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Growth and Production of Citrinin by *Penicillium viridicatum* Westling and their Control by Propolis

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In the current study, the possible role of environmental conditions (incubation period, incubation temperature, pH value) and nutritional requirements (carbon source, nitrogen sorce) for maximum production of citrinin by *Penicillium viridicatum* Westling were investigated. The optimum conditions for citrinin production were incubation for 8 days at 30°C on buffered broth medium (pH 5.0) contain sucrose and yeast extract as carbon and nitrogen sources, respectively. Propolis caused significant decrease in both growth and citrinin production of *P. viridicatum*. The alteration in mycelial amino acids content of *P. viridicatum* due to propolis indicated cellular damage by enhancing the instability and harmful hydrolysis of proteins towards catabolism.

Key words: Citrinin, *Penicillium viridicatum* Westling, propolis, mycelial amino acids.

Citrinin, an antibiotic and nephrotoxin[1], is produced by many species of Penicillia involve *P. viridicatum*[2] on many food and feed materials[2,3] causing many agricultural and medicinal problems lead to serious economic impact causing losses of millions of dollars in terms of production and reproduction. Citrinin causes alteration of amino acids uptake and their incorporation into protein by plant cotyledons[4], several cytological abnormalities, including chromosome breakage, polyploidy, Anaphase Bridge as well as Laggard of onion[5] and decreased seed germination[6].

Knowledge about the impact of environmental conditions and nutritional requirements on the fungal growth and citrinin production could be useful to prevent and control spoilage and contamination of this mycotoxin. The growth and mycotoxins production by fungal flora greatly influenced by several environmental factors as well as nutritional requirements [7,8]. The usage of systemic chemicals is primary method to control of mycotoxigenic molds attack seeds, food and feeds. Currently several chemical fungicides have been rejected and removed from the markets due to possible toxicological risks[9]. Consequently, there is urgent need to develop non-chemical alternative strategies to control mycotoxigenic molds and bioremediation of their mycotoxins [8,10]. Propolis (PR) is a resinous mixture that honeybees collect from tree buds, sap flows, and other botanical sources. This resin is masticated, salivary enzymes added, and the partially digested material is mixed with beeswax and used in the hive. The main function of PR is to prevent the decomposition of organic matter within beehive by inhibiting microbial growth and activity[11]. Many biological activities of PR have been reported such as medicinal[12]; antibacterial[13] and antifungal[14]. Several

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different mechanisms have been suggested to discuss the antifungal potential of PR[15].

It has been established that amino acids are a potentially building blocks for proteins and being key metabolic intermediates in biological membranes[16]. Concerning fungal amino acid biosynthesis, simple nitrogenous compounds such as ammonium may be assimilated into amino acid families, the carbon skeletons of which originate from common precursors of intermediary carbon metabolism[17]. Importance of amino acid alterations in fungal growth has been reported as sensitive monitor for antifungal resistance[18] and fungal activity such as mycotoxin production[19, 20].

The present study was aimed to investigate the environmental conditions and nutritional requirements for growth and citrinin production by *P. viridicatum*. Also, the current research investigates effectiveness antifungal mechanism of PR on growth, citrinin production and amino acid metabolism of *P. viridicatum* as citrinin producer model.

MATERIALS AND METHODS

The experimental mold

The experimental mold (*Penicillium viridicatum* Westling) was isolated from Egyptian rice grain sample and its production of citrinin mycotoxin was confirmed previously[2].

General culture conditions

The growth and citrinin production by *P. viridicatum* were studied using glucose-ammonium nitrate salts broth medium[21]. Erlenmeyer flasks (250 ml) containing 100 ml culture medium were autoclaved, inoculated with one agar disc (10 mm) of each fungal culture (7 days old), and incubated under static culture conditions. At the end of the incubation period, the cultures were filtered (Whatman No.1 filter paper used for collect the mycelial growth) and filtrates used for citrinin estimation. Mycelium growth was washed carefully with distilled water, dried at 105°C up to two successive constant weights, then the mycelia dry weight was recorded. The basal culture medium was incubated at different incubation periods and temperatures. The pH value of the basal medium was adjusted using citrate-phosphate buffer according to Malik and Singh[22] to study the effect of pH value on growth and citrinin production. The carbon and nitrogen sources of the basal culture medium were supplemented with other different sources in equivalent.

