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INHIBITORY EFFECT OF CLOVE (*SYZYGIUM AROMATICUM*) ON THE GROWTH OF *PENICILLIUM CITRINUM* AND CITRININ PRODUCTION

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ABSTRACT

Clove was studied for its effect on the growth of *Penicillium citrinum* and citrinin production in culture medium and rice. The minimum concentration at which clove inhibited fungal growth and citrinin production was determined and recorded as 1.8 mg/mL in yeast extract sucrose broth. All concentrations of clove inhibited the fungal growth; however, citrinin production was inhibited significantly only at higher concentration. At 1.6 mg/mL, clove inhibited fungal growth and citrinin production by 80 and 58%, respectively; hence, this concentration was used to study the relationship between *P. citrinum* and citrinin production. Clove inhibited the growth of *P. citrinum* in culture media by 60–70%, throughout the observation period of 14 days. Along with the fungal growth inhibition, citrinin production was inhibited significantly. In rice, clove delayed the growth of *Penicillium* by 3 days; however, after 5 days of incubation, fungal growth and citrinin production were equivalent to the control.

PRACTICAL APPLICATIONS

Citrinin is a nephrotoxin and found as a common contaminant in rice, wheat and red yeast rice. This study showed the potential of clove in controlling *P. citrinum* and citrinin in culture media and rice. As spices are commonly used in many food ingredients, clove may be recommended for use in processed and ready-to-eat foods as a preservative. The use of whole clove eliminates the need for extracting essential oils and is more economical.

INTRODUCTION

Citrinin is a mycotoxin produced by *Penicillium citrinum*, *Penicillium expansum*, *Monascus purpureus*, *Monascus ruber*, *Aspergillus ochraceus*, *Aspergillus niveus* (Hetherington and Raistrick 1931; Kurata 1990; Blanc *et al.* 1995). Few citrinin-producing fungi also produce ochratoxin A and patulin, and co-occurrence of citrinin with these toxins has been reported (Vrabcheva *et al.* 2000; Martins *et al.* 2002). These fungi are found as natural contaminants in a wide variety of food and feed grains, fermented and processed food (Pattanagul *et al.* 2008; Li *et al.* 2012; Zaied *et al.* 2012). Red yeast rice, which is used as dietary supplement, food preservative and colorant in many Asian foods, is reported to be often contaminated with citrinin (Gordon *et al.* 2010; Li *et al.* 2012). Consumption of such contaminated food is hazardous to both humans and animals. Citrinin is hepatotoxic and nephrotoxic to a number of animal species (Bilgrami *et al.* 1988). It primarily affects the kidney, and the possible role of citrinin and ochratoxin A in the Balkan endemic nephropathy was implicated (Vrabcheva *et al.* 2000). Although there are no prescribed regulations for citrinin contamination, the European Food Safety Authority has determined a level of no concern for nephrotoxicity of 0.2 μg/kg body weight per day for citrinin (EFSA 2012).

The ubiquitous nature of fungi and mycotoxins makes them unavoidable contaminants. A number of methods have been developed for removing them from food and feed. Botanicals such as medicinal plants, herbs and spices have been exploited as antimicrobial agents and for inhibiting the synthesis of various mycotoxins. Extracts and essential oils of plants have been used for inhibiting fungi and

mycotoxins in culture media and food materials as well. The oil extracted from thyme, anise, cinnamon and spearmint was reported to inhibit aflatoxins, ochratoxin A and fumonisins in wheat grains (Soliman and Badeaa 2002). The growth of *Aspergillus flavus* and aflatoxin B1 production were inhibited efficiently by clove and cinnamon in culture media and rice (Aiko and Mehta 2013). Very few studies have been carried out for controlling citrinin contamination. The essential oil of *Zataria multiflora* and extracts of *Azadiracta indica, Cymbopogon citratus, Andrographis paniculata* were reported to inhibit the growth of *P. citrinum* and citrinin synthesis (Mossini and Kemmelmeier 2008; Reddy *et al.* 2010; Noori *et al.* 2012). The components of clove and thyme, i.e., eugenol and thymol, respectively, have been reported to inhibit *P. citrinum* growth and citrinin production as reported by Vazquez *et al.* (2001).

