

Efficacy of Inorganic salts and Organic acids against Colony Growth of *Aspergillus flavus* and Their use to control Aflatoxin level in Post Harvest Maize

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Abstract: Inorganic salts and organic acids are known in the feed industry as an effective, affordable and non-toxic tool to control of mold growth, as the fungicides are toxic chemicals and prohibited for direct use in grains to be used for consumption directly as feed/food. There is a need to evaluate the efficacy of these compounds against pathogens responsible for mycotoxin production in post harvest maize. A trial was conducted to evaluate efficacy of six non-toxic chemicals viz Sodium carbonate, Sodium bicarbonate, Potassium carbonate, Ammonium carbonate, Acetic acid and Sodium propionate, screened in different concentration (5 mM, 10 mM, 20 mM, 30 mM, 4 mM and 50 mM) for inhibition of radial growth of toxic isolates of *A. flavus* (Af 4) *in vitro*. Out of them Ammonium carbonate, Potassium carbonate and Sodium carbonate were found very effective in reducing the radial growth of *A. flavus* at 20 mM conc and fungitoxic at 30 mM conc. In post harvest condition these inorganic salts were tested in ambient storage condition @ 4 gm/kg grain in maize genotypes HM-4 and HQPM – 1 at 13.8 % grain moisture for their efficacy in controlling the aflatoxin contamination. All these chemicals were effective in reducing the concentration of aflatoxin up to 88 % as compared to control. In case of organic acids viz, Propionic acid, Acetic acid and Sodium propionate were tested @ 4 ml/kg grains on maize cvs. Bio 9681, 900M and BH-2187. These were found effective in reducing the aflatoxin concentration up to 69.4 % at 13 to 14 % grain moisture in ambient storage condition for eight months duration.

Key words; *A. flavus*, Aflatoxin, Inorganic salts, organic acids, carbonates, maize

Introduction

Aflatoxin production by *A. flavus* and *A. parasiticus* is a serious problem. The discovery that the aflatoxin is a potent hepatotoxin produced by *A. flavus* (Sargeant, *et. al* 1961) has led to intensive studies on the presence of mycotoxin in food stuff (Borker, *et. al* 1966; Ciegler and Lillehoj. 1968; Hesseltine, *et. al* 1966 Wogan 1965). The ubiquitous fungus *A. flavus* is capable of growing over wide temperature and moisture ranges. Conditions favouring molds growth as well as invasion include grain moisture levels higher than 12 percent, warm temperature, the presence of oxygen and prolonged storage time (Garlich *et. al* 1976; Tabib *et. al* 1981; Good & Hamilton 1981). The incidence and level of aflatoxin in grains varies with environmental conditions, genotype and locations. Molds in food and feed have been a problem for many years but aflatoxin focused attention on the problem of molds toxin and gave tremendous impetus to research in this area. Aflatoxin is important because of the potential threat it poses to human and animal health.

Several management tools such as the use of molds inhibitors and drying of grains are available to control mold invasion. Incidence of grey molds can be reduced through manipulation of crop management practices and through uses of traditional chemicals, fungicides, and non toxic chemicals. Several investigators (Landers *et. al* 1967; Sanders *et. al* 1982; Wilson *et. al* 1975) have found that carbon-dioxide- modified atmosphere storage inhibits aflatoxin production, because sodium bicarbonate decomposes to carbon dioxide under high temperature and relative humidity condition that normally favours aflatoxin production (Anderson *et. al* 1975; Northolt *et. al* 1982). In a study Thomas *et. al* (1987) hypothesized that Sodium bicarbonate may inhibit aflatoxin production on corn kernels during storage and it is inexpensive, easy to handle and generally recognized as safe for use in foods. Traditional chemical fungicide may be restricted to be used in food and feed in storage condition as well as due to food quality protection act. Some alternative means of safe control must be developed. Organic acids are known in the feed industry as an effective and affordable tool to the control molds growth. A variety of organic acids such as acetic acids, lactic acids, propionic acids etc. are used

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to help control mold invasion (Higgins and Brnkhaus 1999). Neem oil, sulphur dioxide, phosphates and bicarbonates have exhibited potential for disease control in other disease system (Anonymous.1983; Barchietto, *et. al* 1992; Belanger, *et. al.* 1995; Carter, and Locke, 1994; Clark, and Gilein, 1991; Horst, *et. al* 1992; Mustonen 1992; Reuveni, *et. al.* 1994; and Thompson, D.P. 1989) Bicarbonates and other salts are generally regarded as safe by the environmental protection agency.

