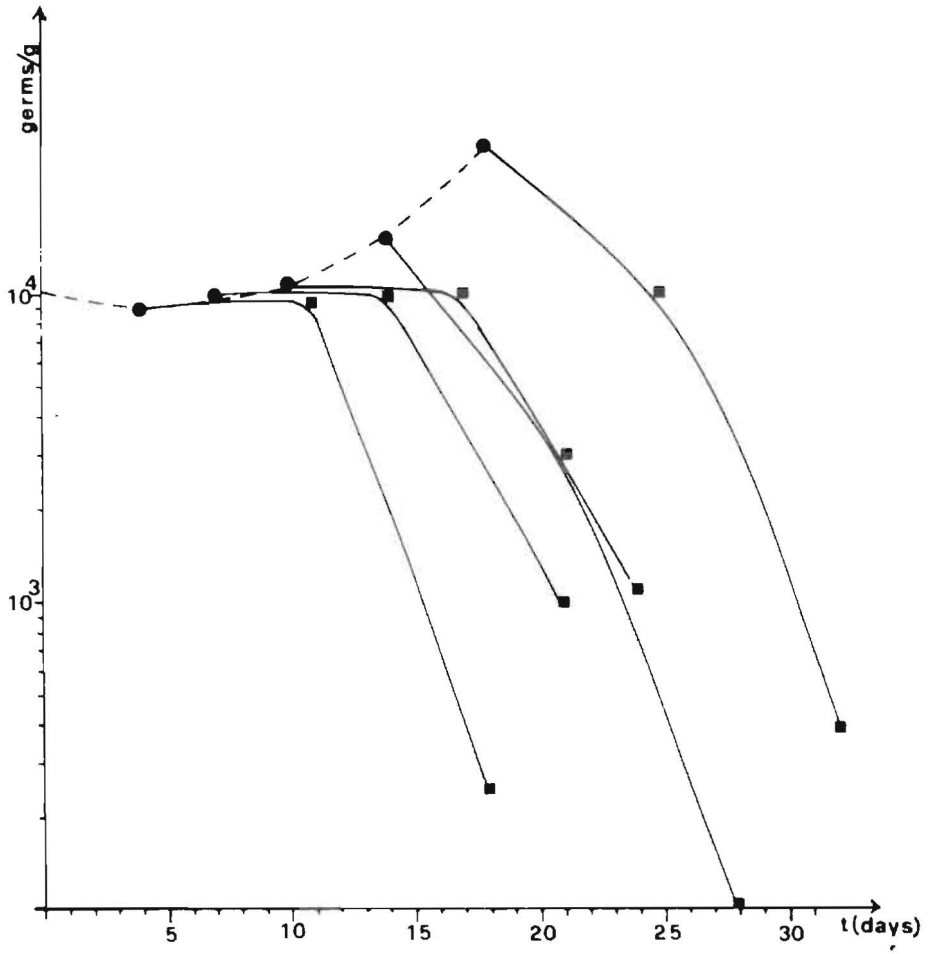


Fig.3. Effect of exposure to nitrogen for 7 and 14 days at different phases of the growth curve of A. flavus in air.

complete anoxia.

Further, we carried out experiments to measure the duration of the inhibiting effect after two different exposure periods to nitrogen on wheat seeds infected with A.flavus, bringing the samples back into an air flow.

The data in Table 2 show that, when the fungus is brought back into air after a 30-days exposure period to nitrogen, its growth appears to be immediately stimulated. After 60 days in nitrogen, the fungus grows more slowly when brought back into air. On the 7th day there is, in fact, a further decrease of the number of germs per gram, while on the 14th day there is a clear increase of fungal growth.