The objective of the present study was to study the efficacy of whole clove in controlling the growth of *P. citrinum* and production of citrinin. The relationship between growth of *P. citrinum* and citrinin production with time in the presence of clove was studied.

MATERIALS AND METHODS

Spice and Fungal Strain Used

Clove (*Syzygium aromaticum*) was purchased from a retail outlet of Vellore, India. The spice was checked and found free from fungal and mycotoxin contamination.

A citrinin-producing strain of *Penicillium* was isolated from the herb *Glycyrrhiza glabra*. The fungus was identified at the Agharkar Research Institute, Pune, India, as *P. citrinum* Sopp 1910 by partial DNA sequence (504 bases) similarity with NCBI sequence accession HQ232482.1.

Effect of Clove on the Growth of *Penicillium citrinum*

Clove was cut into small pieces of approximately 3 mm in mesh size. Various concentrations of clove ranging from 0.2 to 2.0 mg/mL were added to 20 mL yeast extract sucrose (YES) culture broth in 100 mL Erlenmeyer flask and autoclaved at 121C for 15 min. A spore suspension of 10^6/mL in sterile distilled water was prepared by collecting the spores from 7-day-old *P. citrinum* culture. One hundred microliters of the spore suspension was inoculated into the medium and incubated at 28C for 7 days. A control set without clove was maintained under the same condition. The antifungal activity was determined after 7 days of incubation in terms of mycelia biomass (dry mycelium weight). The mycelium in the medium was filtered through Whatman paper, and the dry weight was determined by drying at 60C in hot air oven until weight became constant. The minimum inhibitory concentration (MIC) of clove against *P. citrinum* was determined after 7 days of incubation.

Effect of Clove on Citrinin Production

Citrinin was extracted from the culture broth with equal volume of chloroform (20 mL). The extraction was performed in a separating funnel and shaken vigorously by wrist action movement. The chloroform layer was collected, pooled and analyzed. Qualitative analysis of citrinin was performed by thin layer chromatography (TLC) as given by Golinski and Grabarkiewicz-Szczesna (1984). Ten microliters of the extract along with the standard citrinin was spotted on a TLC plate (Silica gel 60, Merck, Whitehouse, NJ, USA) and developed in a toluene/ethyl acetate/formic acid (6:3:1 v/v/v) solvent system. The plates were then observed in an ultraviolet cabinet at long wavelength for the characteristic yellow fluorescence of citrinin. The amount of citrinin produced was quantified using a UV spectrophotometer (Shimadzu 1800, Kyoto, Japan) by measuring the absorbance at a wavelength of 330 nm. The percentage inhibition of citrinin production was calculated following Tian *et al.* (2011).

$$\text{Inhibition \%} = (1 - \text{mean concentration of citrinin in treatment/mean concentration of citrinin in control}) \times 100$$

Relationship between the Growth of *P. citrinum* and Citrinin Production in the Presence of Clove

The growth of *P. citrinum* and citrinin production in the presence of clove was studied up to 14 days. Clove at 1.6 mg/mL concentration was used to study the relationship between the growth of *P. citrinum* and citrinin production following the procedure mentioned in the Effect of Clove on the Growth of *Penicillium citrinum* section. The flasks were incubated at 27C for 14 days. Every second day, the growth of the fungi was determined by dry mycelium weight, and citrinin production was quantified as mentioned in the Effect of Clove on the Growth of *Penicillium citrinum* and Effect of Clove on Citrinin Production sections. A control set was maintained without the addition of clove under the same condition. The percentage inhibition was calculated as mentioned earlier.

