International Research Journal of Biological Sciences _____ ISSN 2278-3202
Vol. **3(10),** 52-59, October **(2014)** *Int. Res. J. Biological Sci.*

Effects of Vitamins A, C and E on Growth and Colonial Morphology of Aspergillus flavus

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Available online at: www.isca.in, www.isca.me
Received 14th May 2014, revised 30th June 2014, accepted 21st August 2014

Abstract

Contamination of the aflatoxin-producing Aspergellus flavus occurs in several economical significant crops causing decrease in crop yield and pose health problems. This study was designed to evaluate the ability of vitamins A, C, E, and its combinations to inhibit fungal growth with corresponding colonial morphology changes. This was done by adding a specific concentration of the different vitamins to a culture media and a single point inoculation was employed. It was then incubated for seven (7) days after which growth inhibition, total dry weights, and radial growth were obtained. Results of showed that both growth and mycelia biomass of A.flavus were affected by the vitamins used and its combinations. Among all the vitamins used, at higher concentrations, vitamin C (ascorbate) has complete inhibitory effects to the growth. Decreasing concentrations of thse vitamins in the medium led to an increasing amount of fungal growth as well as increasing fungal biomass and radial growth rates. It was observed that vitamin A, C, and E inhibit fungal growth in a similar way though its combinations exhibited lesser inhibitory effects. In addition, significant concentration-dependent inhibition of growth, mycelia biomass and radial growth rate was evident for selected vitamins. Thus, vitamins A, C, and E can be used to control fungal growth in important crops and processed agricultural products.

Keywords: Biomass, contamination, mycelia, vitamins.

Introduction

The fungi *Aspergillus flavus* synthesize a group of structurally related secondary metabolite named aflatoxins[1]. Aflatoxins are genotoxic and carcinogenic and can cause both acute and chronic toxicity in humans[2]. The naturally produced ones are Aflatoxins B1, B2, G1 and G2[3]. However the interest in aflatoxin has focused on aflatoxin B1 because it is being dominantly produced by the fungi and possesses extreme toxic activity on humans and animals[4].

Aflatoxins are naturally found in soil, decaying vegetation, hay and grains undergoing microbiological deterioration where it invades all types of organic susbtrates. Contamination of aflatoxin-producing *Aspergelli* occurs in several important crops such as maize, meanuts, cottons, tree nuts, corn, and other variety of foods and feedstuffs. Aflatoxin is 200 times carcinogenic than that of benzopyrene. Based on the experimental models, aflatoxin exposure has been seen to cause common gene mutations associated with the majority of human cancers[5]. In this regard, the need for protection from foods and feedstuffs against aflatoxin producing fungi are universally recognized already and several approaches have been suggested[6].

Several studies have suggested that there are approximately more than 100 compounds that have been demonstrated to inhibit aflatoxin production by means of inhibiting the fungal growth and biosynthesis. The use of dietary carotenoids has shown significant decrease in the amount of aflatoxins produced by *A.flavus*[7,8] and beta-carotene in the reduction of incidence of cancer by inhibiting or attenuating the onset of chemical-induced cancers in various target tissues[9]. Vitamin C is a water soluble antioxidant agent that is reported to have reduced DNA damage probability, prevents cancer and heart disease, and reduces toxic effects of nano zinc oxide[10].

The study aimed to determine the effects of vitamins A, C, E, and their combinations to the growth and morphology of *A.flavus*. In determining the response, measurement of the radial growth rate of the colonies and comparison of the total dry weights of the different colonies treated with the vitamins after seven days of incubation was done.

Material and Methods

Vitamins Used: The vitamins used were retinol palmitate (Vitamin A) with 25,000 IU; ascorbic acid (Vitamin C) 100mg/ml which was in liquid form; and d-alpha tocopherol (Vitamin E) with 400 IU. Both vitamins A and E were bought in a soft gel form. The liquid inside the capsule was removed using a sterile syringe and was transferred in a sterile vial.

Fungal strain: The most toxigenic *A.flavus* isolate of wild strain used throughout this study was obtained from the culture collection of the Department of Biological Sciences, MSU-Iligan Institute of Technology.

