British Microbiology Research Journal
2(2): 108-122, 2012

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Review on Citrinin: Synthetic Methods, Molecular Biosynthesis and Effect of Plant Extracts

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Review Article

Received 6th March 2012
Accepted 22nd May 2012
Online Ready 19th June 2012

ABSTRACT

Introduction: Citrinin is a mycotoxin originally isolated from *Penicillium citrinum*. It has been found to be produced by a variety of other fungi (*Aspergillus niveus, Aspergillus ochraceus*, *Aspergillus oryzae*, *Aspergillus terreus*, *Monascus ruber*, *Monascus purpureus* and *Penicillium camemberti*) which are found or used in the production of human foods (Abramson et al., 1999). The inhibitory effect of plant extracts on citrinin biosynthesis have been examined (Mossini and Kemmelmeier, 2008; Reddy et al., 2010). They found that all the tested plant extracts reduced the citrinin production. Shimizu et al. (2005, 2007) found that the *pksCT* gene was essential for citrinin biosynthesis in *M. purpureus.* Also Sakai et al. (2008) reported that introducing additional copies of an activator gene (ctnA), controlled by the *Aspergillus nidulans* trpC promoter, into the citrinin-cluster-containing transformants enhanced the transcription of all the genes in the cluster and resulted in an almost 400-fold higher citrinin production compared to that of the parental transformant.
Aims: To give idea on physicochemical properties of citrinin, its production, effects of some plant extracts on it and gene involved in citrinin biosynthesis.
Study Design: Review study.
Place and Duration of Study: Department of Biology, Faculty of Science, Taif- Saudi Arabia and Department of Botany, Faculty of Science, Tanta-Egypt. 2011-2012.
Methodology: Citrinin was produced in liquid potato-dextrose medium (PD) or in glucose medium. The citrinin was extracted three times with chloroform (1:1 v/v), pooled and concentrated in vacuo at 40ºC using a rotary evaporator. The crude extract was diluted in minimum amount of chloroform (2 ml) and citrinin was estimated by thin layer chromatography (TLC). Effects of some plant extracts like neem leaf extract and some medicinal plants were determined.
Conclusion: This review was written with the aim of demonstrating the scope of citrinin production, various analytical techniques in citrinin detection and estimation and effects of

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some plant extracts and genes on citrinin biosynthesis. It was found that plant extracts can be used as a potential source of sustainable ecofriendly botanical fungicides to protect food grains from toxigenic *P. citrinum* and citrinin accumulation under storage conditions.

Keywords: Citrinin; penicillium; aspergillus; monascus; plant extract; genes.

1. INTRODUCTION

Mycotoxins are a group of structurally diverse secondary metabolites produced by various fungal species. Citrinin is a mycotoxin originally isolated from *Penicillium citrinum*. It has been found to be produced by a variety of other fungi (*Aspergillus niveus, Aspergillus ochraceus*, *Aspergillus oryzae*, *Aspergillus terreus*, *Monascus ruber*, *Monascus purpureus* and *Penicillium camemberti*) which are found or used in the production of human foods (Abramson et al., 1999). As a mycotoxin, citrinin possesses antibiotic, bacteriostatic, antifungal and antiprotozoal properties. It is also known as a hepato-nephrotoxin in a wide range of species (Hanika et al., 1983; Bilgrami et al., 1988; Berndt, 1990). Contaminations of citrinin were reported in a number of agricultural commodities, foods, fruits and juices (Abramson et al.,1999; Allah and Ezzat, 2005; Bailly et al., 2002; CAST, 2003; Comerio et al., 1998; Gimeno and Martins, 1983; Gordon et al., 2010; Heber et al., 2001; Jackson and Ciegler, 1978; Kpodo et al., 1995; Martins et al., 2002; Meister, 2004; Nguyen et al., 2007; Reddy et al., 2010; Tabata et al., 2008; Tangni and Pussemier, 2006; Xu et al., 2006).

The inhibitory effect of plant extracts on citrinin biosynthesis have been examined (Mossini and Kemmelmeier, 2008; Reddy et al., 2010). They found that all the tested plant extracts reduced the citrinin production.

During biosynthesis of secondary metabolites like citrinin, many biosynthetic enzymes are required and should function coordinately in the synthesis of these structurally complex metabolites, many of which are polyketides. Polyketides, such as pigments and mycotoxins, are structurally diverse and often complex compounds that are at least partially synthesized by multifunctional enzymes called polyketide synthases (PKSs). The genes encoding these enzymes have often been reported to localize in an adjacent region or to form a gene cluster (Brown et al., 1999, Kennedy et al., 1999), similar to the situation of biosynthetic gene clusters for secondary metabolites in prokaryotic actinomycetes. Shimizu et al. (2005, 2007) found that the *pksCT* gene was essential for citrinin biosynthesis in *M. purpureus.* Also Sakai et al. (2008) reported that introducing additional copies of an activator gene (ctnA), controlled by the *Aspergillus nidulans* trpC promoter, into the citrinin-cluster-containing transformants enhanced the transcription of all the genes in the cluster and resulted in an almost 400-fold higher citrinin production compared to that of the parental transformant.