Estimation of citrinin

The extraction and clean-up of citrinin were carried out according to Jackson and Ciegler[23]. Fifty ml of culture filtrate was shaken with an equal volume of chloroform for 30 min. The chloroform layer was separated over a bed of anhydrous sodium sulfate. Clean-up was carried out using concentrated HC1 and 0.1 M $NaHCO_3$[23]. The quantitative determination of citrinin was carded out fluorometrically according to Trantham and Wilson[24] using spectrofluorometer Varian model 330 with detector (Varian Associates, Houston, TX, USA). The excitation wavelength of the fluorometer was 330 nm and the emission wavelength was 500 nm. The chemical and biological confirmation were carries out according to Hald and Krogh[25] and Abd_Allah and Ezzat[2], respectively. Citrinin-sensitive strain of *Bacillus brevis* was kindly provided by Dr. Gamal El-Didamony (Botany Department, Faculty of Science, Zagazig University, Egypt) was used as biological indicator.

Preparation of PR extract

Propolis was collected from colonies at Abha city, Saudi Arabia and it was scrapped-off the top of frames and inner wall boxes of bee colonies. PR was extracted with aqueous:ethanol (10:90, v/v)[15]. Based on preliminary experiment, three concentrations (0.2, 0.4 and 0.6; g/100ml) of PR were selected. The composition of growth medium was according to the optimum composition obtained for maximum citrinin production.

Extraction and estimation of amino acids

The extraction of mycelial free amino acids was carried out according to Malik and Singh[22]. The qualitative and quantitative determination of amino acids was carried out using LKB 415 alpha plus Amino Acid Analyzer (AAA)[26]. Standard amino acids (BHD Chemicals, Poole, UK) were used as reference.

Statistical analysis

All experiments were repeated at least three times. The data were statistically analyzed using analysis of variance for a completely randomized design[27].

RESULTS AND DISCUSSION

The maximum mycelial dry weight and citrinin production was obtained after incubation for 6 and 8 days, respectively. The further incubation more than the optimum incubation periods was accompanied with gradual decrease in both growth and citrinin production by *P. viridicatum* (Table 1). Our results are in agreement with other authors [2,28] who reported that the maximum citrinin production was attained during the stationary phase. It was observed that no correspond between both growth and production curves in many fungi[2,29]. These aspects are unequivocally supportive of our findings with respect to relationship between growth and production curve of *P. viridicatum*.

The optimum incubation temperature (°C) for maximum growth and citrinin production of *P. viridicatum* was 35°C and 30°C, respectively (Table 2). No citrinin was detected after incubation at both 10°C and 45°C. In the same connection, it has reported that the optimum incubation temperature for citrinin production was 30°C[7]. On the other hand, the optimum temperature for citrinin production by other fungal species was 20°C[30].

Table 3 shows that the growth and citrinin production by *P. viridicatum* increased with increasing pH value of the culture medium until it reached a maximum at pH 5.5 and 5.0 respectively,

Table 1. Effect of different incubation period (day) on growth (g/100 culture medium) and production of citrinin (ug/100 culture medium) by *P. viridicatum*.

Incubation period (day)	Mycelial growth (g/100 culture medium)	Citrinin production (ug/100 culture medium)	Specific production of citrinin*
2	0.5992	98.2	165.83
4	1.0652	155.8	146.25
6	1.7641	183.0	103.84
8	1.5125	230.4	152.45
10	1.4660	203.2	138.96
12	1.1922	167.4	147.95
14	0.9729	152.6	156.83
LSD at: 0.05	0.2402	45.7	

*= Citrinin production (ug/100 culture medium)/ Mycelial growth (g/100 culture medium).

Table 2. Effect of different incubation temperature (oC) on growth (g/100 culture medium) and production of citrinin (ug/100 culture medium) by *P. viridicatum*

Incubation temperature (°C)	Mycelial growth (g/100 culture medium)	Citrinin production (ug/100 culture medium)	Specific production of citrinin*
10	0.4462	ND	ND
15	0.7298	50.2	70.25
20	1.0108	133.4	132.11
25	1.2850	193.8	151.68
30	1.5124	257.2	170.18
35	1.1841	227.8	193.46
40	0.7334	97.0	134.72
45	0.3215	ND	ND
LSD at: 0.05	0.2167	61.2	

*= Citrinin production (ug/100 culture medium)/ Mycelial growth (g/100 culture medium).
ND= Not detected under the experimental conditions.

and then contrary impression was observed gradually with further increment in pH value. The optimum pH value for citrinin production by many fungi was 5.0[31]. The concentration of hydrogen ion (pH value) in culture medium is an important and vital factor in growth and activity of fungi in agriculture; industry and during storage of food as well as feed commodities[9].

As sole carbon sources for the growth and citrinin production of *P. viridicatum*, fructose, glucose, sucrose, mannose, lactose, CMC, cellulose, starch, apple pectin and rice powder were evaluated in the same equivalent amount. The growth and citrinin production with glucose and sucrose respectively, were significantly higher; however the minimum growth and citrinin production were shown with apple pectin (Table 4). Such results are in agreement with those of other researcher[7]. The maximum specific production of citrinin was observed with rice powder. The superiority of rice powder over the other tested carbon sources may be attributed to the presence of many elements and vitamins (in addation to starch) might playing stimulatory effect

Table 3. Effect of different hydrogen ion concentration (pH value) on growth (g/100 culture medium) and production of citrinin (ug/100 culture medium) by P. viridicatum.