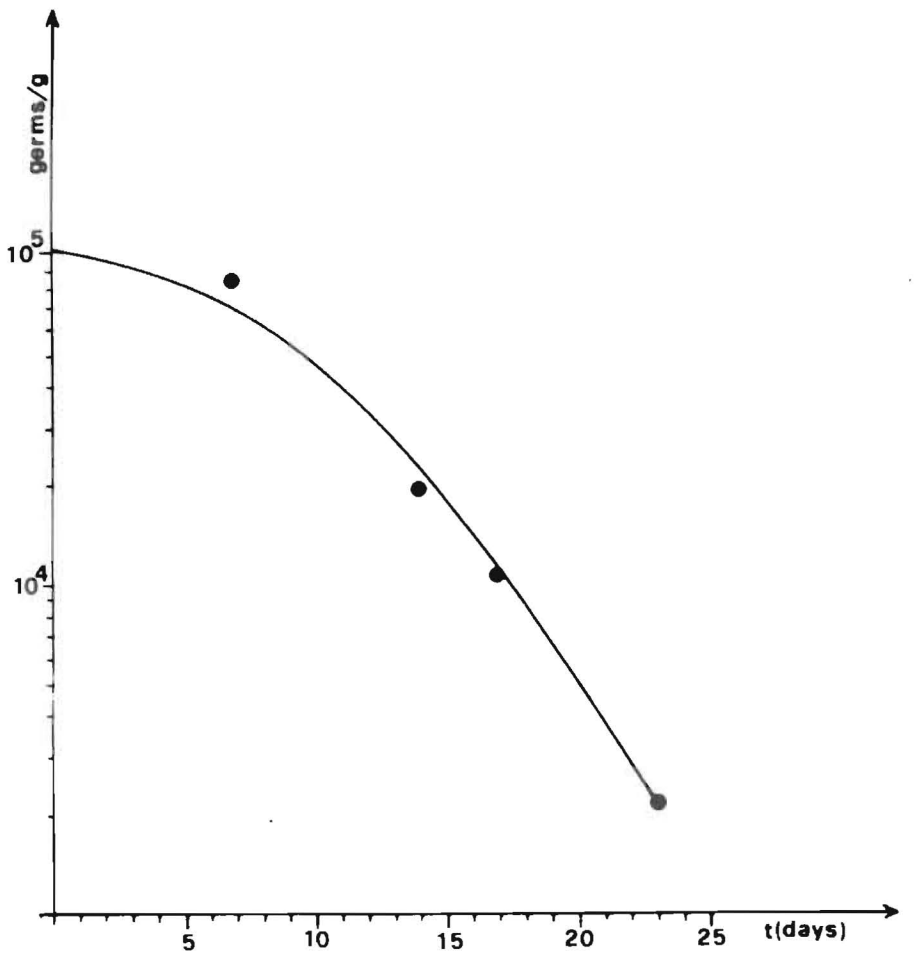
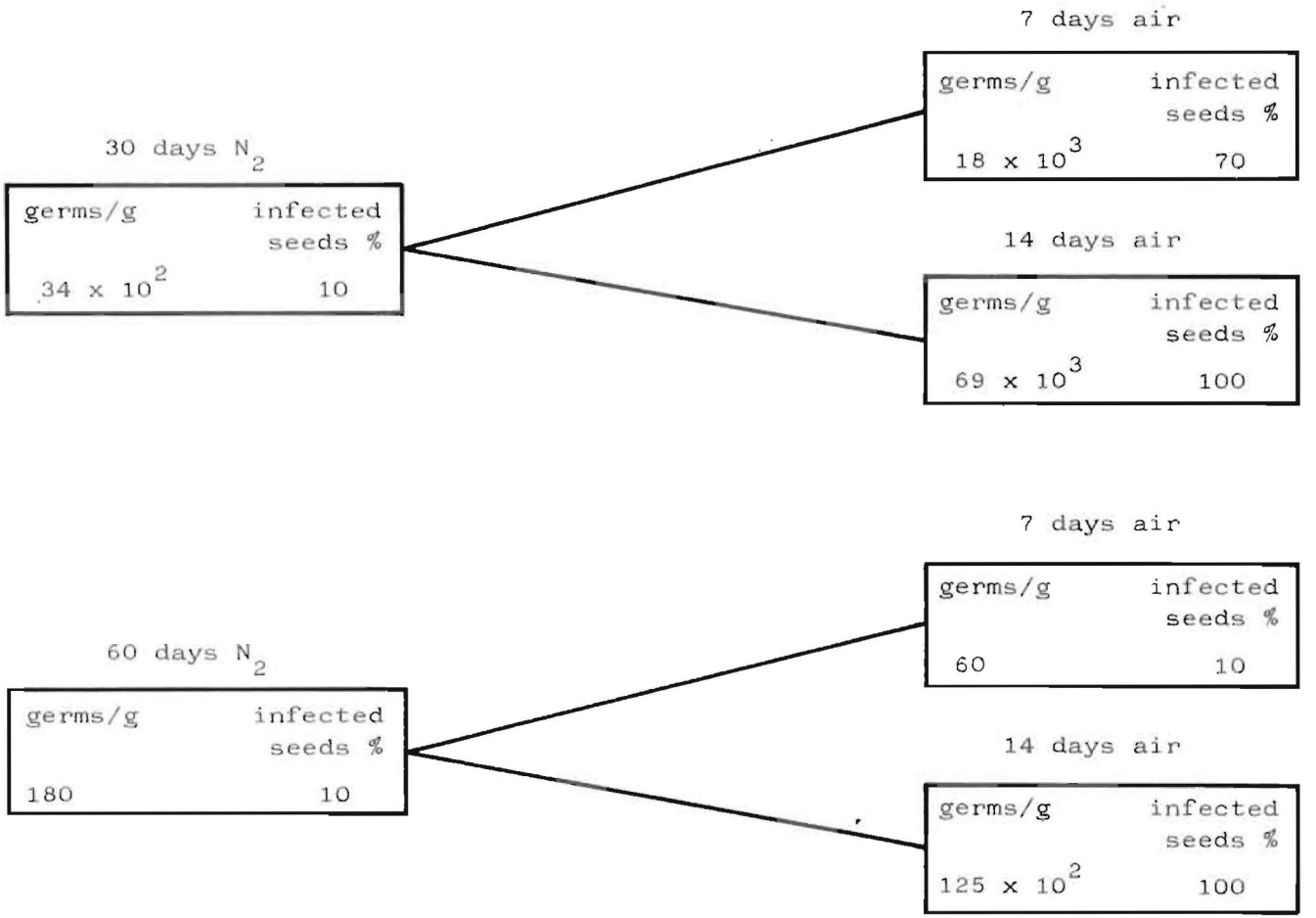