2. PHYSICOCHEMICAL PROPERTIES OF CITRININ

Citrinin [$C_{13}H_{14}O_5$, IUPAC: (3R, 4S)-4, 6-dihydro-8-hydroxy-3, 4, 5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid; CAS No.: 518-75-2] (Fig. 1), is an acidiclemon-yellow crystal with maximal UV absorption at 250 nm and 333 nm (in methanol), melting at 172ºC (Xu et al., 2006). It is sparingly soluble in water but it is very soluble in organic and inorganic solvents (Deshpande, 2002).

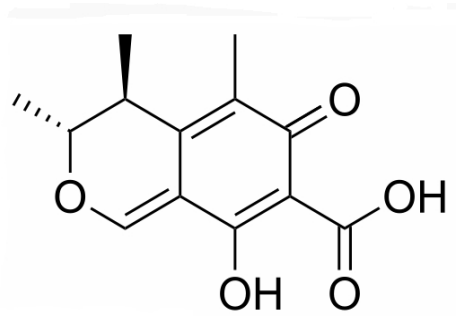

Fig. 1. Structure of citrinin (Deshpande, 2002)

Jackson and Ciegler (1978) reported that heat degrades citrinin, but toxicity is retained in a fraction that is chloroform soluble and $NaHCO_3$ insoluble. This would agree with findings by Chu (1946) that antibiotic potency is retained after autoclaving. The explanation for the appearance of the unknown toxin during heating is that the toxin is a degradation product of citrinin. Because the toxin was not removed by $NaHCO_3$ like citrinin, it seemed probable that the heat had caused decarboxylation of the citrinin to a compound such as decarboxycitrinin (Fig. 2a), which is a natural metabolite of *P. citrinum* (Curtis et al., 1968) or decarboxydihydrocitrinin (Fig. 2b).

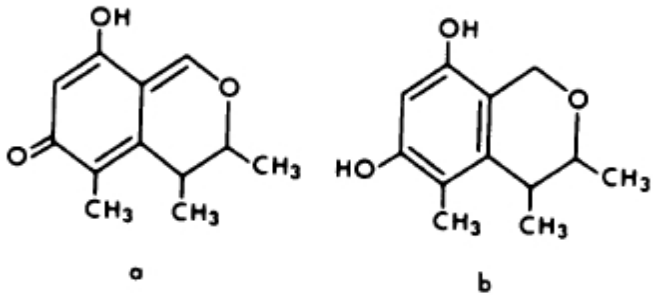

Fig. 2. (a) Decarboxycitrinin; (b) decarboxydihydrocitrinin (Jackson and Ciegler, 1978)

Citrinin is a quinine methide with two intramolecular hydrogen bonds (Xu et al., 2006). Citrinin crystallizes in a disordered structure, with the p-quinone and o-quinone two tautomeric forms in a dynamic equilibrium in the solid state (Fig. 3).

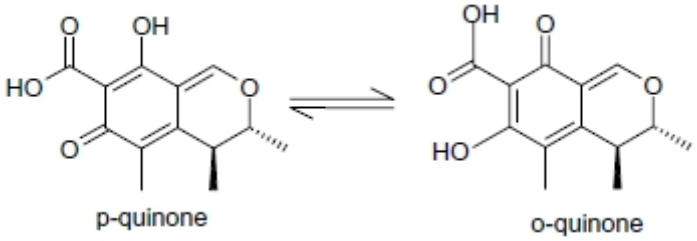

Fig. 3. Structural formula of citrinin isomers (Xu et al., 2006)

Yao et al. (2011) found that investigation of a microbial fermentation organic extract of *Penicillium* sp. H9318 led to the isolation of a new isoquinolinone alkaloid, (5*S*)-3,4,5,7-tetramethyl-5, 8-dihydroxyl-6(5*H*)- isoquinolinone (1) along with four known citrinin derivatives (2-5) (Fig. 4). Citrinin (2) exhibited significant inhibitory activity against *Streptomyces* 85E in the hyphae formation inhibition (HFI) assay, while compounds 1, 3-5 were not active when tested at 20 mg/disk in the HFI assay.

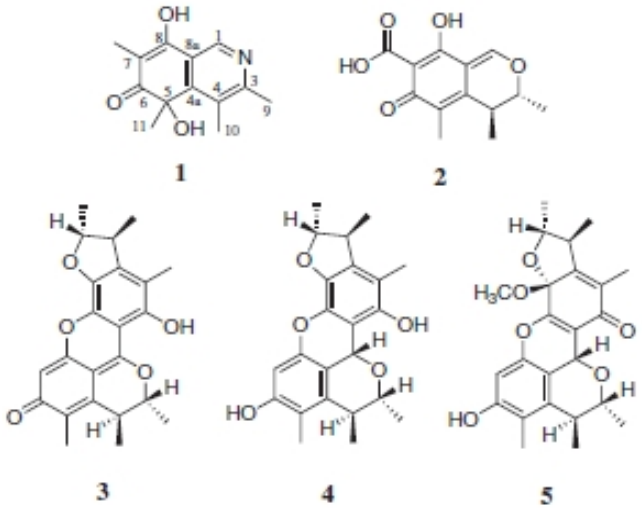

Fig. 4. Isoquinocitrinin A (1) and four known compounds, citrinin (2), penicitrone A (3), penicitrinols A (4) and B (5) (Yao et al., 2011)