Hydrogen ion concentration (pH value)	Mycelial growth (g/100 culture medium)	Citrinin production (ug/100 culture medium)	Specific production of citrinin*
4.0	0.8023	ND	ND
4.5	1.0622	77.0	72.78
5.0	1.2869	265.8	207.78
5.5	1.7937	235.2	131.09
6.0	1.6245	169.4	104.46
6.5	1.2849	116.8	91.1685
7.0	1.0473	66.8	64.31
LSD at: 0.05	0.4371	32.4	

*= Citrinin production (ug/100 culture medium)/ Mycelial growth (g/100 culture medium).
ND= Not detected under the experimental conditions.

Table 4. Effect of different equivalent carbon sources on growth (g/100 culture medium) and production of citrinin (ug/100 culture medium) by *P. viridicatum*

Carbon sources	Mycelial growth (g/100 culture medium)	Citrinin production (ug/100 culture medium)	Specific production of citrinin*
Fructose	1.6926	254.6	150.51
Glucose	2.0081	278.8	138.92
Sucrose	1.7812	328.0	184.63
Mannose	0.7229	142.2	197.86
Lactose	0.8667	97.2	112.20
Manitol	0.6492	59.2	91.62
CMC	0.5651	58.2	103.47
Cellulose	0.3933	50.2	128.14
Starch	0.6989	80.2	114.68
Apple Pectin	0.3087	32.6	105.41
Rice powder	0.8985	209.4	232.96
LSD at: 0.05	0.2906	27.4	

*= Citrinin production (ug/100 culture medium)/ Mycelial growth (g/100 culture medium).
CMC= Carboxymethly cellulose.

for citrinin production which discuss the natural contamination of rice grains with high level of citrinin during storage[2,7,32].

The investegated nitrogen sources (sodium nitrates, potasium nitrates, ammonium nitrate, ammonium chloride, ammonium sulphate, ammonium phosphate, urea, peptone, and yeast extract) were different in their effect on growth and citrinin production by *P. viridicatum* under the experimental conditions. The results indicated clearly that yeast extract was the most favourable nitrogen source for maximum growth and citrinin production, however the minimum ones were obtained with ammonium sulphate and urea, respectively as compared with all tested nitrogen sources (Table 5). Such results are parallel with other findings[7]. The hydrolysis of natural complex (yeast extract) nitrogen source lead to intermediate compounds might be act as stimulators and precursors essentially for maximum growth and citrinin production[7]. In another connection, the hydrolysis of ammonium sulphate and urea lead to significan alteration (decrease and increase, respectively) in hydrogen ion concentration of the growth medium[33].

Both growth and citrinin production of *P. viridicatum* were increasingly decreased with the increase of concentration of PR in the medium in directly proportionally (Table 6). The inhibitory effect of PR on fungal growth and mycotoxin production recorded in our study was agreed with those reported of recent studies on other fungi such as *Candida albicans*[34] and *Aspergillus parasiticus*[15]. The antifungal mechanism of PR is still not complete clarity. The phenols[35] and flavonoids[36] has been considered the main carriers

Table 5. Effect of different equivalent nitrogen sources on growth (g/100 culture medium) and production of citrinin (ug/100 culture medium) by *P. viridicatum*

Nitrogen sources	Mycelial growth (g/100 culture medium)	Citrinin production (ug/100 culture medium)	Specific production of citrinin*
Sodium nitrate	2.2457	276.8	123.39
Potassium nitrate	2.0831	232.6	111.71
Ammonium nitrate	1.9148	284.6	148.67
Ammonium chloride	1.3596	195.2	143.56
Ammonium sulfate	1.3111	187.8	143.15
Ammonium phosphate	1.6927	222.8	131.59
Urea	1.7781	157.0	88.40
Peptone	2.9136	299.6	102.86
Yeast extract	3.1067	313.8	101.04
LSD at: 0.05	0.5012	31.2	

*= Citrinin production (ug/100 culture medium)/ Mycelial growth (g/100 culture medium)

Table 6. Effect of different concentrations (w/v) of propolis on growth (g/100 culture medium) and production of citrinin (ug/100 culture medium) by *P. viridicatum*

Concentration of propolis (g/100 ml)	Mycelial growth (g/100 culture medium)	Citrinin production (ug/100 culture medium)	Specific production of citrinin*
Contro	3.1067	313.8	101.04
0.2 % (w/v)	2.0403	226.6	111.12
0.4 % (w/v)	1.4227	190.2	138.02
0.6 % (w/v)	0.7302	119.8	169.54
LSD at: 0.05	0.6831	52.7	

*= Citrinin production (ug/100 culture medium)/ Mycelial growth (g/100 culture medium).
ND= Not detected under the experimental conditions.

of antifungal properties of PR. In this regard, it was reported that PR caused an inhibition in cell division hence suggested that PR might inhibit DNA replication[37]. The kinetic production of citrinin increased gradually with increasing PR concentrations (Table 6) indicating that the response of fungal (*P. viridicatum*) growth was more sensitive to PR concentrations than citrinin production.

