# CITRININ ACCUMULATION UNDER STRESS OF CAMPHORE AND BLUE-GUM EXTRACT

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*SUMMARY: The effect of camphore and the aqueous leaves extract of blue-gum (Eucalyptus globulus L.) on the growth of Aspergillus terreus var. aureus and the formation of citrinin was studied. The low levels 0.05 and 0.5% of camphore and leaves extract, respectively, were found to be the most effective on citrinin synthesis during all periods of incubation. The high level of camphore (0.5%) inhibited the mycelial growth and citrinin production after 5 days of incubation. However, after 10 and 15 days of incubation, citrinin increased, but was still below the control level. In replacement culture, camphore increased citrinin accumulation with rise in respiration. There was no increase in citrinin accumulation at a defined level of stress and this depended on the pre-grown mycelia and condition of their incubation.*

*Key Words: Camphore, leaves extract, citrinin, respiration.*

## INTRODUCTION

Citrinin occurred as a co-contaminant in cereals associated with porcine nephropathy (12). It has established teratogenic (7), renal tumors (3) and inducing enlargement and tubular necrosis of the kidney. Therefore, citrinin regarded as an important mycotoxin which may be ingested by man and animals. Extensive studies have been made on the action of different chemicals in controlling citrinin production by Penicillium spp. (5,9,11).

Blue-gum oil has established, anti-aflatoxigenic (16) and anti-dermatophytes (15). However its effect on growth and citrinin biosynthesis has not been reported. The current study was made to assess the fungicidal as well as anti-toxigenic properties of aqueous leaves extract of blue-gum and camphore (the major active ingredient of blue-gum) against a known toxigenic strain of *Aspergillus terreus* var. aureus.

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## MATERIALS AND METHODS

**Mould, medium and treatments**

A toxigenic strain of *A. terreus* var. aureus No.21, isolated from faba bean in this laboratory, was used in the study.

The effect of camphore and aqueous leaves extract of blue-gum on cultures initiated from conidia were studied. Spore suspension of 1-week-old culture of the mould was made and 1 ml (approx 106 spores) was added to 50 ml (containing 30 g sucrose, 5 g yeast extract, 2 g $KNO_3$, 1 g $K_2HPO_4$, 0.5 g KCl and 0.5 g $MgSO_4$. $7H_2O$ in liter distilled water) medium. Three levels of camphore in ethanol viz. 0.05, 0.1 and 0.5% and sterile extract of fresh blue-gum (*Eucalyptus globulus L.*) leaves in water viz. 0.5, 1.0 and 5.0% were added to the inoculated culture medium. The same volume of ethanol and water were adjusted in all treatment and the control. Three replicates were prepared in each case. The cultures were agitated in a rotary shaker (150 c.p.m.) at 28°C for 15 days and periodically analyzed for growth and citrinin production.

The effect of camphore and aqueous leaves extract of blue-gum on cultures initiated by pre-grown mycelial pellets were studied. After sequential culturing in rotary shaker (150 c.p.m) for 5

Table 1: Effect of camphore and aqueous extract of fresh blue-gum leaves on mycelial growth, $CO_2$ evolution and citrinin production by the replacement culture of A. terreus var. aureus with agitation.

| Substances used | Concn. % | Mycelium dry weight (mg) | $CO_2$ evolution (mg/g dry wt/24 h | Specific production of citrinin (μg/g dry wt) |
|---|---|---|---|---|
| | Z | 400 | - | 750 |
| | 0.00 | 921 | 32.6 | 1086 |
| Camphore | 0.05 | 890 | 40.2 | 1236 |
| | 0.50 | 412* | 159.7* | 1456* |
| Leaves extract | 0.50 | 973 | 41.2 | 822 |
| | 5.00 | 834 | 57.2* | 1079 |

Z: Values at time of transfer.

*: Significant difference compared to the control at 5% level.

days at 28°C, the mycelial pellets were transferred to 250 ml flasks containing 50 ml of medium. 0.05 and 0.5% of camphore or 0.5 and 5% of leaves extract were added. All flasks were subsequently shaken at 150 c.p.m. at 28°C for 48 hours and analyzed for $CO_2$ and citrinin production.