Culture Conditions: Stock cultures were grown initially on Saboraud Dextrose agar (SDA) slants for 24 hours at 25^0C. Reference stock cultures were stored at room temperature. Working stock cultures were obtained by sub- culturing mycelia from a covered (SDA) slant. The fungal culture used for spore development and subsequent susbtrate inoculation was obtained from working stock cultures after seven (7) days of incubation at room temperature stored in a dark room.

Spore Suspension: Conidia were harvested by adding 5 ml of sterile distilled water to a 14-day old sporulated fungal culture in a SDA plate. The surface of the agar plate was gently and aseptically scraped and was transferred in sterilized 20 ml test tubes. One ml of these spore suspensions was transferred to the five (5) centrifuge tubes. The tubes were centrifuged for 5 minutes in 25,000 rpm under 25^0C. After the centrifugation, the tubes were vortexed for 30 seconds and were prepared for inoculation.

Preparation of the Solid Media*:* A sterilized Saboraud Dextrose agar was used. Before the agar solidifies, a 75ml was transferred to four (4) Erlenmeyer flask. Then, 20ml, 10 ml, 5 ml, and 2.5 ml of vitamin A were added to each flask. The liquid medium was poured to plates with three (3) replicates and was allowed to solidify for 15 minutes. The same procedure was followed in using vitamins C and E and using its combination. Table 1 shows the different volumes and combinations of each vitamin.

Table-1
The mean diameter, growth inhibition, total dry weight, and radial growth rate of *Aspergillus flavus*

Vitamin(s)	Volume (ml)	Mean Diameter (mm)*	Growth inhibition (%)*	Total Dry Weight (mg)*	Radial Growth Rate (µm/hr)*
A	20	58.33	25.22	81	182
	10	71.33	8.55	127	212
	5	74.67	4.27	153	221
	2.5	80	NI	208	244
C	20	0	100	0	0
	10	23.33	70.09	54	96.5
	5	45.33	41.88	91	136
	2.5	59.67	23.50	130	177
E	20	31.33	59.83	108	107
	10	37.67	51.71	117	113
	5	45.00	42.30	155	138
	2.5	56.33	27.78	236	158
A and C	20	43.00	44.87	103	152.78
	10	58.00	25.64	105	180.58
	5	71.67	8.12	128	210.04
	2.5	78	NI	131	227.80
A and E	20	48.33	38.04	130	134.57
	10	64.00	17.95	157	194.52
	5	69.67	10.68	169	200.02
	2.5	78.33	NI	177	233.92
C and E	20	36.33	53.42	70	122.77
	10	41.67	46.58	79	146.73
	5	59.33	23.94	101	182.75
	2.5	66.00	15.38	105	211.24
A, C, and E	20	48.66	37.62	127	158.97
	10	69.66	10.69	177	207.03
	5	71.66	8.13	179	231.43
	2.5	75.00	3.85	198	233.83

* Mean for three (3) replicates ** NI= No inhibitions

Inoculation and Growth Conditions: After the medium was supplemented with each different volume of vitamins, the media were allowed to dry and were inoculated with the fungal test species. A 100 µl of the fungal spore suspension was used for one-point inoculation at the center of each plate. It was then incubated in a dark room for seven (7) days.

Measurement of Colony Diameter: The colony diameters were measured using a standard ruler after seven days. The zone of inhibition was determined as follows:

Growth Inhibition (%) = [(NT-T)/ T] x 100

where NT is the colony diameter of non-treated and T is the colony diameter of the treated. The mean diameters of colonies from the three (3) replicates were obtained.

Measurement of Biomass of the Colonies: After the last measurements of the fungal colonies, when the colonies reached above 40 millimeters in diameter, the dry weights of the colonies were determined by cutting out a colony and melting the gel in a 100-200 ml of distilled water at 60^0C for 10 minutes and collecting the colony on a pre-weighed Whatman filter paper. The filter paper and filter holder were pre-warmed by adding 50 ml of sterile water with temperature of 60^0C to the filtration unit approximately 30 seconds before filtering. The same volume of water was used for washing. The filters were allowed to stand for 10 minutes and dried at 80^0C for 12 hours in a dry bench. Uninoculated plates were used as controls to correct for any precipitations from the medium or resolidification of gel material. Filters were weighed after incubation.