The results revealed the presence of 16 free amino acids namely amino butyric acid; alanine; aspartic acid; cysteine; glutamic acid; glycine; isoleucine; lysine; methionine; ornithine; phenylalanine; proline; serine; tryptophan; tyrosine and valine in mycelia of *P. viridicatum* (Table 7). The alteration in amino acids composition of molds can be used as sensitive monitor for mold resistance against abiotic stress of chemical fungicide[38] and biofungicids from plant origin[20]. The present data shown that, 0.2% (w/v) of PR caused significant increase in amino butyric acid, glutamic acid, proline, phenylalanine, serine as well as valine and appereance of histidine, arginine as well as threonine, however alanine, aspartic acid, cysteine, glycine, isoleucine, lysine, methionine, ornithine, tryptophan as well as tyrosine have been decreased as compared with amino acids of control treatment of *P. viridicatum*. The further concentration of PR (0.4%, w/v) caused significant increase in histidine, arginine as well as threonine as compared with PR concentration of 0.2% (w/v). At highest concentration of PR (0.6%, w/v) caused disapperance of alanine, isoleucine, lysine, ornithine, phenylalanine, serine, tyrosine and valine. Generally the total free amino acids incerased with the lower concentration of PR (0.2%, w/v), however the two successive concentrations (0.4 & 0.6%, w/v) caused reverse effect proportionally. The accumulation of glutamate amino acids (glutamic acid and proline) in mycelial growth of *P. viridicatum* due to PR means activation of their biosynthesis from glutamate via both glutamate kinase followed by ^{3}- semialdehyde dehydrogenase (for proline) and glutamine synthetase (for glutamic acid), respectively[19]. On the other hand, the consumption of such glutamate amino acids decreased due to PR to support more energy (Adenosine Tri-phosphate, ATP) required for mold resistance against antifungal potential of PR[39]. In the same connection, proline amino acid was

Table 7. Effect of different concentrations of propolis (g/100 ml) on mycelial amino acid content (mg/g dry weight) of *P. viridicatum*.

Concentration	Amino acids (mg/g dry weight)																					
of propolis	amino BA	ala A	arg R	asp D	cys C	glu E	gly G	his H	ile I	lys K	met M	orn O	phe F	pro P	ser S	thr T	trp W	tyr Y	val V	Total
Control	0.341	0.852	ND	0.893	1.054	0.613	0.752	ND	0.142	1.273	0.614	0.328	0.397	0.521	0.412	ND	0.815	0.152	0.375	9.534
0.2 % (w/v)	0.596	0.713	0.279	0.625	0.831	0.893	0.627	0.281	0.027	1.012	0.429	0.114	0.573	0.835	0.571	0.251	0.682	0.027	0.681	10.047
0.4 % (w/v)	0.929	0.392	0.637	0.471	0.714	0.971	0.421	0.412	ND	0.371	0.237	ND	0.782	0.981	0.836	0.327	0.417	ND	ND	8.898
0.6 % (w/v)	1.236	ND	1.164	0.237	0.531	0.257	0.203	0.591	ND	ND	0.103	ND	ND	1.203	ND	0.435	0.263	ND	ND	6.223
LSD at:	0.05	0.194	0.127	0.471	0.203	0.175	0.192	0.097	0.273	0.137	0.162	0.176	0.216	0.207	0.163	0.137	0.089	0.192	0.076	0.294

ND: Not detected under the experimental conditions.
Abbreviation: Amino butyric acid, Amino BA; Alanine, ala A; Arginine, arg R; Aspartic Acid, asp D; Cysteine, cys C; Glutamic Acid, glu E; Glycine, gly G; Histidine, his H; Isoleucine, ile I; Lysine, lys K; Methionine, met M; Ornithine, orn O; Phenylalanine, phe F; Proline, pro P; Serine, ser S; Threonine, thr T; Tryptophan, trp W; Tyrosine, tyr Y, and Valine, val V.

shown to minimize cellular damage by enhancing the stability of proteins and biological membrane[40] as suggested mechanism of PR.

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