3. SYNTHESIS OF CITRININ

3.1 Conditions for Citrinin Culturing

Citrinin was produced by *Penicillium citrinum* in liquid potato-dextrose medium (PD) as described by Mossini and Kemmelmeier (2008). Inocula containing 10^5 spores of each citrinin producing isolates of *Penicillium citrinum* were added to PD medium (3 mL) in 25 mL flasks (20x100mm) and incubated at 26±0.5ºC for 21 days. Citrinin was extracted from cultures three times with 10 ml of chloroform, treated with anhydrous sodium sulfate, filtered and evaporated to dryness (Betina, 1984). Residues were dissolved in 0.1 mL chloroform.

Also Hajjaj et al. (1997) described the production of citrinin as follows: A strain of *Monascus ruber* (ATCC 96218) was grown at 28ºC in a chemically defined medium. A suspension of 10^8 spores was used to inoculate a 1-liter baffled Erlenmeyer flask containing 200 ml of glucose medium. The presence of red pigments and citrinin in the culture broth was determined spectrophotometrically. For the ^{13}C labeling of citrinin, 1 ml of an aqueous solution (99.2% enriched with [1-^{13}C] [2-^{13}C] sodium acetate [20 mg·ml^{-1}] or 98.6% enriched with [1, 2-^{13}C] sodium acetate [10 mg · ml^{-1}]) was added after 3, 4, 5, 6, and 7 days to a 200-ml culture.

3.2 Detection of Citrinin

Citrinin was isolated from the medium by filtration of the mycelium cultures of *Monascus ruber* on M14 membranes (0.8 µm porosity). The filtrate was lyophilized, resuspended in 60 ml of water, and extracted three times with water saturated with *n*-butanol. The organic phase was dried and vacuum concentrated, and the residue was dissolved in 50 ml of acidified water (pH 2.0). This solution was treated twice with 120 ml of ethyl acetate, and the retained organic phase was extracted twice with 150 ml of 0.4% $NaHCO_3$. The aqueous phase was adjusted to a pH of 3.0 with HCl and again extracted twice with 120 ml of ethyl acetate. The organic phase containing citrinin was evaporated to dryness and resuspended in a minimal volume of water. The toxin was isolated by thin-layer chromatography in chloroform-methanol-water (65/25/4, vol/vol/vol). The band containing the toxin was solubilized in chloroform which was evaporated. Spectra were referenced internally to the solvent for ^{13}C NMR and to trimethylsilyl for ^{1}H NMR (Fig. 5) (Hajjaj et al., 1999).

It was found that the production of citrinin started after 45 h of cultivation, the citirinin concentration was 6.5 µg/ml.

The kinetics of pigment and citrinin production during the growth of *M. ruber* were studied by Hajjaj et al. (1999). After a lag of about 20 h, cell biomass and red pigments increased in parallel to reach 2.3 g (dry weight)/liter and 112 mg/liter, respectively, at the time of glucose exhaustion. The maximal rate of pigment production was observed 20 to 60 h after the initiation of fermentation. The uncoupling in the production of these two polyketide derivatives suggested that they might not follow the same metabolic pathway.

Hajjaj et al. (1999) suggested that the precursor for citrinin formation is a tetraketide arising from the condensation of one acetyl-CoA molecule with three malonyl-CoA molecules instead of a pentaketide (one acetyl-CoA molecule and four malonyl-CoA molecules). Then, an additional acetyl-CoA molecule is added to the tetrakedite to form intermediate 1 (Fig. 6). Furthermore, one cannot exclude the possibility that a malonyl-CoA molecule condenses to the tetraketide and that this is accompanied by a decarboxylation. These experiments confirmed that citrinin arose from the polyketide pathway, by a route apparently similar to that found in *P. citrinum* and *A. terreus* (Barber, Staunton 1980, Sankawa et al., 1983). The occurrence of a tetraketide as a precursor for both citrinin and red pigments may account for the differential production of these two polyketides during the growth of *M. ruber* (Fig. 6).