Measurement of Radial Growth Rae: To ensure that the colony was expanding at a constant rate, colonial diameters were measured seven times while their growth rates were also determined. The rate was calculated by linear regression of each colony radius versus time (in hours).

Results and Discussion

Effects of Vitamins: Table 1 shows the summary of all the results obtained in this study. It showed that the mean diameters for all culture treated with different concentrations of vitamins were directly proportional to the growth inhibitions as well as the total dry weights and radial growth rates. However, there were cultures that showed no inhibitions.

Growth Inhibition of Vitamin A: Vitamin A is considered to be a natural antioxidant produced by fungi[11]. SDA medium with a 20ml- volume of vitamin A showed 25.22% of growth inhibition followed by 8.55% and 4.27% inhibition for 10-ml and 5-ml volumes. There is an increasing profusion of fungal growth in a decreased volume of vitamin. Figure 1 shows the different colony formations of *A.flavus.*

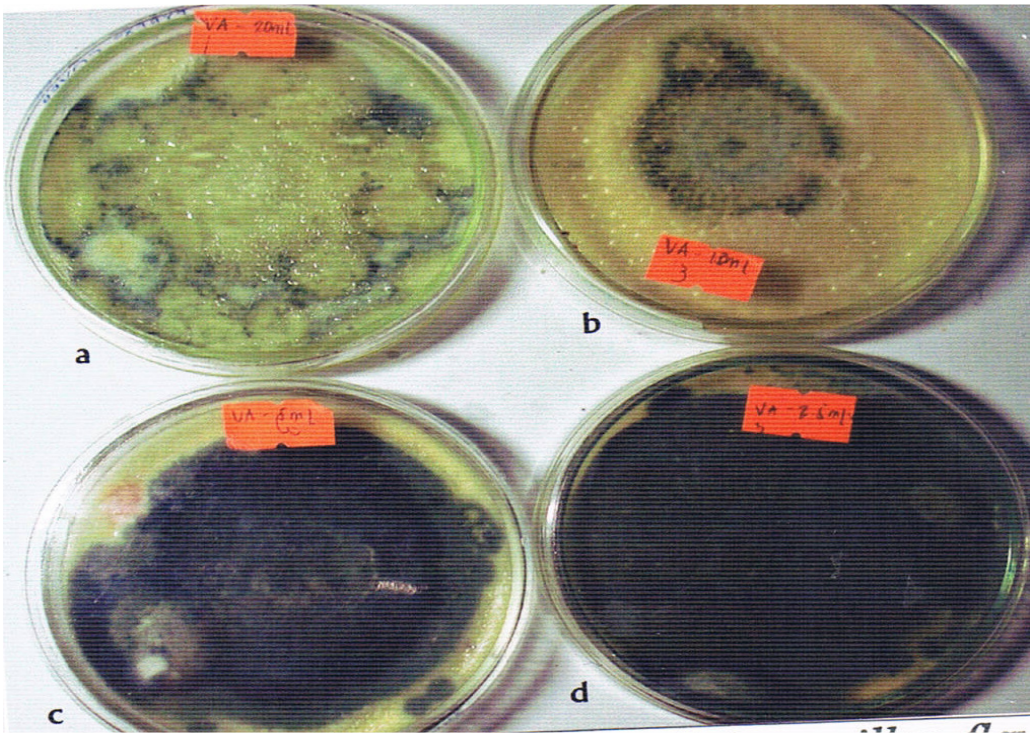

Figure-1
Colony formation of *A.flavus* at different volumes of Vitamin A (a) 20ml; (b) 10ml; (c) 5ml; (d) 2.5 ml

Growth Inhibition of Vitamin C: There is a complete inhibition or 100% for both growth and mycelia biomass at 20ml of vitamin C followed by 70.09% for 10ml, 41.88% for 5ml, and 23.50% for 2.5 ml volume. Results showed that at higher concentrations, vitamin C is inhibitory. Although it is being produced by the fungi[12,13], in a study conducted by Hansberg and Aguirre[14], exogenous ascorbate can inhibit sporulation in fungi and can be fungicidal[15]. Figure 2 shows the colony formation of *A.flavus* at different volumes of vitamin used. The physiological basis for these observations could be in the regulation of oxidative stress during the growth of *A.flavus* which may be similar to that observed in *Sclerotium rolfsii*[16]. The balance between the reduced and oxidized forms of vitamin C is vital for fungal differentiation. The exogenous ascorbate introduced at high concentrations somehow disrupted this balance pushing towards inhibition of fungal growth.

