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***Research Article***

# *IN VITRO* EFFECT OF SELENIUM ON FUNGAL BIOMASS AND AFLATOXIN PRODUCTION BY *ASPERGILLUS PARASITICUS*

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## ABSTRACT
A study was conducted to evaluate the effect of selenium (Se) in organic and inorganic form on fungal growth and aflatoxin (AF) production in liquid medium and cracked maize. Basal media (100 ml) in flask were supplemented without (control) or with Se-yeast or sodium selenite at a concentration of 1 or 3 ppm. Similarly broken maize in conical flasks were supplemented without (control) or with Se-yeast or sodium selenite at 1.5 and 3 ppm level. All the flasks were inoculated with one ml of fungal spore suspension containing $10^6$ to $10^7$ spores of *Aspergillus parasiticus* (NRRL 2999). The liquid medium was incubated at 26±1°C for 10 days and the cracked maize was incubated at 28±1°C for 7 days in a BOD incubator. Each treatment was replicated 6 times. The results of Se supplementation in liquid media revealed that the Se-yeast increased fungal biomass at both levels significantly, whereas sodium selenite did not increase fungal biomass when compared to control. The aflatoxin $B_1$ ($AFB_1$) production in 100 ml media was significantly highest due to Se-yeast at 3 ppm or sodium selenite at 1 ppm level followed by sodium selenite at 3 ppm, Se-yeast at 1 ppm and it was lowest in control. However, the $AFB_1$ production per gram of fungal biomass was significantly increased by sodium selenite at 1 and 3 ppm, followed by Se-yeast at 3 ppm than Se-yeast at 1 ppm or control. Similarly the results of Se supplementation on cracked maize revealed that the $AFB_1$ production in maize amended with Se-yeast at 1.5 ppm or sodium selenite at 3 ppm was significantly higher followed by Se-yeast at 3 ppm or sodium selenite at 1.5 ppm than control. It is concluded that the organic and inorganic Se significantly increased the AF production, while the fungal biomass was significantly higher in organic form than inorganic form of Se.

***Key Words*: *Aflatoxin, Fungal Biomass, Liquid Medium, Selenium.***

## INTRODUCTION
The aflatoxins are group of closely related fungal secondary metabolites produced by certain strains of Genus *Aspergillus.* The aflatoxins are reported to be carcinogenic, mutagenic and teratogenic and toxic which causes severe economic loss in poultry due to poor growth, reduced feed intake, poor FCR and immunosuppression rather than heavy mortality. Several studies have been conducted on dietary supplementation of nutrients for counteracting aflatoxicosis in poultry, such as supplementation of protein (Beura *et al.*, 1993), ∞ - tocopherol, ascorbic acid (Hoehler and Marquardt, 1996), zinc (Hegazy and Adachi, 2000). The selenium (Burguera *et al.*, 1983) supplementation have also been tried to counteract aflatoxicosis with varied response.

Dietary supplementation of any nutrient especially trace minerals that counteract aflatoxicosis can not be used based on *in vivo* experiments alone, because the nutrients which counteract aflatoxicosis *in vivo* may influence the fungal growth and subsequent aflatoxin production *in vitro* in feed ingredient/ mixed feed. If dietary supplementation of any nutrient increases aflatoxin production by the fungi in feed, then its use is limited, even though the nutrient is effective against aflatoxicosis. For example, the supplementation of selenium was found to be effective against aflatoxicosis in poultry (Burguera *et al.*, 1983), but unfortunately, the effect of supplemental selenium on fungal growth and subsequent aflatoxin production

in feed received a little attention. Hence a study was formulated to find out the *in vitro* effect of supplemental selenium on fungal growth and aflatoxin production in liquid media and maize.

## MATERIALS AND METHODS

### *Culture and preparation of inoculum*

The culture of *Aspergillus parasiticus* strain NRRL 2999 maintained in Mycotoxin Lab. of ANFT Division of CARI was used for this study. The spores of the strains were regularly subcultured on potato dextrose agar (PDA) slants. For preparation of inoculum, fresh fungal spores of 7 to 10 days old in PDA slants were harvested in sterile phosphate buffer saline (PBS) containing 0.05 % tween-80 and the spore count was done. The collected spores in sterile PBS were then diluted suitably to give final spore concentration of $10^6$ to $10^7$ spores/ ml of PBS. One ml of this spore suspension was used to inoculate the experimental liquid media and broken maize.

### *Preparation of basal media and broken maize*

A synthetic medium as suggested by Reddy *et al.* (1971) for aflatoxin production was used without addition of trace minerals, as basal medium. The composition of basal medium is given in Table 1. Hundred ml of the media were transferred to individual flasks of similar type with same capacity and surface area. All the flasks were then autoclaved at 15-1bs pressures for 15 minutes and cooled. Similarly, fifty gm of good quality maize, free from possible adulterants was taken in each 250 ml conical flask, plugged with cotton and autoclaved at 15 1b psi for 15 minutes and cooled.

