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EFFECTS OF PHOSPHINE IN THE DEVELOPMENT OF *ASPERGILLUS FLAVUS* AFLATOXIN PRODUCTION IN MAIZE GRAINS STORED AT DIFFERENT MOISTURE CONTENTS

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ABSTRACT

The objective of this research was to investigate the effect of phosphine on the growth of *A. flavus* and aflatoxin production in maize stored at different moisture contents. In this experiment samples were previously inoculated with an *A. flavus* toxigenic strain. The experimental design used surface of response methodology to assess the various effects of different moisture contents. (water activities from 0.85 to 0.98), phosphine concentrations (from 0 to 4 g m^{-3}) and exposure periods (from 1 to 15 days) to the fumigant. Analyses of m.c., water activity, mycological composition, ergosterol content and aflatoxins were carried out on the samples subjected to the different treatments. Previous results showed that as the exposure time increased, more complete control of *A. flavus* was achieved. Mainly at lower water activities an increase in the phosphine exposure period is more important than increasing the concentration to achieve good control of *A. flavus*. As moisture content increases the increase in phosphine concentrations becomes more important. *Fusarium verticillioides* seems to be particularly tolerant of phosphine mainly under the high m.c. conditions which also favours its development. Since this species is a potential fumonisins producer, more information is needed to aid in the formulation of practical control recommendations. The *Penicillium* species tolerant to phosphine were those mainly found in freshly harvested maize. It is encouraging that aflatoxin production was severely affected by phosphine even at low concentrations.

INTRODUCTION

Corn is the main cereal crop grown in Brazil, which is the third major corn producing country in the world after China and the United States. The tropical conditions that prevail during most of the year in Brazil favour fungal growth and mycotoxin production, especially when the freshly harvested grain is kept at a high moisture content (m.c.) while waiting to be dried. The recommended moisture level for safe storage of corn grains is from 12 to 14% m.c. However, when the harvest season coincides with the rainy season, situations exist where grains with high m.c.'s must be held for several days, and the corn cannot be sun dried. Furthermore drying facilities are not available everywhere, or where present, they are unable to fully supply the demands for drying. The occurrence of mycotoxins in corn is a problem well known all over the world. Under Brazilian conditions the aflatoxins deserve special attention not only because of their potential carcinogenic effects but because the species *Aspergillus flavus* and *A. parasiticus* which produce these mycotoxins are very well adapted to the Brazilian climate and are frequently found in Brazil.

Phosphine (PH_3), a fumigant used worldwide for stored-product insect control, has been shown to be a promising alternative for controlling fungi and mycotoxin production in grain stored at high m.c.'s. Its advantages are its low cost, its commercial availability and the fact that it leaves almost no residue in the product. The potential of PH_3 to affect mold growth was first tentatively observed in 1960's, but PH_3 was reported to have little effect on dormant fungi (Raghunatan *et al.,* 1969; Sinha *et al.,* 1967). A decrease in mold development was observed for PH_3-treated wheat (Hocking and Banks, 1991a) and rice grains (Hocking and Banks, 1991b; Castro and Pacheco, 1995). A complete arrest of the aflatoxin production on shelled peanuts was observed with a PH_3 concentration of 0.5 g/m^3 for a period of 14 days in a laboratory-scale experiment (Castro *et al.,* 1992). The effect of the fumigant was also tested in a warehouse-scale experiment on unshelled peanuts with m.c.'s above the recommended level (Castro *et al.,* 1995). Neither *A. flavus* and/or *A. parasiticus* nor aflatoxins were detected in the fumigated stacks immediately after the treatment and a much better control was observed in the fumigated stacks a month after fumigation. Excellent results were obtained in a second experiment conducted in a commercial warehouse located in a peanut producing area in São Paulo, Brazil (Castro *et al.,* 1996). *A. flavus* and/or *A. parasiticus* were either not detected or were isolated in insignificant amounts and the contamination levels of aflatoxins remained unchanged in stacks of freshly harvested peanuts stored with m.c.'s in the range of 18 to 21% treated with PH_3 (3.0 g aluminium phosphide/m^3) for 7 d. The objective of the current research was to investigate the effects of PH_3 on the growth of *A. flavus* and aflatoxin production in corn stored at different m.c.'s. In these experiments samples were previously inoculated with an *A. flavus* toxigenic strain. An experimental design named 'surface of response methodology' was used in order to assess the various effects of the PH_3 on *A. flavus* in corn grains stored at water activities (a_w) from 0.85 to 0.98 (equivalent to m.c.'s from 16.0% to 24.5% w.b.), PH_3 concentrations (from 0 to 4 g/m^3) and exposure periods (from 1 to 15 days) to the fumigant. Analysis of m.c., a_w and fungi were carried out on the samples. PH_3 concentrations were measured by gas chromatography. A factorial design experiment was carried out to verify the effects of PH_3 on aflatoxins production in grains at 0.92 and 0.95 a_w exposed to concentrations of 0.05, 0.1 and 0.2 g/m^3 for 8 and 15 d.