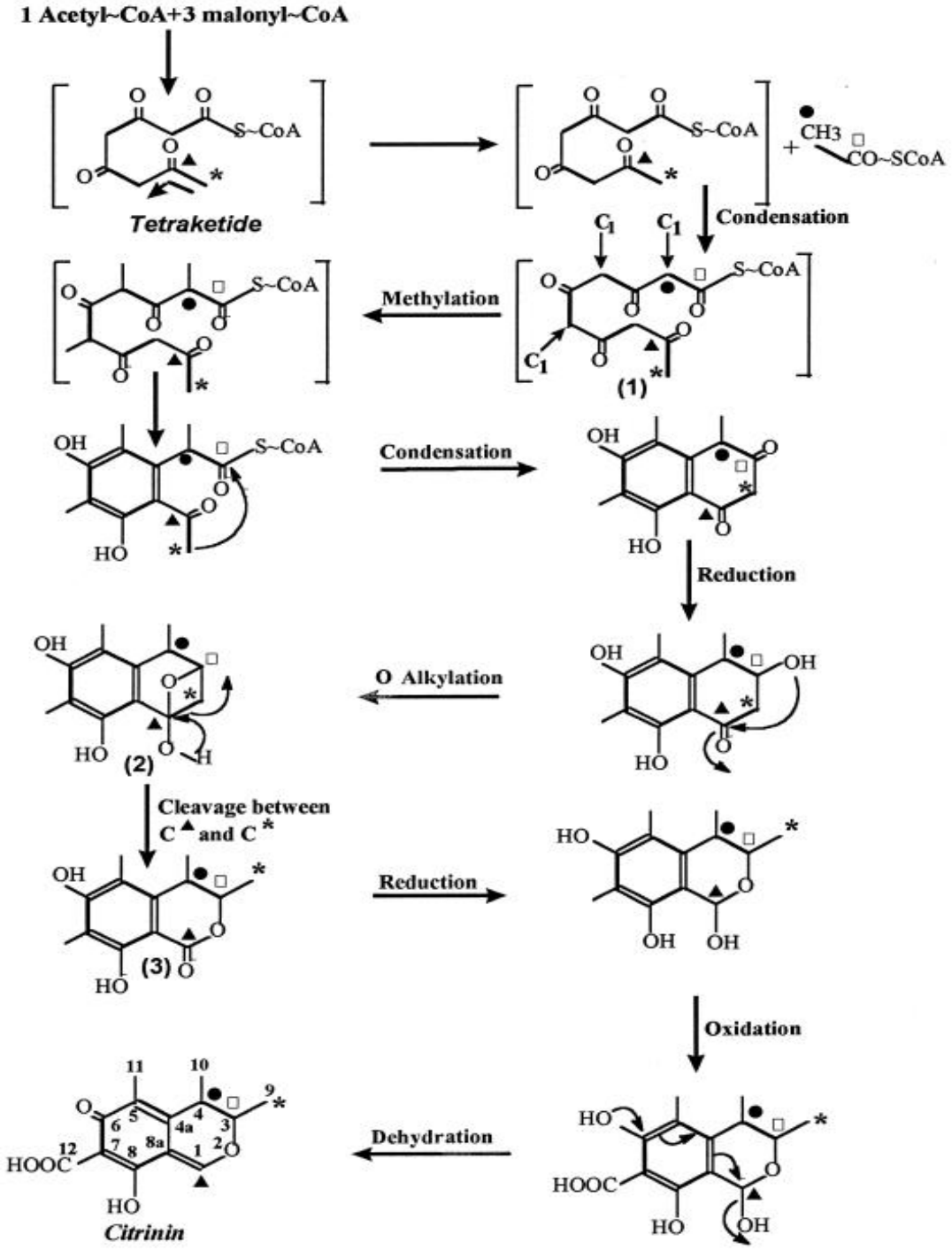

Fig. 5. Scheme of the biosynthesis of citrinin by *M. ruber*. The start of the condensation reaction is indicated by the bent arrow in the upper left panel. Intermediates are numbered. Enrichment of C-1 (▲), C-3 (□), C-9 (*), and C-4 (•) is indicated (Hajjaj et al., 1999).