Inhibitory Effect of Clove in Rice

The inhibitory effect of clove against the growth of *P. citrinum* and citrinin production was studied on white

Clove (mg/mL)	DMW (g)	% Inhibition	Citrinin (mg/g)	% Inhibition
0	1.018 ± 0.031[a]	0	10.77 ± 1.03[a]	0
0.2	0.951 ± 0.015[a]	6.48	14.4 ± 4.4[b]	0
0.5	0.928 ± 0.073[a]	8.84	15.82 ± 1.03[b]	0
0.8	0.643 ± 0.166[b]	36.83	9.02 ± 1[a]	16.24
1.6	0.187 ± 0.007[c]	81.63	4.5 ± 0.39[c]	58.22
1.8*	0 ± 0	100	0 ± 0	100
2	0 ± 0	100	0 ± 0	100

TABLE 1. EFFECT OF DIFFERENT CONCENTRATIONS OF CLOVE ON THE GROWTH OF *PENICILLIUM CITRINUM* AND CITRININ PRODUCTION IN YES MEDIUM AFTER 7 DAYS OF INCUBATION

* MIC at 1.8 mg/mL.

Values are mean ($n = 3$) ± SD.

Values followed by different letters in a column are significantly different in one-way ANOVA ($P < 0.05$).

ANOVA, analysis of variance; DMW, dry mycelium weight; MIC, minimum inhibitory concentration; SD, standard deviation; YES, yeast extract sucrose.

polished rice. Twenty grams of rice in 100 mL Erlenmeyer flask was washed with distilled water and completely drained. One hundred milligrams (5 mg/g rice) of clove was added to the washed rice and autoclaved at 121C for 15 min. One hundred microliters of *P. citrinum* spore suspension (10^6 spores/mL) was inoculated and incubated at 28C for 5 days. Moisture content of the rice was maintained at 23% for optimal fungal growth and toxin production. After incubation, citrinin was extracted from 20 g rice culture with 40 mL chloroform by vigorous shaking and then filtered through Whatman paper. The amount of citrinin produced was quantified as described in the Effect of Clove on Citrinin Production section.

Statistical Analysis

All the experiments were carried out in triplicate and repeated to confirm the results. The data were reported as mean ± standard deviation and subjected to analysis of variance to determine the significant difference at $P < 0.05$ level.

RESULTS AND DISCUSSION

The antifungal activity of clove was studied against *P. citrinum* in YES medium. Fungal growth and citrinin synthesis in the presence of clove are given in Table 1. The result showed that all the concentrations of clove had some inhibitory effect on the growth of fungus in a dose-dependent manner. The growth of *P. citrinum* gradually decreased with increasing concentrations of clove and up to 81% inhibition was observed at 1.6 mg/mL. Clove at concentrations from 1.8 mg/mL and above completely inhibited *P. citrinum* growth in culture media. Although the fungal growth was reduced, lower concentrations of clove (0.2 and 0.5 mg/mL) had enhanced the production of citrinin. However, at higher concentrations (>0.5 mg/mL), the synthesis of citrinin was reduced, and complete inhibition was observed at 1.8 mg/mL of clove. Hence, the MIC of clove was recorded as 1.8 mg/mL in YES medium. These results were in confirmation with the study of Bokhari (2007), who reported complete inhibition of *P. citrinum* and citrinin production by clove in the study of antifungal activity of spices. However, the concentration used in his study was much higher (8 mg/mL) than used in the present study. In a study by Reddy *et al.* (2010), aqueous extract of *C. citratus* and *A. paniculata* also showed the inhibition of *P. citrinum* and citrinin production, which increased with increasing concentration of the plant extracts.

At 1.6 mg/mL, clove inhibited fungal growth and citrinin synthesis up to 80 and 58%, respectively. This concentration was further used to study the relationship between growth of *P. citrinum* and citrinin production over a period of time. The observations were taken every 2 days by obtaining the dry weight of the mycelium. The highest growth was noted on the sixth and eighth days of incubation in both control and in the presence of clove. Although the growth curve was similar in both conditions, the presence of clove inhibited the fungal growth significantly ($P < 0.05$). The growth of *P. citrinum* was controlled efficiently on all the days, with 60–70% inhibition from the 4th until the 14th day of incubation as given in Table 2.