MATERIALS AND METHODS

Raw material

The experiments were conducted with the corn hybrid Cargill 606, freshly-harvested and sun-dried, grown at the Experimental Station of Instituto Agronomico de Campinas, São Paulo, Brazil. The material (approximately 50 kg) was cleaned, homogenized in a Boerner homogenizer and kept in the freezer at -20°C until assays were set up. The corn was rehumidified up to the desired level, for each a_w, before the PH_3 tests. The rehumidification was performed by adding amounts of distilled water previously calculated, in order to increase the initial water activities of the product up to 0.85, 0.87, 0.92, 0.95 and 0.98 a_w (equivalent to m.c.'s of 15.3%, 16.2%, 19.2%, 21.6% and 24.5% w.b.).

Source of phosphine

Tablets of the commercial aluminium phosphide Gastoxin™ were used.

Selection of the toxigenic strain of *Aspergillus flavus*

Samples of corn from different localities were plated on AFPA agar medium (Pitt *et al.,* 1983). The strains of *A. flavus* detected were isolated in assay tubes of Czapek culture medium and incubated at 25°C for 10 d in an incubator. The growing cultures were transferred to plates of coconut agar medium (Lin and Dianese, 1976) and observed under ultra-violet light. Aflatoxin quantification was carried out in the cultures, which showed the highest fluorescence. The culture medium and the colony were homogenized in a mixer and the aflatoxin extracted with chloroform. The extract was filtered and concentrated in a rotary evaporator to almost complete dryness. The quantification was made by thin layer chromatography. A strain isolated from the Taiuba variety was chosen due to its high fluorescence on the coconut agar medium. In YES medium this strain produced 94 ppm and 2.7 ppm, respectively, of B_1 and B_2 aflatoxins.

Experimental designs

Two experiments were carried out. The first was to verify the effects of PH_3 on *A. flavus* control, and the second one to evaluate the effects of this fumigant on production of aflatoxins.

Experimental design and statistical analyses for fungal analyses: A rotational design of second order with three factors and six repetitions at the central point was used by applying the surface response methodology technique (Box *et al.,* 1978). The factors and the level of each factor used in this study are shown in the design table (Table 1) and include: a_w's from 0.85 to 0.98, exposure times from 1 to 15 d, and fumigant concentrations from 0.0 to 4.0 g/m^3. The range intervals for the various factors were selected based on practical and published data.

TABLE 1
Experimental design used

Coded				Real Values		
Assays	Water activity	Time (days)	PH_3 (g/m^3)	Water activity	Time (days)	PH_3 (g/m^3)
1	-1	-1	-1	0·87	3·25	0·8
2	-1	-1	1	0·87	3·25	3·2
3	-1	1	-1	0·87	12·75	0·8
4	-1	1	1	0·87	12·75	3·2
5	1	-1	-1	0·95	3·25	0·8
6	1	-1	1	0·95	3·25	3·2
7	1	1	-1	0·95	12·75	0·8
8	1	1	1	0·95	12·75	3·2
9	-1.682	0	0	0·85	8	2·0
10	1.682	0	0	0·98	8	2·0
11	0	-1.682	0	0·92	1	2·0
12	0	1.682	0	0·92	15	2·0
13	0	0	-1.682	0·92	8	0·0
14	0	0	1.682	0·92	8	4·0
15	0	0	0	0·92	8	2·0
16	0	0	0	0·92	8	2·0
17	0	0	0	0·92	8	2·0
18	0	0	0	0·92	8	2·0
19	0	0	0	0·92	8	2·0
20	0	0	0	0·92	8	2·0