Growth Inhibition of Vitamin E: At 20ml volume, a growth inhibition of 59.83% was observed in SDA plate with vitamin E, 51.71% at 10ml, 43.30% at 5ml, and 27.78% at 2.5ml volume (figure 3). Reduction of fungal growth for all the concentrations of vitamin E was possibly due to interference. Kumar and Prasad[17] suggested that growth and aflatoxin production by *A.flavus* are proportionate process. In addition, a study presented by Nair and Verma[18] showed that vitamin E has a protective effect on lipid peroxidation in the testis of aflatoxin-treated mice considering that this vitamin is a potent biological antioxidant and its antioxidative function is mainly due its reaction with membrane phospholipid bilayers to breah the chain reaction initiated by hydroxyl radical. In addition, vitamin E has a higher affinity to aflatoxin and acts by reducing its bioavialbility through the formation of stable association. It implies then that vitamin E has the capability to reduce and impede aflatoxin formation and with the reduction of such, consequently, fungal formation will be inhibited as well. Nonetheless, growth and aflatoxin production by *Aspergellus parasiticus* was observed to be an independent phenomenon[19].

Growth Inhibition of the Combinations of Vitamins A, C, and E: Figure 4 shows the different results for the growth inhibition of the combination of each of the vitamins. Individually, the three vitamins were shown to be inhibitory at higher concentrations. Thus, when in combination, the inhibitory effect seems to be lesser or decreased. In a study conducted on vitamin C and E interactions, it was shown that vitamin C restored the lipid antioxidant properties of vitamin E[20-22]. This phenomenon may be applied to the present study where the synergistic effect of each vitamins A, C, and E were more of the restoration of the antioxidant properties, thus, canceling out inhibitory effects oxidative balance may also be restored when these vitamins were added in combinations causing continuance of fungal growth. These results are very significant since no other study has ever been reported on the novel effects of vitamins A, C, and E combinations on the growth dynamics of the aflatoxin-producing fungus *A.flavus*.