Keeping this in view a study was undertaken to elucidate the potential of non toxic organic acids and inorganic salts to inhibit the growth of *Aspergillus* as well as effect of these chemicals to control aflatoxin build up in post harvest stages.

Material and Methods

Isolates of *A. flavus* were, obtained form different maize grains of different genotypes. All the isolates were characterized on the basis of toxin production, as highly toxic; moderately toxic and non toxic. These isolates were characterized with the help of Ammonia vapour test (Kumar *et. al* 2006) and maintained at DMR (Directorate of Maize Research) laboratory on slant of PDA. Out of them one toxic isolate (Af No. 4) was selected to undertake this experiment. Six chemicals (inorganic salts and organic acids) were evaluated in different concentration (5 mM, 10 mM, 20 mM, 30 mM, 40 mM and 50 mM) for inhibition of radial growth of toxic isolates of *A. flavus* in vitro by food poisoning technique. Details about the chemicals are given below;

- Sodium propionate_(Riedel-de Haen) ($C_3H_5 NaO_2$)
M = 96.06 g/mol
- Sodium bicarbonate ($Na_2 HCO_3$) – 99.5% (sigma)
M = 84.01 g/mol
- Sodium carbonate (Na_2CO_3) – 99.0% (sigma)
M = 105.99 g/mol
- Ammonium carbonate ($(NH_4)_2CO_3$ (Merck)
M = 157.13 g/mol
- Acetic acid (CH_3COOH) – 100% (Merck)
M = 60.05 g/mol
- Potassium carbonate (K_2CO_3) – 99% sigma Aldrich
M = 138.21 g/mol

Potato dextrose Agar (PDA) at 20 gm/litre was autoclaved. Prior to cooling and solidification, supplement (inorganic salts and organic acids) to PDA in above mentioned concentration were added. Six mm disc of the pathogen grown on PDA plate was inoculated on the poisoned medium (PDA) aseptically and sealed with parafilm. 4 Plates per treatment was taken to maintain 4 replications. These plates were inoculated and one check was maintained by non poisoned PDA. All the plates were incubated at 28°C. The colony growth diameter was measured after every 24 hours. The chemicals were tested in concentrations from 0 (Control)

to 50 mM to determine whether they have fungistatic or fungitoxic effect on *A. flavus*. The data were analyzed using statistical programme super ANOVA Analysis performed included one-way and two-way ANOVA to determine significance of treatment in altering colony growth.

Effect of inorganic salts and organic acids on post harvest storage of maize in reducing the mycotoxin concentration

Apart from the effect of these chemicals on radial growth of *A. flavus* was observed, some of the non toxic chemicals were evaluated for reducing the aflatoxin concentration in post harvest ambient storage condition in maize. The chemicals evaluated for reducing the concentration of aflatoxin in ambient storage are; Sodium carbonate, Sodium bicarbonate, Potassium carbonate, Ammonium carbonate, Propionic acid, Acetic acid Sodium propionate. These chemicals were tested on grains of maize genotypes HQPM – 1 and HM – 4, 900 M, Bio 9681, BH-2187 @ 4 gm/kg grains, whereas Propionic acid, Acetic acid were tested @ 4 ml/kg grains. 2½ kg of each genotype was taken to start the storage experiment and estimation of aflatoxin concentration these grain samples were done prior to start the experiment. The grains of these genotypes were inoculated with toxic isolate of *A. flavus*, (Af no. 4 isolate) before 24 hours @ 10 mg/kg grain of spore biomass of *A. flavus* and kept in a separate gunny bags in ambient storage condition for 8 months duration. The estimation of aflatoxin from these maize grain samples was done by using Enzyme-Linked Immunosorbant Assay (ELISA) method after eight months storage period. The direct competitive ELISA method followed Training manual ICRISAT (A Training Course 2005). The statistical analysis was done by using the OD values obtained for AFB1 standards draw a curve, taking AFB1 concentrations on the X-axis and OD values on the Y-axis