For each a_w the flasks containing the inoculated samples were distributed in desiccators of 9-L capacity (1 flask/desiccator), with approximately 500 mL of appropriate saturated salt solution for humidity control. The salts used were potassium dichromate (0.98), lead nitrate (0.95), sodium potassium tartrate (0.92), sodium tartrate (0·87) and potassium chloride (0.85) in different concentrations in order to obtain saturated solutions and create equilibrium environments of controlled relative humidity ranging from 85 to 98% r.h. and a free space volume of 8.7 L. The desiccators were kept in a chamber at a controlled temperature of 25±2°C. According to the experimental design previously established, the samples were submitted to different PH_3 treatments. Each assay was run three times. Immediately after the PH_3 fumigation period, the samples were subjected to the various analyses.

Experimental design and experimental set up for the aflatoxins analysis: A factorial design experiment was carried out in order to verify the effects of PH_3 on grains stored at 0.92 and 0.95 a_w (equivalent to 19.2 and 21.6% m.c. w.b.) and exposed to PH_3 concentrations of 0.0, 0.05, 0.1 and 0.2 g/m^3 for 8 and 15 d. For each a_w the flasks containing the inoculated samples were distributed in desiccators of 9-L capacity (1 flask/desiccator), with approximately 500 mL of saturated salt solution. The salts used were lead nitrate (0.95) and sodium potassium tartrate (0.92) in different concentrations in order to obtain saturated solutions and create equilibrium environments of controlled r.h. of 92% and 95%. The desiccators were kept in a chamber with a controlled temperature of

25±2°C and opened after holding for 8 and 15 d. Three repetitions of each treatment were carried out. Immediately after the PH_3 fumigation period, the samples were collected for aflatoxins analyses.

Analyses

Water activity and moisture content: The a_w of the samples were determined in a DECAGON apparatus model 2X-T. The m.c. determination was carried out with 15 g of grains, in an aerated oven at 120°C for 72 hours (Anon. 1976).

PH3 concentration: The PH_3 was obtained from the Gastoxin tablets in a generator, according to the FAO method No. 16 (Anon. 1975). The gas concentration inside the generator was analysed by gas chromatography. A cylinder of PH_3 of 99.99% purity was used as a standard to measure the PH_3 concentration obtained from the tablets in the generator. The percentage of PH_3 from the tablets was obtained by comparing the areas of a pure PH_3 obtained from the cylinder with the areas obtained from the tablets. This concentration of PH_3, was assessed as 86%, and this value was used to calculate the required concentrations for the assays, considering that 1 mL of PH_3 (99.9%) is equivalent to 1.39 mg of gas.

At the end of the exposure periods the gas concentrations inside the desiccators were measured again. A PH_3 standard curve was used to assess the PH_3 concentrations.

The gas chromatographic conditions were: gas chromatograph HP 6890 with a flame photometric detector, temperature of 150ºC, with a phosphorous filter (525 nm), hydrogen flow of 150mL/min and air flow of 110 mL/min. Capillary column 1909/j413 HP-5 with phenyl methyl siloxane 5% (30m x 320 μm x 0.25 μm) with a nitrogen constant flow of 2.0 mL/min and a split injector (50:1) with make up at 60mL/min, both at 150ºC. Sampling volume was 20 □L and 100 □L. The chromatography was linked to computer software (Chemstation) HP Vectra XA.

Fungal analyses: Direct plating methodology was used, in order to evaluate the percentage of grains internally infected with different fungi in addition to *A.□flavus* experimentally inoculated. The grains were immersed in 0.4% sodium hypochloride solution for 1 min and then rinsed with sterile distilled water. Fifty grains were plated (10 grains/plate) on the AFPA culture medium (Pitt *et al.*, 1983) for the *A. flavus* detection and on DG18 medium (Hocking and Pitt, 1980) for the detection of xerophilic species. The fungal identification was carried out according to the methodology described by Samson *et al.,* (1981).

Aflatoxins analysis: The methodology described by Soares and Rodrigues-Amaya (1989), which uses thin layer chromatography was used.

RESULTS AND DISCUSSION

Fungal analyses

The results of the fungal analyses are shown in Table 2.