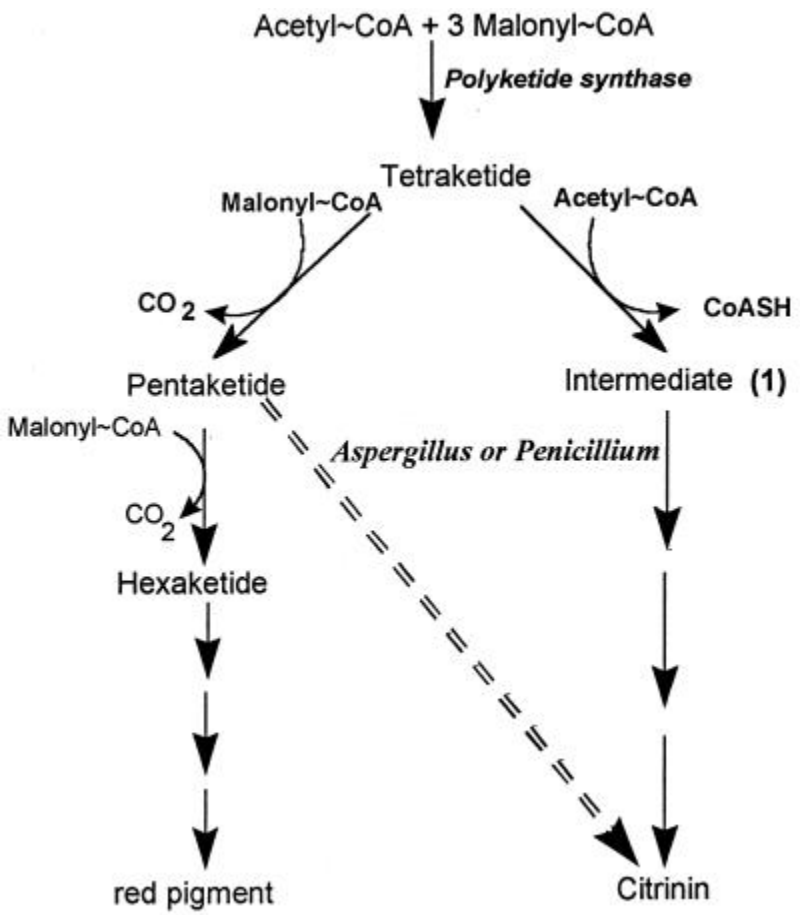

Fig. 6. Biosynthesis of citrinin and red pigment in *M. ruber*. The toxin pathway in *Aspergillus* and *Penicillium* is indicated by the dashed arrow (Hajjaj et al., 1999).

Mossini and Kemmelmeier (2008) analyzed the citrinin as follows: Dried extract of *Azadirachta indica* leaves was dissolved in chloroform. The extract then underwent thin layer chromatography (TLC) on 20 x 20 cm Aluminium plates, with toluene-ethyl acetate-formic acid (6:4:0.5, v/v). After development the plates were exposed to 365 nm ultraviolet light (UV). Citrinin appeared as a fluorescent yellow spot. The phenolic group in citrinin, estimated by Folin-Phenol reagent (Amandioha, 2000), gave a linear relationship with concentration over the 5-100 µg/mL range. HPLC analysis was performed to confirm spectrophotometric results. Residues were dissolved in an appropriate volume of mobile phase (1 mL for all samples), filtered through a 0.45 µm disposable syringe filter prior to injection into the chromatograph. Aliquots (20 µL) were injected on HPLC column and analyzed. Comparison of sample retention times with that of the standard identified the presence of citrinin in the samples. The relationships between peak height and area and the amount injected were linear over the ranges 2.5-50 ng. The citrinin production by the three isolates (K1, K4 and k8) of *P. citrinum* was 2.17×10^{-2}, 4.25×10^{-2} and 6.3×10^{-2} µg respectively as measured spectrophotometrically.

Also Reddy et al., (2010), extracted and determined citrinin as follows:

The culture filtrates (10 ml) of *P. citrinum*, isolated from rice grain, was used for extraction and estimation of citrinin. The citrinin was extracted three times with chloroform (1:1 v/v), pooled and concentrated in vacuo at 40ºC using a rotary evaporator. The crude extract was diluted in minimum amount of chloroform (2 ml) and citrinin was estimated by TLC according to Razak et al., (2009) with minor modifications. Briefly, different volumes (1 to 5 µl) of sample extracts were applied to precoated TLC plates (TLC Silica gel 60 F254, Merck, Germany) along with standard (containing citrinin at 0.5 µg/ml). The plates were developed in toluene/ethyl acetate/formic acid (6:4:0.5 v/v) in glass tanks covered with aluminum foil. After development, the plates were dried and observed under long wavelength (365 nm). Citrinin appears as a fluorescent yellow spot. The intensity of the sample spots was compared with that of the standard spot. The citrinin concentration was 9.2 µg/ml.

3.3 Effect of Selected Plant Extracts on Citrinin Production

3.3.1 Neem leaf extract (NLE)

Dried leaves of *Azadirachta indica* A. Juss (Meliaceae) were extracted by maceration in distilled water (100 g/L) and stirred for five hours in the dark, at room temperature. At the end of the extraction, the material was sieved through Whatman 1 filter paper, freeze-dried, and preserved in dark flasks. A 10% aqueous extract of the residue was prepared and used (Mossini et al., 2004). Treatments in four replicates consisted of 10% freeze-dried aqueous neem leaf extract (NLE) at concentrations 3.12, 6.25, 12.5, 25 and 50 mg/mL added to the PD, before autoclaving and inoculation (Mossini and Kemmelmeier 2008). After 21 days, quantitative determination of the extracts from liquid culture media demonstrated inhibition of citrinin production by three isolates of *P. citrinum* on media with NLE. Neem extracts of 3.12 mg/mL reached 87.16% inhibition on K4 and 85.86% inhibition on K1 and 94.86% inhibition on K8 citrinin production at NLE 6.25 mg/mL (Fig. 7).