The effect of camphore on cultures initiated by pre-grown mycelial mats were studied. After sequential culturing in medium without agitation at 28°C for 5 days, the disrupted mycelia were transferred to 250 ml flasks containing 50 ml of medium and 0.5 ml of ethanol containing the appropriate amount of camphore. All flasks were subsequently incubated without agitation at 28°C for 48 hours and then analyzed for $CO_2$ and citrinin production.

**Growth and citrinin analysis**

The resulting mycelia were collected by filtration and dry weights were determined after washing and drying to constant weight at 80°C. Other cultures (mycelium + filtrate) were extracted for citrinin with chloroform (9). Quantitative determination of citrinin was done according to Damodaran *et al.* (8).

**Determination of $CO_2$ evolved**

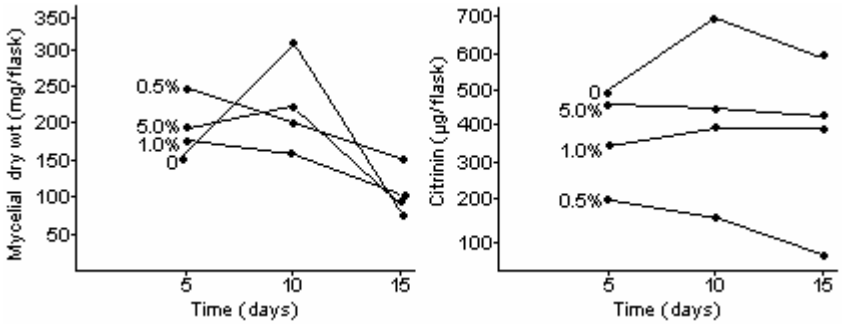
Mycelial respiration was measured by continuous air current method adopted by Kyo Sato (13). $CO_2$ evolved from mycelia of treated and untreated fungal species was absorbed in 0.5 N NaOH solution. Titration was carried out for the un-neutralized NaOH with 0.5 N HCl after addition of an excess amount of 3 N $BaCl_2$.

## RESULTS AND DISCUSSION

The toxigenic strain of *A. terreus* var. *aureus* reached their maximum growth and citrinin production after 10 days and gradually decreased after 15 days of incubation (Figure 1). The potential of aqueous leaves extract of blue-gum and camphore for inhibition of growth and citrinin production was investigated.

The most essential oils of blue-gum were presented in the leaves. The aqueous leaves extract was tested at three levels (0.5, 1.0 and 5.0%) (Figure 1). The low level increased the mycelial growth after 5 days of incubation and gradually decreased with time, but their level was still higher than the control after 15 days of incubation. Synthesis of citrinin was inhibited by about 60, 78.6 and 91.7% after 5, 10 and 15 days, respectively. Ahmed and Agnihotri (1) and Eman (10) noticed the inhibitory activity of extract from *Eucalyptus citriodora* and *E. globulus* against fungal growth, aflatoxin and sterigmatocystin production. Masood and Ranjan (14) noticed that the aqueous leaf extracts of some plants inhibited mycelia growth and aflatoxin production by *A. flavus*.

Figure 1: Effect of aqueous extract of fresh Blue-gum leaves on mycelial growth and citrinin production by *A. terreus* var. *aureus* after three incubation periods.



Camphor was tested at three levels 0.05, 0.1 and 0.5% (Figure 2). The low level inhibited citrinin production by about 80% after 5 days of incubation. This inhibition was related to mycelia growth, whereas the growth of the fungus highly promoted. After 10 days of treatment, inhibition of citrinin production increased (85.7%) and this inhibition was related to mycelial growth depletion. The high levels inhibited both growth and citrinin production after 5 and 10 days of treatment. The mycelial growth and toxin production increased with incubation time and so the inhibitory effect was gradually decreased. This phenomenon was more detected by the increasing of camphor concentration. Tiwari *et al.* (16) reported that eucalyptus oil decreased the growth and aflatoxin production. However, Ansari and Shrivastava (2) noticed after 12 days, of oil incorporation, aflatoxin production was greater than the control.