Fig.4. Effect of nitrogen atmosphere on non-sterilized wheat seeds microflora in time.

TABLE 2 Effect of two different exposure times in nitrogen on successive growth in air of A. flavus on wheat.



Finally we carried out an experiment on non-sterilized wheat seeds. Wheat at 13% moisture content, with $13x10^4$ germs/gram was used. The wheat was moistened to 19% and then kept in air for 10 days. Whereupon it was placed in a nitrogen flow for different periods of time (7, 14, 17, 23 days). The results (Fig.4) showed that nitrogen has an inhibiting effect on fungal growth. Moreover, among the fungal strains found in the seeds, the Penicillia and Aspergilla result the most sensitive to anoxia while sterile mycelia increase in time.

Effect of nitrogen on aflatoxin production.

The determination of aflatoxins produced by A.flavus inoculated on wheat seeds shows that the strain used in our experimental conditions produces only aflatoxin B_1 and that the total lack of oxygen inhibits the production of aflatoxin.

Data in Table 3 show that, in our experimental conditions, there is a direct connection between fungal growth and the production of aflatoxin: in fact the production of aflatoxin B_1 increases in connection with fungal growth.

TABLE 3 Growth of A.flavus in air and nitrogen on wheat and production of aflatoxin B_1.

Time (days)	germs/g air	germs/g N_2	aflatoxin B_1 (μg/kg) air	aflatoxin B_1 (μg/kg) N_2
0	$10.5x10^3$	$10.5x10^3$	-	-
7	$10\ x10^3$	$7.0x10^3$	8.03	-
14	$18\ x10^3$	$4.9x10^3$	80.00	-
21	$71\ x10^3$	$3.7x10^3$	118.04	tr
27	$72\ x10^3$	$3.5x10^3$	120.00	-
34	$72.5x10^3$	$3.3x10^3$	119.00	-

At the first control (7 days) the number of germs was inferior to those inoculated as a consequence of an adaptation period of the fungus and the lack of viability of some of the inoculating conidia. The inoculated conidia did not contain aflatoxins which explains why the high number of germs present at the beginning of the experiment did not give rise to aflatoxin.

A further confirmation of the nitrogen inhibiting effect on the production of aflatoxin B_1 appears in Fig.5, in which samples were first kept in air for 10 days, with consequent production of aflatoxin B_1 and later submitted to complete anoxia for 7, 14 and 21 days with progressive decrease of aflatoxin B_1.

The results obtained on the inhibiting effect of nitrogen atmosphere on the production of aflatoxins confirm those presented by other researchers on wheat seeds and peanuts (Wilson and Jay, 1975; Diener and Davis, 1969). On the contrary it was reported that the decrease in respiratory activities stimulates the production of aflatoxins (Shih and Marth, 1974).

CONCLUSION

On the basis of the results shown, we can conclude that the conditions of total anoxia prove to be an efficient means of limiting the growth of the tested fungal species growing in favourable conditions of moisture and temperature on stored seeds. This confirms the data of other researchers about the inhibiting effect of a nitrogen atmosphere with low oxygen content both on the field and storage microflora. The inhibiting effect appears to be efficient in a rather short storage period, of about one month, in our experiments.