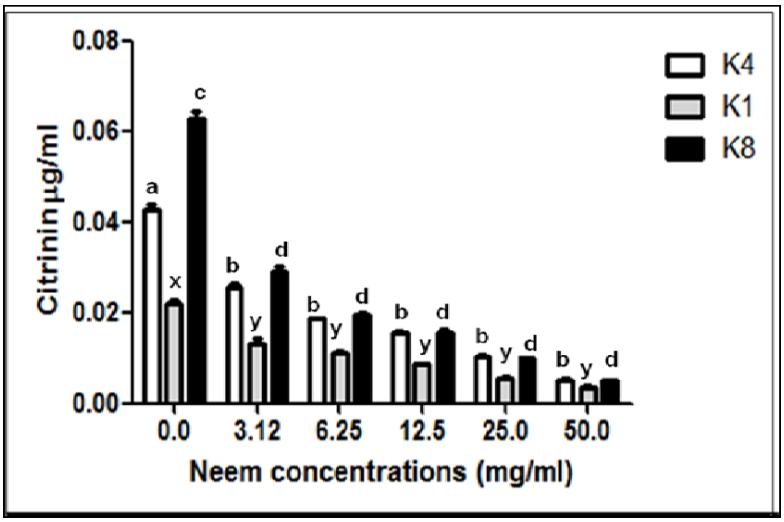

Fig. 7. Citrinin production from *P. citrinum* isolates determined by spectrophotometric assay. Bars indicate standard deviation for experiments carried out in four replicates. Different small letters over the columns indicate statistically significant differences ($p<0.05$) for isolate K4 (a, b), K1 (x, y) and K8 (c, d) (Mossini and Kemmelmeier, 2008).

3.3.2 Aqueous extracts of five medicinal plants

Leaves of five medicinal plants (*Andrographis paniculata, Cymbopogon citratus, Eurycoma longifolia, Kaempferia galanga* and *Orthosiphon aristatus*) were collected and washed under tap water. The leaves were dried in hot air oven at 60ºC for 4 days and fine ground to fit in 20 mesh sieve. Ten grams of ground powder were shaken in 100 ml distilled water at 200 rpm for 5 h at room temperature (Razak et al., 2009). The insoluble material was filtered by Whatman No.1 filter paper and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and passed through 0.22 µm membrane filter and stored at -20ºC until use. Various concentrations (2.5, 5.0, 7.5 and 10.0 mg/ml) of aqueous plant extracts were added to cooled liquid broth. A 10 µl volume of suspension containing 10^5 spore/ml of fungus was inoculated in each flask and shaken at 200 rpm for 10 days at 25 ± 2ºC. The control contained PDB broth and 10 µl of fungal suspension (Reddy et al., 2010). It was found that all plant extracts effectively reduced the citrinin production ranging from 42.3 to 91.3% at 10 mg/ml concentration in liquid media. Among the plant extracts tested, *C. citrates* effectively inhibited the citrinin production by *P. citrinum* ranging from 22.8 to 91.3% followed by *A. paniculata* ranging from 8.6 to 83.6% at all concentrations tested. Other plant extracts showed less reduction ranging from 42.3 to 54.3% even at higher concentration (10 mg/ml).

3.4 Gene Involved in Citrinin Biosynthesis

Shimizu et al. (2005) cloned a polyketide synthase (PKS) gene for citrinin (CT) (pksCT) from *M. purpureus*. They reported that PCR with primer pair KS and LC5c (Fig. 8) yielded a single distinct product, and the PCR product (430 bp) was very close to the expected size, 420 bp, calculated from the sequences of other fungal PKSs. The deduced amino acid sequence was 49%, similar to the aflatoxin biosynthetic PKS from *A. parasiticus* and 43% similar to the bikaverin biosynthetic PKS from *G. fujikuroi*. These similarities suggested that the PCR fragment was part of a PKS gene responsible for the biosynthesis of multi-aromatic-ring polyketides.

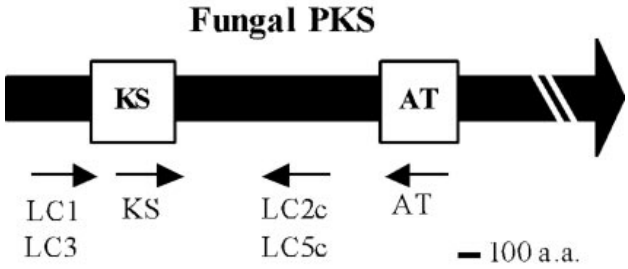

Fig. 8. Primer positions in the flanking regions of KS and AT domains of a consensus fungal PKS. The boldface arrow and open boxes show the fungal PKS gene and regions encoding the KS and AT domains, respectively. The small arrows indicate the primers' positions and directions (Shimizu et al., 2005).

The complete PKS gene (7,838 bp; DDBJ accession no. AB167465) encoding a 2,593-amino-acid protein (Fig. 9) was obtained from the *M. purpureus* genome by three rounds of colony hybridization by using probe A to identify a BamHI (1)-BamHI (2) fragment and an EcoRI-SalI fragment and probe B to clone a KpnI (2)-KpnI (3) fragment.