The production of citrinin by *P. citrinum* at different days in the presence and absence of clove is illustrated in Fig. 1. In control, citrinin was produced from the second day and reached a maximum on the sixth day, after which a decline in the production was noted. A similar observation was reported by Vazquez *et al.* (2001) where citrinin was produced to a maximum on the 12th day and after which the production declined. In the presence of clove, the concentration of citrinin produced was significantly lesser than the control ($P < 0.05$). The amount of citrinin produced on the second day of observation was equivalent in both control and clove. As the incubation period increased, the production of citrinin was inhibited from the fourth day onward (Table 2). Clove inhibited both the growth and citrinin production

TABLE 2. PERCENT INHIBITION OF *PENICILLIUM CITRINUM* GROWTH AND CITRININ PRODUCTION AT DIFFERENT DAYS BY CLOVE IN YES MEDIUM

Day	Inhibition of mycelia growth %	Inhibition of citrinin synthesis %
2	26.97	0
4	70.22	89.36
6	73.19	87.5
8	70.95	79.99
10	68.27	71.66
12	62.29	64.68
14	64.53	46.53

Concentration of clove used was 1.6 mg/mL.
YES, yeast extract sucrose.

TABLE 3. PRODUCTION OF CITRININ BY *PENICILLIUM CITRINUM* IN THE PRESENCE OF CLOVE IN RICE

Test	Rice (g)	Clove (mg)	Citrinin (μg/g)
Control	20	0	29.5 ± 3.53
Clove	20	100	34 ± 5.65

Values are mean (n = 3) ± SD.
Concentration of clove used was 5 mg/g.
SD, standard deviation.

significantly as given in Fig. 1. A direct correlation between fungal growth and citrinin production was observed. Reduction in fungal biomass resulted in decreased production of citrinin. Kumar *et al.* (2008) also reported a direct correlation in the case of *A. flavus* growth and aflatoxin production in the presence of thyme essential oil. The results obtained suggest that inhibition of *P. citrinum* growth resulted in the subsequent inhibition in citrinin production.

Various substrates such as rice, wheat, corn, etc., have been used for the production of citrinin (Damodaran *et al.* 1973; Jackson and Ciegler 1978). Li *et al.* (2012) have reported the natural occurrence of citrinin in 52% of red yeast rice and related product samples analyzed for citrinin. These food stuff proved to be good substrates for the growth of fungi and mycotoxin production. Hence, the need for an efficient, safe and natural method for controlling these contaminants in food is required. In this experiment, clove was used to study its inhibitory effect on *P. citrinum* and citrinin production in rice. The results showed that the presence of clove in rice delayed the growth of *P. citrinum* for 3 days; however, the fungus started to grow from the fourth day. At the end of the 5-day incubation, fungal growth was equivalent to the control. The amount of citrinin produced in the presence of clove and control, as given in Table 3, did not show inhibition of citrinin production by clove at 5 mg/mL. These results were in contrast with the observations of the authors, where clove inhibited the production of aflatoxin B1 in rice by 99% without inhibiting mycelia growth of *A. flavus* (Aiko and Mehta 2013), indicating that the mechanism of inhibition by clove may vary from organism to organism. The inhibitory effect of clove against *P. citrinum* both in culture media and rice was similar. In both conditions, citrinin production was found to be directly related to the fungal growth, where inhibition of growth ultimately resulted in inhibition of toxin production. Clove at lower concentration, i.e., 1.8 mg/mL, completely inhibited fungal growth and citrinin production in culture media; however in rice, even 5 mg/mL did not exhibit any inhibitory effect. The antifungal property of clove can be attributed to eugenol, which is the main constituent of clove. Eugenol has been used for inhibiting the growth of *A. flavus* and *P. citrinum* and their mycotoxins (Bullerman *et al.* 1977; Vazquez *et al.* 2001; Xing *et al.* 2012). *M. purpureus*, commonly used for the fermentation of red yeast rice, is also a potent citrinin producer (Pattanagul *et al.* 2008). Hence, further studies may be carried out using clove and other common spices against *M. purpureus*.