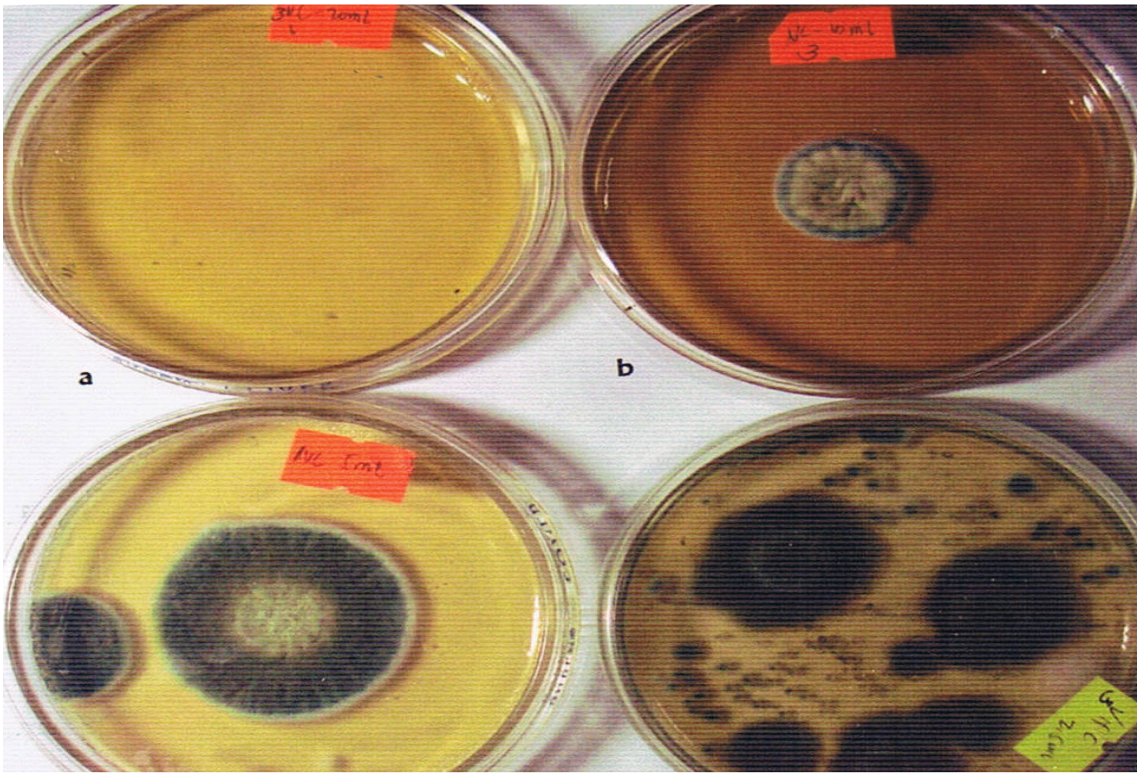

Figure-2
Colony formation of *A.flavus* at different volumes of Vitamin C (a) 20ml; (b) 10ml; (c) 5ml; (d) 2.5 ml