TABLE 2
Results of the surface response methodology

Assays	a_w	Time	PH_3	*A flavus* AFPA (%)	% infected grains DG18	*A. flavus* DG18 (%)	F. *verticillióide* (%)	*Penicillium sp* (%)	Yeast and yeast-like fungi (%)	Others
T1	0.87	3.25	0.8	13	80	25	38	11	0	0
T2	0.87	3.25	3.2	28	83	34	34	10	0	0
T3	0.87	12.75	0.8	3	40	6	18	7	7	7
T4	0.87	12.75	3.2	4	27	14	8	0	2	6
T5	0.95	3.25	0.8	91	93	74	41	4	31	0
T6	0.95	3.25	3.2	34	89	55	45	3	9	4
T7	0.95	12.75	0.8	47	100	53	82	9	76	2
T8	0.95	12.75	3.2	7	100	12	63	2	76	1
T9	0.85	8	2	9	79	23	24	13	0	22
T10	0.98	8	2	6	100	7	100	2	73	0
T11	0.92	1	2	16	82	30	38	20	17	3
T12	0.92	15	2	7	94	6	51	4	60	0
T13	0.92	8	0	100	100	100	16	30	0	1
T14	0.92	8	4	3	60	8	49	6	2	3
T15	0.92	8	2	2	68	0	50	10	8	4
T16	0.92	8	2	1	85	9	57	14	19	0
T17	0.92	8	2	8	70	4	44	14	8	4
T18	0.92	8	2	6	82	16	66	4	8	0
T19	0.92	8	2	0	82	6	70	12	2	0
T20	0.92	8	2	8	98	10	26	4	52	0

In addition to *A. flavus* which was experimentally inoculated, the fungal species also present in the samples that were detected on DG18 agar medium, were *Fusarium verticillioides*, *Penicillium* spp. (*P variabile*, *P. citrinum, P. funiculosum* and *P. pinophilum*), others filamentous fungi, yeasts and "yeast-like fungi" (*Geotrichum* sp. and others not identified). These fungi are commonly found in freshly harvested grains. *F. verticillioides* is a potential fumonisin producer and together with the yeasts and yeast-like fungi are generally present in grains with high moisture contents. Yeast and yeast-like fungi cause rancidity and off odour. No *Eurotium* spp. were detected in the samples. These species are xerophilic fungi which are often the primary invaders of stored grains and in the range of a_w of our experiment are less competitive.

The models and the determinant coefficients (r^2) for the responses studied are shown in Table 3. In general the determinant coefficients were high showing that the models aptly described the experimental data obtained in the intervals of a_w, exposure time and PH_3 concentrations studied.

Figure 1 shows the percentage of grains internally infected by *A. flavus* detected on AFPA agar medium obtained when a_w was fixed at 0.89, 0.92 and 0.95. As can be observed PH_3 concentration is more important than exposure time for *A. flavus* control. As a_w increases higher PH_3 concentrations are needed to reach the minimum percentage of grain internally infected by this specie.

TABLE 3
Models and determinant coeficients for the responses studied

Response	Model (p<0,05)	(R^2)
Aspergillus flavus (%) (AFPA)	199.81 – 1258.56 Aw + 1272.45 Aw^2 + 20.77 t + 0.19 t^2 + 211.27 PH3 + 12.34 $PH3^2$ – 28.51 Aw_xt – 301.82 Aw_xPH3 + 0.08 t*PH3	82.37%
% of infected grains	= 995,23 – 1598,50 Aw + 665,30 Aw2 – 81,25 t + 0,03 t2 – 10,53 PH3 – 1,67 PH32 + 87,83 Aw*t 16,03 Aw*PH3 – 0,30 t*PH3	67.80%
Aspergillus flavus (%) (DG18)	= 1340,01 – 3426,55 Aw + 2272,71 Aw2 12,15 t + 0,26 t2 + 132,12 PH3 + 12,15 PH32 - 19, 26 Aw*t - 205,66 Aw*PH3 – 0,58 t*PH3	76.33%
Fusarium verticillióides	1508,36 – 3154,30 Aw + 1641,33 Aw2 – 68,52 t – 0,21 t2 + 32,42 PH3 - 5,64 PH32 + 80,90 Aw*t – 2,67 Aw*PH3 – 0,73 t*PH3	78.53%
Penicillium spp	= -1093,84 + 2596,93 Aw - 1509,00 Aw^2 - 12,15 t -0,04 t^2 - 5,16 PH3 + 1,03 $PH3^2$ + 13,87 Aw*t - 0,00 Aw*PH3 - 0,30 t*PH3	58.50%
Yeasts and " yeast-like fungi"	3916,80 – 8568,15 Aw + 4699.67 Aw^2 – 77,20 t + 0,45 t^2 + 52,33 PH3 – 3,91 $PH3^2$ + 79,36 Aw*t – 45,41 Aw*PH3 + 0,43 t*PH3 com	87.61%