CONCLUSION

The present study has shown that clove inhibits the growth of *P. citrinum* and citrinin production efficiently in culture medium. A direct correlation between the growth of *P. citrinum* and citrinin production in the presence of clove was noted. In rice, clove inhibited the fungal growth as well as citrinin production up to 3 days. The inhibitory effect of clove in rice is significantly important because of citrinin contamination in fermented rice products. Spices are commonly used as preservatives, medicine or as flavoring and antimicrobial agent. The antifungal property of spices may

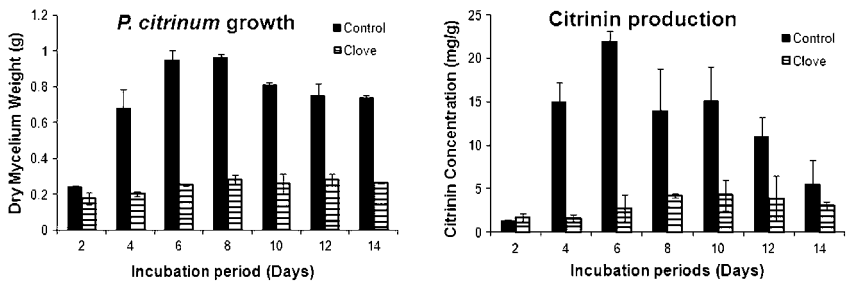

FIG. 1. GROWTH OF *PENICILLIUM CITRINUM* AND CITRININ PRODUCTION IN THE PRESENCE OF CLOVE IN YEAST EXTRACT SUCROSE MEDIUM AT DIFFERENT DAYS

be applied to food and feed during the various stages of processing as a measure for quality control.

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REFERENCES

AIKO, V. and MEHTA, A. 2013. Control of *Aspergillus flavus* and aflatoxin production with spices in culture medium and rice. World Mycotoxin J. *6*(1), 43–50.

BILGRAMI, K.S., SINHA, S.P. and JESWAL, P. 1988. Nephrotoxic and hepatoxic effects of citrinin in mice (*Mus musculus*). Proc. Indian Natl. Sci. Acad. *B54*, 35–37.

BLANC, P.J., LORET, M.O. and GOMA, G. 1995. Production of citrinin by various species of *Monascus*. Biotechnol. Lett. *17*(3), 291–294.

BOKHARI, F.M. 2007. Spices mycobiota and mycotoxins available in Saudi Arabia and their abilities to inhibit growth of some toxigenic fungi. Mycobiology *35*(2), 47–53.

BULLERMAN, L.B., LIEU, F.Y. and SEIER, S.A. 1977. Inhibition of growth and aflatoxin production by cinnamon and clove oil, cinnamic aldehyde and eugenol. J. Food Sci. *42*(6), 1107–1109.

DAMODARAN, C., RAMADOSS, C.S. and SHANMUGASUNDARAM, E.R.B. 1973. A rapid procedure for the isolation, identification and estimation of citrinin. Anal. Biochem. *52*, 482–488.

EUROPEAN FOOD SAFETY AUTHORITY (EFSA). 2012. EFSA Panel on Contaminants in the Food Chain (CONTAM); Scientific opinion on the risks for public and animal health related to the presence of citrinin in food and feed. EFSA J. *10*(3), 2605.

GOLINSKI, P. and GRABARKIEWICZ-SZCZESNA, J. 1984. Chemical confirmatory tests for ochratoxin A, citrinin, penicillic acid, sterigmatocyctin, and zearalenone performed directly on thin layer chromatographic plates. J. AOAC Int. *67*(6), 1108–1110.

GORDON, R.Y., COOPERMAN, T., OBERMEYER, W. and BECKER, D.J. 2010. Marked variability of monacolin levels in commercial red yeast rice products: Buyers beware! Arch. Intern. Med. *170*(19), 1722–1727.

HETHERINGTON, A.C. and RAISTRICK, H. 1931. Studies in biochemistry of microorganism XI. On the production and chemical constitution of a new yellow colouring matter, citrinin, produced from glucose by *Penicillium citrinum* Thom. Philos. Trans. R. Soc. Lond. B. Biol Sci. *220*, 269–297.