The inhibitory effects of both leaves extract and camphor were more pronounced at the low level after all periods of incubation. However, this effect decreased by increasing the incorporation levels. Citrinin is synthesized via polyketide metabolic pathway. The low dose of camphor and leaves extract of blue-gum may inhibit a key enzyme (s) in polypeptide pathway.

Increase of citrinin production surmised can be as the spores/mycelium was initially under stress showing low growth and toxin production followed by high toxin production in the late phase of incubation. These results are in agreement with the finding of Bauer *et al.* (4), Tsai *et al.* (17), Masood and Ranjan (14) and Ansari and Shrivastava (2). They reported stimulation of aflatoxin production at sub lethal levels of inhibitors specially after advanced incubation.

The following experiments are conducted to establish the correlation between stress and citrinin production in replacement cultures.

In replacement medium, the pre-grown mycelial pellets, re-incubated under shaken in medium containing various concentrations of camphor and aqueous leaves extract (Table 1) indicated that camphor inhibited mycelial growth while the specific production of citrinin and $CO_2$ evolution increased at 0.5% concentration. The aqueous leaves extract did not significantly affect mould growth and citrinin production.

In order to examine the detailed effect of camphor as stress on $CO_2$ and citrinin production while minimizing fungal growth, the replacement culture technique of Buchanan *et al.* (6) was employed (Table 2). Camphor caused increase in $CO_2$ evolution especially at 0.1% concentration. An apparent relationship between the rise in respiratory activity observed and the synthesis of citrinin. No increase in citrinin accumulation was observed at 0.5 and 1.0% whereas, respiration delayed at these concentrations.

The irregularities in metabolic pathways exerted by stress conditions might affect mould survival and adaptation. Stimulation of respiration under stress may be due to the increased availability of respiration substrates and/or probably due to the increased energy required for osmotic adjustment and this limits growth. The decrease in growth

Figure 2: Effect of camphore on mycelial growth and citrinin production by *A. terreus* var. *aureus* after three incubation periods.

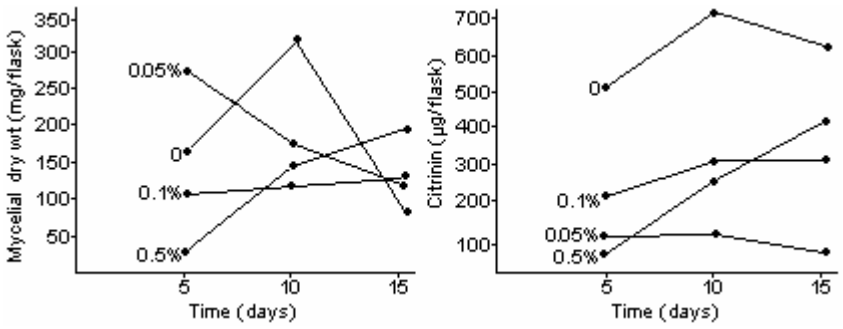


Table 2: Effect of camphore on mycelia growth, $CO_2$ evolution and citrinin production by the replacement culture of *A. terreus* var. *aureus* without agaitation.

| Camphore % | Mycelium dry weight (mg) | $CO_2$ evolution (mg/g dry wt/24 h | Specific production of citrinin (μg/g dry wt) |
|---|---|---|---|
| Z | 214 | - | 467.3 |
| 0.00 | 282 | 48.9 | 627.7 |
| 0.05 | 270 | 59.3 | 674.1 |
| 0.10 | 224* | 69.2* | 910.7* |
| 0.50 | 122* | 24.8* | 618.0 |
| 1.00 | 126* | 25.7 | 630.2 |

Z: Values at time of transfer.

*: Significant difference compared to the control at 5% level.

of stressed mould is probably associated with the required osmotic regulations which cause a diversion of metabolites from the synthesis of cell constituents into the synthesis of the osmoregulants which in addition, could be inhibitory. Also, it could be due to the increase in the consumption of assimilates by a mould at the time that uptake decreased as a result of the present experimentation.

One possibility is that stress affects the mitochondrial ATP ase, depressing the transport of metabolites into the organelle. This could result in an elevation of the cytoplasmic level of two-carbon precursors for citrinin synthesis, and accordingly increase production of the toxin. Evaluation of these hypothesis will require additional research.

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