### *Preparation of experimental media and broken maize*

The experimental details are given in Table 2. In Experiment 1, the basal media was added without (control – T1) or with selenium as selenium yeast to give final concentration of 1 and 3 ppm (T2, T3) and selenium as sodium selenite to give final concentration of 1 and 3 ppm (T4, T5). Similarly, in Experiment 2, ten ml of sterilized distilled water was added to the maize without (control – T1) or with selenium as selenium yeast to give final concentration of 1.5 and 3 ppm (T2, T3) and selenium as sodium selenite to give final concentration of 1.5 and 3 ppm (T4, T5). Each treatment in both experiments is replicated by six times.

### *Inoculation and incubation*

All the experimental media and maize were inoculated with one ml of fungal spore suspension. The experimental media were incubated at $26\pm1^0$ C. for 10 days and broken maize samples were incubated at $28\pm1^0$ C. for 7 days in a BOD incubator.

### *Study of response criteria*

After the incubation period, the following parameters were studied.

*(a) Fungal biomass in liquid media:*

The fungal biomass as influenced by various treatments was estimated by the method as suggested by Cuero and Ouellet (2005). Briefly, the mycelial mat was separated from liquid media and washed with distilled water. The mycelia were then subjected to drying in hot air oven at $40^0$ C for 3 to 4 days and the weight of mycelia was noted down and expressed as dry mycelia weight in grams.

*(b) Aflatoxin production in liquid media:*

The AF production in liquid media as influenced by various treatments was determined by Thin Layer Chromatography (TLC) by the method as suggested by Tsai *et al*. (1984), by direct spotting of media on TLC plates. Liquid media of different concentration like 2 µl, 4 µl were directly spotted on TLC plates using micropipettes. AF $B_1$ (M/S Sigma Aldrich Chemical Ltd., U.S.A) standard were also spotted on the same plates. The plates were then developed in Toluene: Ethyl acetate: Formic acid (9:3:1) solvent system and the AF $B_1$ content was calculated by comparing the fluorescent intensities of the sample spots with standard spots.

*(c) Aflatoxin production in cultured maize:*

After incubation period, the cultured maize in flasks were again autoclaved, cooled, dried and then grinded to powder form. The extraction of aflatoxin from the cultured maize was done as per the

procedure described by Pons *et al.* (1966) and quantification of aflatoxin was done by Thin Layer Chromatography (TLC) method by comparing fluorescent intensities of the sample spots with aflatoxin standard spots after developing the plates in Toluene: Ethyl acetate: Formic acid (9:3:1) solvent system.
All the data collected during the experiments were subjected to standard statistical analysis as per Snedecor and Cochran (1989).

## RESULTS AND DISCUSSION

The fungal growth measured as mean mycelial dry weight (gm), the aflatoxin $B_1$ production in liquid media and aflatoxin $B_1$ production in cultured maize as influenced by selenium is given in Table 3.

***(a)Fungal biomass in liquid media***

The result revealed that mycelial dry weight was significantly ($P<0.01$) increased in basal media with added selenium at both levels as selenium yeast (T2, T3) than other groups, whereas the media amended with selenium as sodium selenite (T4, T5) did not differ significantly with control. The result implies that the selenium yeast increased the fungal growth, whereas addition of sodium selenite could not increase the fungal growth. The increase in fungal growth by selenium yeast might be due to the yeast portion selenium yeast rather than the selenium. Yeast extract sucrose broth in which yeast extract was added at 2% was used for growth of *Aspergillus* and aflatoxin production (Tsai et al. 1984). The selenium as sodium selenite did not influence the fungal growth and hence, it can be suggested that sodium selenite might not be needed for the fungal growth.

***(b) Aflatoxin $B_1$ production in liquid media***

The result of study revealed that the aflatoxin production in 100 ml of media was significantly higher in media amended with 3 ppm selenium as selenium yeast (T3) and 1 ppm selenium as sodium selenite (T4) followed by 3 ppm selenium as sodium selenite (T5) and 1 ppm selenium as selenium yeast (T2) than control. However, aflatoxin production per gm of fungal biomass was found to be increased by 1 and 3 ppm selenium as sodium selenite (T4, T5) followed by 3 ppm se as selenium yeast (T3) than 1 ppm se as selenium yeast (T2) or control (T1).