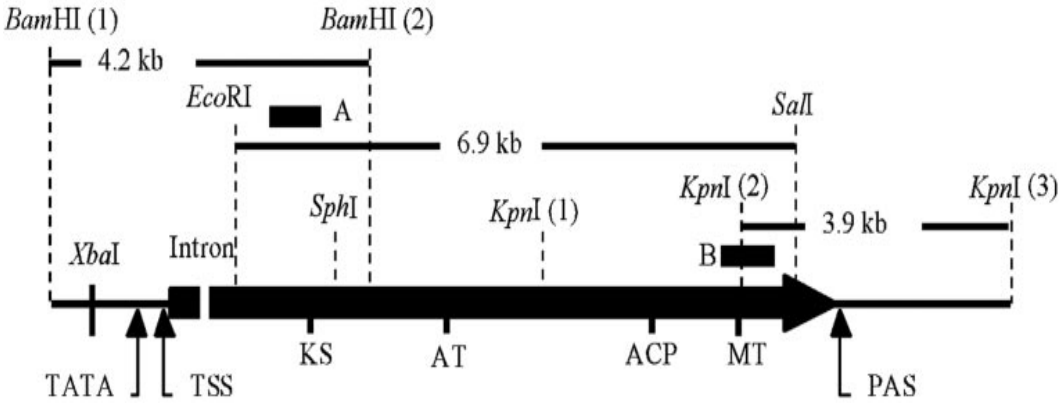

Fig. 9. Restriction map of the cloned 12.9-kb BamHI (1)-KpnI (3) fragment and structure of the cloned PKS. The black boxes indicate the locations of probes A and B used to obtain the BamHI (1)-BamHI (2) and EcoRI-SalI fragments and the KpnI (2) KpnI (3) fragment, respectively, by colony hybridization. The thick black arrow indicates the deduced open reading frame for pksCT in the cloned DNA sequence. The small arrows indicate the TATA box, the transcriptional start site (TSS), and polyadenylation site (PAS), respectively (Shimizu et al., 2005).

Shimizu et al., (2007) cloned the genes in the vicinity of pksCT to obtain new genes involved in CT biosynthesis. An activator gene essential for the efficient production of CT was found in the upstream region of pksCT, and they demonstrated that an extremely low-CT producer can be created by disrupting the gene.

The transcription patterns of the five ORFs were examined by RT-PCR with RNA samples prepared from mycelia cultivated for 2, 4, or 6 days under CT production conditions. pksCT was transcribed from the 2-day cultivation, at which point CT production started in the wild-type strain (Fig. 10A). Similarly, the transcripts of the four other plausible genes (orf2, encoding a regulator; *orf3*, encoding an oxygenase; orf4, encoding an oxidoreductase and orf5, encoding a transporter) were detected from the 2-day cultivation, whereas no transcription of orf1 was detected. Northern blot analysis (Fig. 10B) indicated that the orf2 product is essential for activation of of pksCT transcription (Shimizu et al., 2007).

Shimizu et al. (2007) suggested that, as in the case of AflR in the aflatoxin biosynthetic cluster (Ehrlich et al., 1999), the orf2 product (designated CtnA for being the first factor related to CT biosynthesis other than PksCT) acts as an activator at least on pksCT and (orf5*)* transcription. This occurs probably through binding to specific DNA sequences in the upstream region of each of the two genes, although no definite conserved sequence was discovered in the promoter regions of pksCT and orf5.

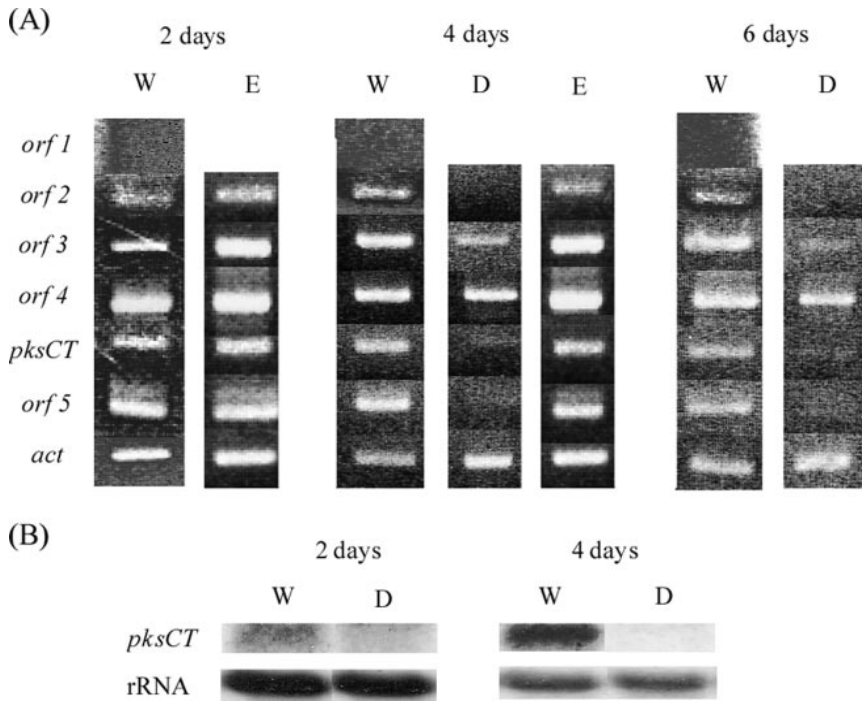

Fig. 10. Transcriptional analysis of putative CT biosynthetic genes. (A) RT-PCR was performed against RNA samples extracted from mycelia harvested from MC liquid medium after the indicated period of cultivation. (B) Northern blot analysis of the pksCT gene. The fragment amplified by RT-PCR, with the primers pksCT F and pksCT R used as a probe. W, wild-type strain; D, ctnA disruptant; E, ctnA-complemented ctnA disruptant; *act* (encoding actin), control (Shimizu et al., 2007).