At 0.89 a_w the lowest percentage of grains infected with *A flavus* is achieved with an exposure period of 7 to 15 days and PH_3 concentrations of 1.5 to 3.0 g/m^3. When water activity is fixed at 0.92 a_w the best conditions for *A. flavus* control are exposure periods from 8 to 15 days and PH_3 concentrations from 1.8 to 3.5 g/m^3. At 0.95 a_w the lowest percentage of infected grains by *A. flavus* is achieved with exposure periods from 8 to 15 days and PH_3 concentrations from 2.0 to 4.0 g/m^3. Hocking and Banks (1991b) verified that the development of *A. parasiticus* was less rapid in PH_3 treated rice than in freshly harvested paddy rice, equilibrated to 0.92 a_w that was inoculated with a mixture of *A. parasiticus* and *E. chevalieri* and exposed to 0.1 g/m^3 of PH_3 for 14 and 28 d at 28°C.

Figure 2 shows the percentage of grains internally infected by fungi detected on DG18 agar medium.

Figure 1

Figure 2

Fumigation conditions leading to minimum percentage of grains internally infected by fungi were more restricted than for *A. flavus* control and varied with a_w of the grains. At 0.89 a_w exposure time was more important than PH_3 concentration. At 0.92 a_w both exposure time and PH_3 concentration affected fungal growth. Under these conditions the best control was achieved in the range of maximum PH_3 concentrations and exposure period. At 0.95 a_w exposure period was again more relevant than PH_3 concentration but, in this case, minimum percentage of infected grains was obtained with the highest PH_3 concentrations though for the shortest periods of time. This may be attributed to the rapid increase of yeasts and yeast like fungi at high a_w's and long exposure periods mainly at low PH_3 concentrations. Castro and Pacheco (1995) also observed a decrease of total infected grains as the fumigant concentration increased when freshly harvested paddy rice, with initial a_w's of 0.84 and 0.90 was exposed to 0.0; 0.5; 1.0 and 1.5 g/m^3 PH_3 at 28°C for 7 and 14 d.

Aflatoxin analyses

The results of the factorial experiment to verify the effects of PH_3 on aflatoxin production are shown in the Figs. 3 and 4.

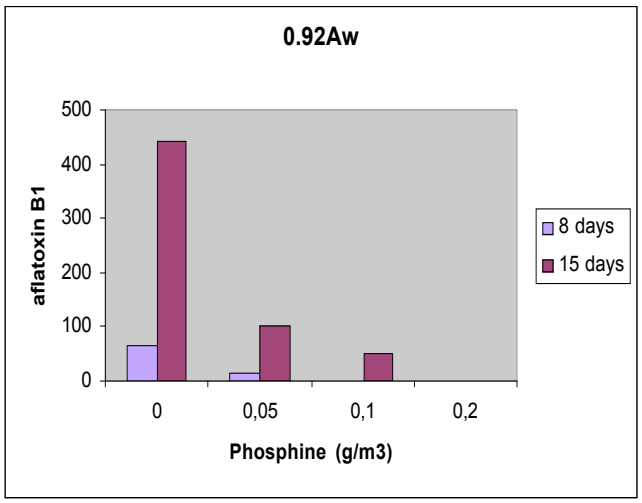

Fig. 3. Aflatoxin B_1 (□g/kg) in corn grains stored at 0.92 a_w and exposed to 0.05, 0.1 and 0.2 g/m^3 of phosphine for 8 and 15 days.