AFB1 ($\mu\text{g/kg}$): $(Ax Dx E)/G$

A = AFB1 concentration in sample extract (ng/ml)

D= Times dilution with buffer

E = Extraction solvent volume used (ml)

G = Sample weight (g)

Result and Discussion

Among the salts tested Ammonium carbonate was found most effective in reducing the radial growth of *Aspergillus flavus* as compared to other salts. Radial growth of *A. flavus* was restricted up to 1.80 cm at 20 mM concentration followed by Potassium bicarbonate (3.32 cm) as compared to control 5.13 cm after 192 hrs Overall Potassium carbonate and Sodium carbonate were found very effective by complete inhibiting the radial growth of *A. flavus* at 30 mM. The optimal concentration for completely inhibition of growth is 30 mM. No colony

expansion was measurable with these salts at 30 mM conc. while the molarity for complete radial growth inhibition with sodium bicarbonate was 50 mM. In case of acetic acid the radial growth was 3.77 cm at 10 mM. In sodium propionate the growth was not affected even at 50 mM concentration. Ammonium carbonate inhibited colony formation at 20 mM concentration Ammonium carbonate, Potassium carbonate and sodium carbonate were also fungitoxic at 30 mM, and no colony was observed even after 168 hrs (Table 1). After colony were transferred fungistatic or fungitoxic activity was not determined with acetic acid and sodium propionate as some colonies were observed even at 50 mM concentration after 16.8 hrs. In case of sodium bicarbonate no colony expansion was recorded at 50 mM concentration which shows its fungitoxic activity at higher concentrate (Fig 1).

Over all the study showed that *A. flavus* has differential sensitivity to these inorganic salts at different concentration as demonstrated by its varying rates for complete inhibition of colony growth. Ammonium carbonate, Potassium bicarbonate and Sodium carbonate have similar effect in reducing the radial growth of *A. flavus*. It was observed that the toxic isolate of *A. flavus* is highly sensitive to Ammonium carbonate by reducing the radial growth even at 20 mM. Ricker *et. al.* (1991)

observed that sodium bicarbonate could completely inhibit the growth of *Rhizoctonia carotae* at 100 mM concentration of and post harvest application of this salt also reduce the losses caused by *R. carotae*. In another study, bicarbonates of ammonium, Potassium and sodium inhibited the colony growth of *Altemaria cucumerina*, observed by Ziv *et. al* (1992) and it was also demonstrated by them that the application of these salts were effective in reducing the lesions formed on cucumber leaves. The preventive application of sodium carbonate and sodium bicarbonate significantly reduces the development of dry rot in potato tuber studied by Mélanie *et. al.* (2002), they also observed the fungitoxic activity of sodium benzoate, sodium metabisulfite, potassium sorbate, trisodium phosphate and aluminum salts. They also hypothesized that the concentration for control of gray mold in field/post harvest situation may be similar to concentration needed for complete inhibition of radial growth of *A. flavus*. Mills *et. al* (2004) studied on effect of salt concentration on mycelial growth, sporulation and germination of post harvest pathogens of potato, they observed that the sodium metabisulfite and propyl - paraben were effective in inhibiting the mycelial growth whereas aluminium compound was highly effective in inhibiting the spore germination.