JACKSON, L.K. and CIEGLER, A. 1978. Production and analysis of citrinin in corn. Appl. Environ. Microbiol. *36*(3), 408–411.

KUMAR, A., SHUKLA, R., SINGH, P., PRASAD, C.S. and DUBEY, N.K. 2008. Assessment of *Thymus vulgaris* L. essential oil as a safe botanical preservative against post harvest fungal infestation of food commodities. Innov. Food Sci. Emerg. Technol. *9*, 575–580.

KURATA, M. 1990. Mycotoxins and mycotoxicoses. In *Microbial toxins in foods and feeds* (A.C. Pohland, V.R. Dowell and J.L. Richards, eds.) pp. 249–259, Plenum Press, New York, NY.

LI, Y., ZHOU, Y.C., YANG, M.H. and OU-YANG, Z. 2012. Natural occurrence of citrinin in widely consumed traditional Chinese food red yeast rice, medicinal plants and related products. Food Chem. *132*(2), 1040–1045.

MARTINS, M.L., GIMENO, A., MARTINS, H.M. and BERNARDO, F. 2002. Co-occurrence of patulin and citrinin in Portuguese apples with rotten spots. Food Addit. Contam. *19*(6), 568–574.

MOSSINI, S.A.G. and KEMMELMEIER, C. 2008. Inhibition of citrinin production in *Penicillium citrinum* cultures by neem [*Azadirachta indica* A. Juss (Meliaceae)]. Int. J. Mol. Sci. *9*, 1676–1684.

NOORI, N., YAHYARAEYAT, R., KHOSRAVI, A., ATEFI, P., BASTI, A.A., AKRAMI, F., BAHONAR, A. and MISAGHI, A. 2012. Effect of *Zataria multiflora* Boiss. essential oil on growth and citrinin production by *Penicillium citrinum* in culture media and mozzarella cheese. J. Food Saf. *32*(4), 445–451.

PATTANAGUL, P., PINTHONG, R., PHIANMONGKHOL, A. and THARATHA, S. 2008. Mevinolin, citrinin and pigments of adlay angkak fermented by *Monascus* sp. Int. J. Food Microbiol. *126*, 20–23.

REDDY, K.R.N., NURIJATI, S.B. and SALLEH, B. 2010. Efficacy of aqueous medicinal plant extracts on growth and citrinin production by *Penicillium citrinum* isolated from rice grains. Afr. J. Microbiol. Res. *4*(23), 2562–2565.

SOLIMAN, K.M. and BADEAA, R.I. 2002. Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. Food Chem. Toxicol. *40*, 1669–1675.

TIAN, J., HUANG, B., LUO, Z., ZENG, H., BAN, X., HE, J. and WANG, Y. 2011. The control of *Aspergillus flavus* with *Cinnamomum jensenianum* Hand-Mazz essential oil and its potential use as food preservative. Food Chem. *130*(3), 520–527.

VAZQUEZ, B.I., FENTE, C., FRANCO, C.M., VAZQUEZ, M.J. and CEPEDA, A. 2001. Inhibitory effects of eugenol and thymol on *Penicillium citrinum* strains in culture media and cheese. Int. J. Food Microbiol. *67*, 157–163.

VRABCHEVA, T., USLEBER, E., DIETRICH, R. and MARTLBAUER, E. 2000. Co-occurrence of ochratoxin A and citrinin in cereals from Bulgarian villages with a history of Balkan endemic nephropathy. J. Agric. Food Chem. *48*(6), 2483–2488.

XING, Y., XU, Q., LI, X., CHE, Z. and YUN, J. 2012. Antifungal activities of clove oil against *Rhizopus nigricans, Aspergillus flavus* and *Penicillium citrinum* in vitro and in wounded fruit test. J. Food Saf. *32*(1), 84–93.

ZAIED, C., ZOUAOUI, N., BACHA, H. and ABID, S. 2012. Natural occurrence of citrinin in Tunisian wheat grains. Food Control *28*(1), 106–109.