Sakai et al. (2008) found that in the region flanking pksCT, there are four more ORFs in addition to ctnA. The estimated gene products of these ORFs showed high similarity to a dehydrogenase (orf1), an oxygenase (orf3), an oxidoreductase (orf4), and a transporter (orf5) respectively (Fig. 11A). Therefore, all four genes, including pksCT and ctnA, appear to constitute a CT biosynthetic gene cluster. Because the putative CT biosynthetic cluster is relatively small (around 20 kb) it provided a suitable cosmid insert.

Characterization of *A. oryzae* transformants with CossCT containing the CT biosynthetic gene cluster was carried out as follows: After a partial BamHI digestion, the fragment containing the 20-kb CT biosynthetic cluster with sufficient flanking regions (4 kb on one side and 9 kb on the other) was recovered from SuperCos CT-1 and transferred into the CossC *Aspergillus-E. coli* shuttle vector creating CossCT. CossCT was then transformed into the heterologous host *A. oryzae* NS4. The transformants were selected on sC medium and their genotypes were confirmed by Southern blot using part of orf4 as a probe (Fig. 11A, B). Sakai et al. (2008) reported that the two transformants (strains 1-1 and 16-2) contained the entire putative CT biosynthetic gene cluster, although strain 16-2 displayed an additional band with a lower molecular weight than the expected size of 16.1 kb. This band likely arose from a truncated form of the cluster (Fig. 11B).

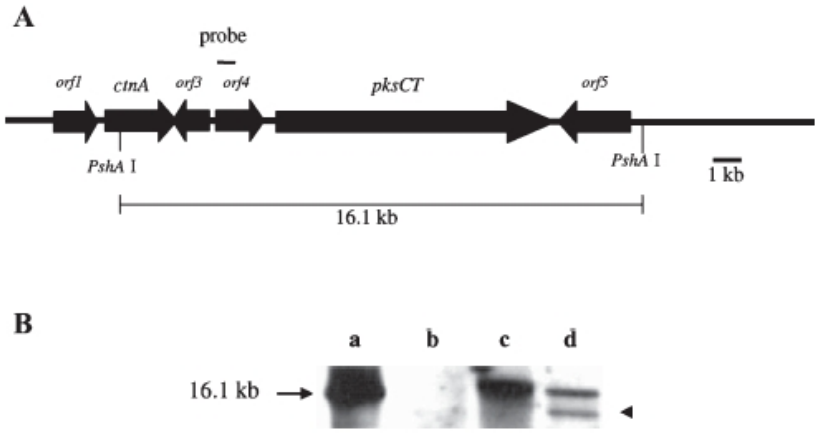

Fig. 11 (A). Organization of the putative CT biosynthetic gene cluster in *M. purpureus*: orfl encoding the putative dehydrogenase; ctnA encoding a positive regulator; orf3 encoding a plausible oxygenase; orf4 encoding a plausible oxidoreductase; pksCT encoding CT polyketide synthase; orf5 encoding a plausible transporter. The bar above orf4 indicates the position of the probe used in panel B. (B) Southern blot analysis of the transformants. Southern blot analysis was carried out against PshAI-digested genomic DNA using the PCR fragment (orf4-f and orf4-r primers) from orf4 as the probe. The arrow indicates the position of a 16.1-kb band corresponding to the PshAI-fragment. An arrowhead indicates the position of a 13.5-kb band corresponding to an apparently truncated cluster in lane d. Lanes: a, CossCT vector; b, *A. oryzae* NS4; c, strain 1-1; d, strain 16-2 (Sakai et al., 2008).

4. CONCLUSION

TLC and HPLC are the most widely used techniques for citrinin characterization. Filamentous fungi are considered promising resources for the development of novel bioactive compounds because of their great potential to produce various kinds of secondary metabolites. This concept is supported by the fact that genome sequencing has revealed the presence of 40 to 50 putative biosynthetic gene clusters in the fungal genome. It was found that *A. oryzae* transformants containing only the CT cluster produced minimal quantities of CT, but introducing an additional activator gene (ctnA) enhanced the transcriptional level of each biosynthetic gene in the cluster, elevating CT production more than 400-fold. The occurrence of a tetraketide as the precursor for both citrinin and red pigments may account for the differential production of these two polyketides during the growth of *M. ruber*.

The identification of an activator and an apparent CT biosynthetic gene cluster in *Monascus* may aid in the identification of corresponding gene clusters in different fungal species and in the development of strategies for suppressing CT production through the application of detailed knowledge of the regulation of CT biosynthesis.

The aqueous extracts obtained from medicinal plants were effective in reducing growth and citrinin production by *P. citrinum* under *in vitro* conditions in liquid media. So, these plant extracts can be used as a potential source of sustainable ecofriendly botanical fungicides to protect food grains from toxigenic *P. citrinum* and citrinin accumulation under storage conditions.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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