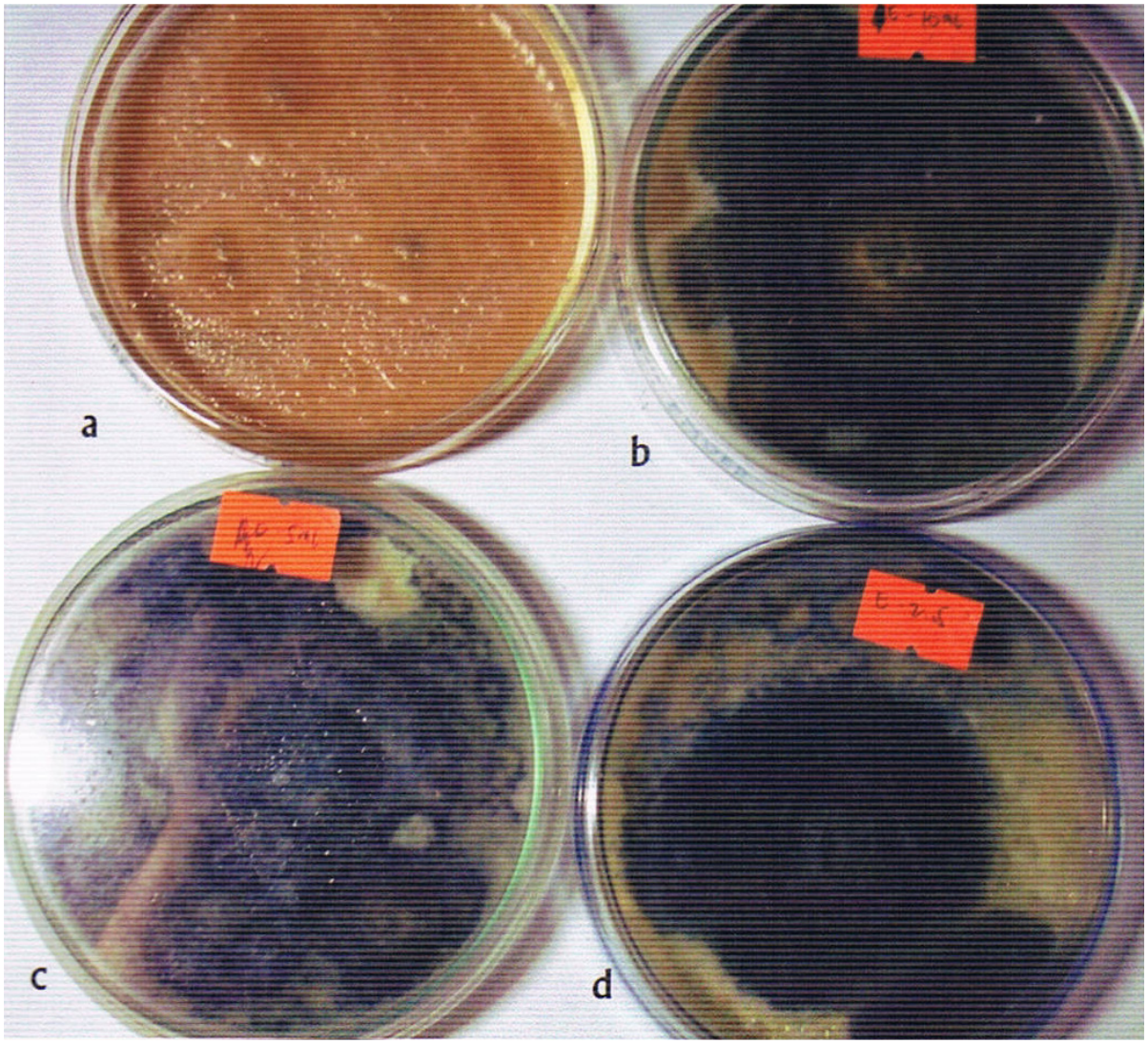

Figure-3
Colony formation of *A.flavus* at different volumes of Vitamin E (a) 20ml; (b) 10ml; (c) 5ml; (d) 2.5 ml

Colonial Morphology Effects: At 2.5ml volume, vitamin A, combination of vitamin A and C, and vitamin A and E stimulated growth of *A.flavus* and showed no inhibition as well as increased mycelia biomass. Vitamins C, E, C and E, and A, C, and E, at an identical concentration, reduced growth of fungi but still have higher mycelia biomass.

At 20 and 10ml volumes, vitamin C had the greatest activity with respect to the growth inhibition as it showed 100% and 70% inhibition of the fungal species. This could imply that with the inhibition in the fungal development subsequently decrease of aflatoxin levels might also be possible. Such claim is supported by the study made by Beneett and Papa[23]when they showed that mutant strains of *Aspergelli* demonstrated relationship between aflatoxin formation and sporulations. Mutants that are deficit in sporulation were unable to produce aflatoxins or with reduced formation. The results could also imply that the vitamins used with its volume-concentrations could serve as antifungal agents against the fungal growth.

Furthermore, growth inhibition for these vitamins was observed to correlate inversely with mycelia dry weight when *A.flavus* was grown on SDA. As the growth inhibition increases, the mycelia dry weight decreases consequently. Likewise, for 5ml volume, vitamin C and E had the greatest activity almost similar with each other as well as in the mycelia biomass.

Radial Growth Rate: Vitamin C has the lowest radial growth rate which signifies that there is a delay in mycelia growth and sporulation. In one study, ascorbate showed decreased sclerotial differentiation in *S.rolfsii*[16]. Ascorbate also delay differentiation and caused decline in its growth. This can also be explained by the fact that ascorbate can act as pro-oxidant.