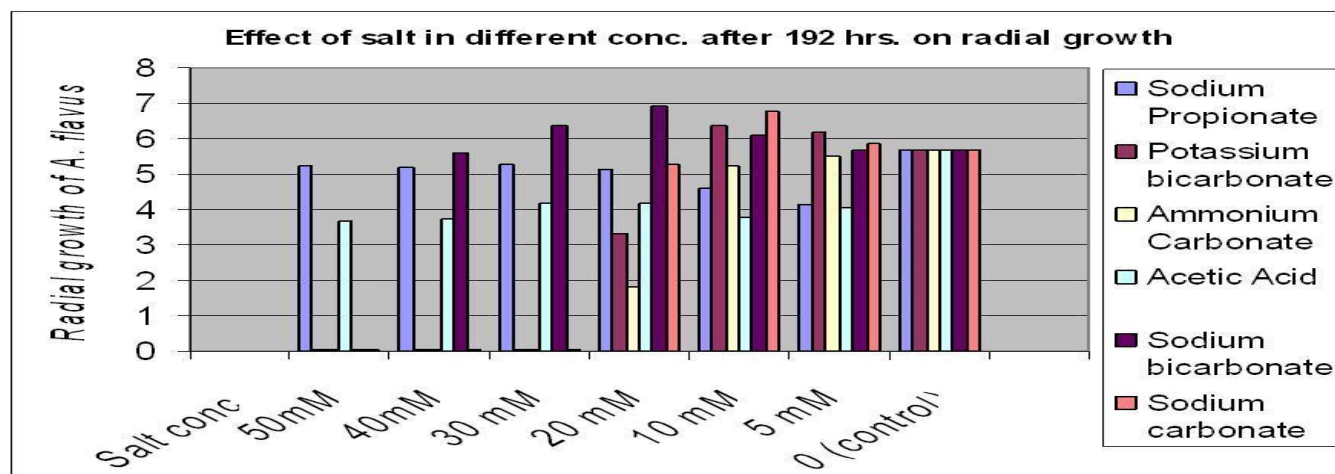


FIG.1

Table 1. Effect of various inorganic salts and organic acids at different concentration on colony growth of *Aspergillus flavus* after 192 hours

Chemical	Sodium Propionate	Potassium bicarbonate	Ammonium Carbonate	Acetic Acid	Sodium bicarbonate	Sodium carbonate	Mean
50MM	5.23	0.05	0.05	3.67	0.05	0.05	1.52
40MM	5.20	0.05	0.05	3.73	5.60	0.05	2.45
30MM	5.27	0.05	0.05	4.17	6.37	0.05	2.66
20MM	5.13	3.32	1.80	4.17	6.93	5.27	4.44
10MM	4.57	6.37	5.23	3.77	6.10	6.77	5.47
5MM	4.13	6.20	5.50	4.03	5.70	5.87	5.24
Mean	4.92	2.67	2.11	3.92	5.13	3.01	
C.D.at 5%		Chemical conc. 0.51	0.51		Che.x conc. 3.63		

Table 2. Effect of inorganic salts and organic acids on post harvest storage of maize in reducing the aflatoxin concentration for eight months duration;

Table; 2 A

S. No.	Treatments @ 4g/kg grains	HQPM – 1*		HM – 4*	
		Conc. of AFB ₁ ppb	% reduction	Conc. of AFB ₁ ppb	% reduction
1.	Sodium Carbonate	11.3	47.9	5.9	81.8
2.	Sodium Bicarbonate	8.9	59.0	4.5	86.1
3.	Potassium Carbonate	14.9	31.4	3.9	88.0
4.	Ammonium Carbonate	3.6	83.4	4.6	85.8
5.	Check (No treatment)	21.7	--	32.5	--
6.	Conc. at initial level ¹	0.001	--	0.013	--

Table: 2 B

S. No.	Treatments @ 4ml/kg grains	900M*		Bio 9681*		BH-2187*	
		Conc. of AFB ₁ ppb	% reduction	Conc. of AFB ₁ ppb	% reduction	Conc. of AFB ₁ ppb	% reduction
1.	Propionic acid	5.6	62.4	5.9	69.6	35.4	47.9
2.	Acetic acid	6.1	59.1	6.2	68.4	40.7	40.1
3.	Sodium propionate @ 4 gm/kg	7.8	47.7	6.8	64.9	39.1	42.4
4.	Check (Untreated)	14.9	--	19.4	--	67.9	--
5.	Conc. at initial level ¹	0.016	--	0.012	--	0.031	--

* Grain samples artificially inoculated with toxic strains of *A. flavus* 24 hours before the application of salts.

Grain sample size of each genotype - 2½ kg and grain moisture ranges between 13 - 41%

¹ Aflatoxin contamination level in maize samples before start of the treatments (initial level).