The result of Experiment 1 suggests that the selenium as sodium selenite increased the aflatoxin production, but without increasing the fungal growth. It is generally agreed that there is no relation between aflatoxin production and the fungal growth. In this study, we found that there existed a difference between sodium selenite and selenium yeast in which the selenium yeast increased aflatoxin production mainly through increased fungal growth and the aflatoxin per gram of fungal biomass did not increased. The induced fungal growth by selenium yeast might be due to yeast portion of selenium yeast. The increased aflatoxin production by sodium selenium might be due to induced lipoperoxidation by the mineral on fungi leading to increased aflatoxin production. Fanelli *et al.* (1984) considered that the aflatoxin production by *Aspergillus parasiticus/ A. flavus* was a system of detoxification for lipoperoxides. Normally, the addition of lipoperoxides causes damage to cells, but in case of *A. parasiticus* it do not cause inhibitory effect on fungal growth, but greatly increase aflatoxin output (Fabbri et al., 1983). This theory was supported by finding of Fanelli et al. (1984) in which supplementation of carbon tetrachloride, which is capable of inducing endogenous lipoperoxidation of internal membrane of fungi, to fungal culture increased the aflatoxin production. In a similar way, we presume that sodium selenite supplementation might also have induced lipo peroxidation and subsequently increased the aflatoxin production. Another possible way of increased aflatoxin production by selenium might be due to its utilization for secondary metabolism for aflatoxin production, as some trace minerals does. For example, production of aflatoxin $B_1$ was increased two folds by copper and four folds by zinc (Cuero and Ouellet, 2005).

***(c) Aflatoxin $B_1$ production in cultured maize***

The result of Experiment II, revealed that the aflatoxin $B_1$ in maize amended with 1.5 ppm selenium as selenium yeast (T2) and 3 ppm selenium as sodium selenite (T5) was significantly higher followed by maize amended with 1.5 ppm selenium as sodium selenite (T4) or 3 ppm selenium as selenium yeast (T3),

than in maize without selenium supplementation. The selenium might have induced aflatoxin production either through its utilization by fungi for aflatoxin production or the selenium might have induced oxidative stress on fungi leading to increased aflatoxin production.

Based on result of the study, it may reasonably be suggested that the supplementation of selenium in feed to counteract aflatoxicosis i*n vivo* should be considered carefully for the effect of selenium on aflatoxin production by contaminating fungi in feed.

**Table 1: Composition of basal medium**

| Ingredients | Chemical formula | Amount (gm) |
|---|---|---|
| Ammonium sulphate | $(NH_4)_2SO_4$ | 3.500 |
| Calcium chloride | $CaCl_2.2H_2O$ | 0.075 |
| L-Asparagine | $C_4H_8N_2O_3H_2O$ | 10.00 |
| Magnesium sulphate | $MgSO_4.7H_2O$ | 0.350 |
| Potassium dihydrogen orthophosphate | $KH_2PO_4$ | 0.750 |
| Sucrose | $C_{12}H_{22}O_{11}$ | 85.00 |

*Distilled water to make up the volume to 1,000 ml (pH adjusted to 4.5 using 0.1N HCl before making up the final volume)*

**Table 2: Details of Experiments**

| Experiment | Treatment | Description of treatment |
|---|---|---|
| | T1 | Basal media (100 ml) (control) |
| | T2 | Basal media + 1 ppm Se as selenium yeast |
| **I.** | T3 | Basal media + 3 ppm Se as selenium yeast |
| | T4 | Basal media + 1 ppm Se as sodium selenite |
| | T5 | Basal media + 3 ppm Se as sodium selenite |
| | T1 | Maize (50 gm) (Control) |
| | T2 | Control + 1.5 ppm Se as selenium yeast |
| **II.** | T3 | Control + 3 ppm Se as selenium yeast |
| | T4 | Control + 1.5 ppm Se as sodium selenite |
| | T5 | Control + 3 ppm Se as sodium selenite |

**Table 3: Effect of selenium supplementation on fungal biomass in media and aflatoxin production in media and maize**

| Treatment | Experiment I | | | Experiment II |
|---|---|---|---|---|
| | Mycelial dry weight (gm) | Aflatoxin $B_1$ (mg/100ml media) | Aflatoxin $B_1$ (mg/ gm fungal biomass) | Aflatoxin $B_1$ (ppm) in maize |
| **T1** | $2.15^a$ | $3.07^a$ | $1.43^a$ | $26.67^a$ |
| **T2** | $3.01^b$ | $4.07^b$ | $1.36^a$ | $38.33^c$ |
| **T3** | $3.17^b$ | $5.87^d$ | $1.85^b$ | $31.67^{ab}$ |
| **T4** | $2.25^a$ | $5.73^d$ | $2.57^c$ | $32.22^b$ |
| **T5** | $2.14^a$ | $5.20^c$ | $2.44^c$ | $38.89^c$ |
| **Pooled SEM** | 0.086 | 0.208 | 0.097 | 1.118 |

*Values bearing different superscripts within a column differ significantly (P<0.05)*

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