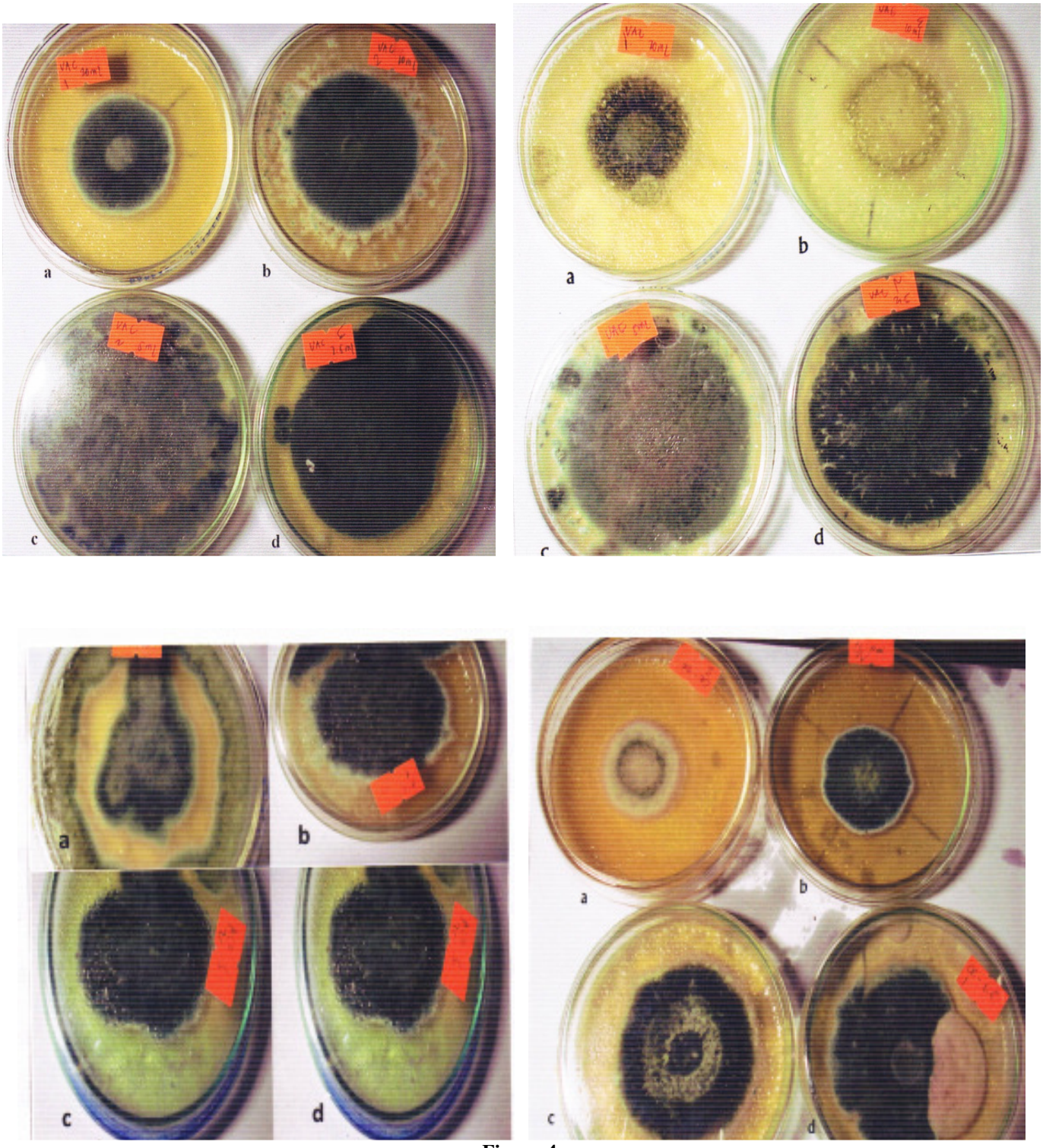

Figure-4

Colony formation of *A.flavus* at different volumes of combination of vitamins; Upper left – Vitamins A and E (a) 20ml, (b) 10ml, (c) 5ml, (d) 2.5 ml; Upper right- Vitamins A and C (a) 20ml, (b) 10ml, (c) 5ml, (d) 2.5 ml; Lower left- Vitamins C and E (a) 20ml, (b) 10ml, (c) 5ml, (d) 2.5 ml; Lower Right- Vitamins A, C, and E (a) 20ml, (b) 10ml, (c) 5ml, (d) 2.5 ml

Conclusion

Results showed that at higher concentrations, vitamin C (ascorbate) showed a total or 100% fungal growth inhibition. Decreasing concentration of vitamin C led to an increasing amount of fungal growth and increasing total dry weight and radial growth rate. The same is true for vitamins A and E. In the different combinations of the three vitamins (AC, AE, CE, and ACE), the patterns of growth inhibition were lesser that at individual dosage.

The successful inhibition of mycelia growth by some concentrations of the different vitamins used in this study indicates the possibility of their uses as fungicides, especially against the growth of toxigenic culture and aflatoxin-producing *Aspergillus flavus*. The information gathered in this study can be used as basis for developing a safe, economically feasible fungal control program in important crops and processed agricultural products.

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