Potential of these organic salts especially Sodium carbonates, S. bicarbonates, Ammonium and Potassium carbonates were studied in controlling the aflatoxin production by *A. flavus* @ 4 gm/kg grains in post harvest condition in ambient storage condition. Among the inorganic salts Ammonium Carbonate was found most effective in reducing the concentration of aflatoxin by 83.8 % in HQPM -1 and 85% in HM-4 whereas Potassium Carbonate was effective in reducing concentration of AFB₁ in HM - 4 by 88% and in HQPM – 1, 62.3% as compared to check. The initial level of aflatoxin level of these maize genotypes were 0.001 ppb in HQPM; 0.013 ppb in cv. HM – 4; 0.016 ppb in cv. 900M; 0.012 ppb in Bio 9681 and 0.031 ppb in BH – 2187 (Table 2 A & B). This indicated that grains were not free from aflatoxin contamination and already carrying infection from field itself and these grains may spoil in storage due to poor storage condition and consequently the aflatoxin level will increase. To avoid further spoilage of grains, application of these inorganic salts during storage is very effective. Overall all the inorganic salts and organic acids were found effective in reducing the concentration of AFB₁ @ 4 gm/kg or 4 ml/kg of grains however the fungitoxic activity of these salts is varying in different concentration. Post harvest application of inorganic salts and organic acids was

studied by Oliver (1998) for suppression of Silver scuff on Potato tuber and he observed there was a significant reduction in disease severity and sporulation of *Helminthosporium soloni*. Marloth (1931) supported that the bicarbonates ion itself has the inhibitory power and this must be ascribed either to its action on the enzymes or enzyme secreting power of the protoplasm or to a direct toxic influence on the protoplasm itself. In a study Dionisio *et. al* (2003) demonstrated that the inorganic salts like sodium carbonate, sodium bicarbonate and calcium chloride etc. had inhibitory effect on post harvest pathogens of banana. The sclerotial germination of *Sclerotium rolfsii* was completely inhibited by Ammonium carbonates and bicarbonates at 50 mM concentration proved by Punja and Grogan (1982). De pasquale and montiville (1990) concluded that ammonium bicarbonates inhibits fungi because the bicarbonate anion supplies the alkalinity necessary to establish an antifungal concentration of free ammonia, in present study may be the ammonia gas contributes to the detrimental activity on *A. flavus*. Palmer (1997) and he started that the bicarbonates are effective in reducing the growth of *B. Cinerea* in as low as 20 mM concentration. He also believed that the bicarbonates may directly interact with membranes to alter normal membrane activity or may disrupt cellular

physiology at this concentration. In another study Montville and P K Goldstein (1987) found that Sodium bicarbonate reduces viability and alters aflatoxin distribution of *Aspergillus parasiticus* in Czapek's agar.

This study shows that the Ammonium carbonate, Potassium carbonate and sodium carbonate are effective against *A. flavus*, in reducing vitro colony growth at 20 mM conc. as well as effective in post harvest stages @ 4 gm/kg grains in ambient storage condition at grain moisture ranges 13 – 14 % by reducing up to 85.8% reduction in production of aflatoxin concentration (Table 2 A.) in case of organic acids Propionic acid was most effective by reducing 69.6 % reduction production of aflatoxin concentration in Bio 9681 (Table 2 B.). This study also supported the data reported by Pelhate (1973) and Sauer *et. al.* (1974).

Data presented herein support the evidence by marloth (1931) that the bicarbonates ion in itself has the inhibitory power and this must be ascribed either to its action on the enzyme or enzyme-secreting power of protoplasm or to be direct toxic influence on protoplasm itself. However, Nobecourt (1992) demonstrated that sodium bicarbonate inactivates extra cellular enzyme in *penicillium* species. In addition bicarbonates may directly interact with membrane to alter normal membrane activities or may disrupt cellular physiology. Recently Samapundo *et. al.* (2007) also concluded in a study that Ammonium bicarbonate could possibly be used as a cheap and easy to apply treatment for use in resource limited developing countries. This study achieved the objectives outlined in the introduction that inorganic salts and organic acids are effective against *A. flavus*, a mycotoxin producing fungi in maize. They are effective in inhibiting the colony growth at concentration as low as 20 mM, Moreover they are safe for consumption and these non toxic chemicals are also effective in post harvest stages @ 4 gm/kg grain up to eight month duration at grain moisture 13 – 14%. In summary, the potential of Ammonium bicarbonate can be used in minimum dose @ 4 gm/kg grain in post harvest maize to be used for food/feed, for protecting stored maize from mold contamination as it was capable of completely inhibiting growth and in reducing the aflatoxin production due to *Aspergillus* isolates. These non toxic chemicals can be used as a cheap, easy to apply and effective treatment to minimize the losses in post harvest maize in developing countries.

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