As can be seen from Fig. 3, at 0.92 a_w, although 0.1 g/m^3 of PH_3 completely arrested production of both aflatoxins for 8 days, a concentration of 0.2 g/m^3 was necessary to control these mycotoxins for a storage period of 15 d. When freshly harvested paddy rice (a_w 0.92) was inoculated with *A. parasiticus* and exposed at 28°C to 0.1g/m^3 PH_3, Hocking and Banks (1991b) also observed that aflatoxins

were still formed although the levels in the PH_3 treated rice were less than half those in the air controls. At 0.95 a_w a concentration of 0.2 g/m^3 completely arrested both aflatoxins production for 8 and 15 days storage. Castro *et al.*, (1992) verified that a PH_3 concentration of 0.5 g/m^3 completely arrested the aflatoxins production in shelled peanuts for a period of 14 days in a laboratory-scale experiment.

There are two Brazilian legislations for aflatoxin limits in food and feeds:

- 30 μg/kg aflatoxins $B_1 + G_1$ (CNNPA 34/76 of the Ministry of Health).
- 20 μg/kg aflatoxins $B_1 + B_2 + G_1 + G_2$ (Regulation 183/96 of the Ministry of Agriculture).

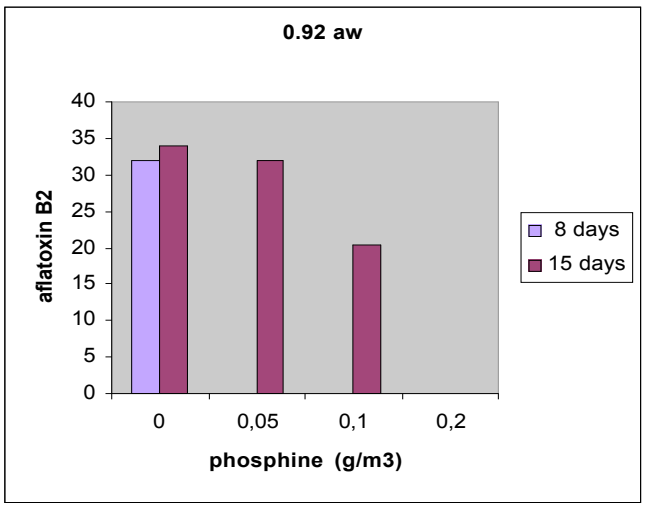

Fig. 4. Aflatoxin B_2 (μg/kg) in corn grains stored at 0.92 a_w and exposed to 0.05, 0.1 and 0.2 g/m^3 of phosphine for 8 and 15 days.

Practical considerations

The fumigation of bagged maize at the necessary concentrations should not be difficult when it is done by fumigating stacks of bags under a gas-proof sheet. For economy of fumigant and to guarantee the concentration and the exposure period it would be necessary to build the stack on a base sheet. This can be rolled together with the covering sheet, or better, the two sheets can be joined with an adhesive. The quality of the sealing can be checked by using a half–life negative pressure test. The fumigation of maize in bulk in a well-sealed silo with circulation can be used, provided that the initial dosage will ensure the necessary concentration and exposure period. An alternative strategy for bulk grain is to put the newly harvested grain directly into large (~2 m^3) strong polyethylene bags supported by re-usable nylon slings (web) (Mills, K.A, pers. comm.). On harvesting, or later on arrival at the grain store, the bags can be dosed and sealed. They can then be unloaded and built into stacks, even in the open air, for the

necessary exposure period. This treatment is intended only as a holding treatment to preserve the quality of grain, which is in excess of the recommended m.c.'s. These methods should give a good distribution of PH_3 with minimal leakage. However, there is no requirement for a totally effective treatment since grain, which is slightly under dosed will be mixed with well-treated grain before drying and the average amount of mycotoxins should be within permitted limits. Clearly, there is still the need for final drying, but the grain can be held in this way until dryer capacity is available.

Lastly, based on the experimental conditions of this study, it can be concluded that:

- When the water activity is constant, the PH_3 concentration has a greater effect than exposure time on *A. flavus* growth.
- PH_3 concentration and exposure time leading to the minimum percentage of infected grains varies with a_w of the product.
- Aflatoxin production is more affected by PH_3 than is *A. flavus* growth and much lower concentrations of the fumigant are needed for aflatoxins control.

The effects of PH_3 on fungal development vary with the type of grain and fungal species to be controlled.

In practical terms PH_3 treatment can be an alternative to maintain grain quality for short-term storage of high moisture corn grains.

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