Inhibition of aflatoxin production was clearly detected and a lowering of the level of the aflatoxin found in infected wheat seeds was observed. This can be a very important factor, considering the high toxicity of these substances produced by A.flavus.

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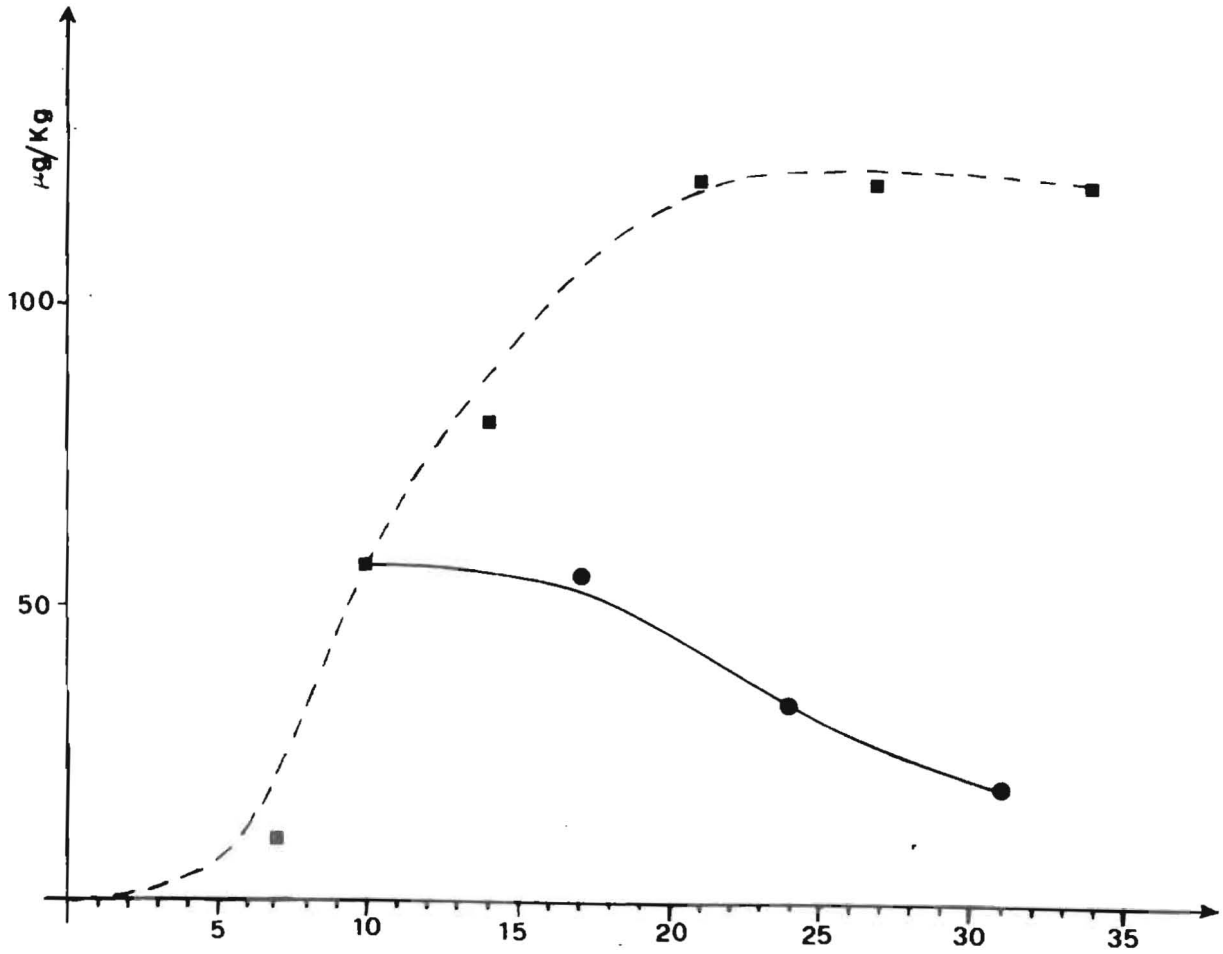

Fig.5. Inhibiting effect of nitrogen on the production of aflatoxin B_1 by a strain of A. flavus grown in air for 10 days. (dotted line: in air, full line: in nitrogen)

their help in setting up the gas flow lines and carrying out inspections.

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