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Factors Affecting the Production of Eremofortin C and PR Toxin in *Penicillium roqueforti*

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Eremofortin C (EC) and PR toxin are secondary metabolites of *Penicillium roqueforti*. Of 17 strains from the American Type Culture Collection that were studied for their ability to produce EC and PR toxin, 13 produced these metabolites. Toxin production by strains grown in solid media (10 cereals and 8 other agricultural products) was also investigated. Production of EC and PR toxin by fungi grown on cereals was greater than production of EC and PR toxin by fungi grown on legumes; fungi grown on corn produced the greatest amount of PR toxin. Addition of corn extracts to the culture medium greatly increased the production of EC and PR toxin in a coordinated manner, with no significant change in mycelial dry weight. The fungi produced the highest levels of EC and PR toxin at 20 to 24°C depending on the strain. Toxin production was higher in stationary cultures than in cultures that were gently shaken at 120 rpm. The optimum pH for production of both EC and PR toxin was around pH 4.0. With regard to spore age, toxin levels did not change significantly when we used spores obtained from fungi that were grown at 24°C for 3 up to 48 days.

Eremofortin C (EC) and PR toxin are secondary metabolites of *Penicillium roqueforti* (7, 20). The chemical structures of EC and PR toxin have been elucidated previously (Fig. 1) (7, 18). The structures of these compound are closely related and differ only in the presence of a hydroxymethyl functional group in EC and an aldehyde functional group in PR toxin at the C-12 position. PR toxin is lethal to rats, mice, and cats when it is administered orally, intraperitoneally, or intravenously (2, 19). PR toxin inhibits RNA and protein synthesis (10, 11), the activities of DNA polymerases α, β, and γ (4, 20), mitochondrial HCO_3^--ATPase activity (3), and mitochondrial respiration and oxidative phosphorylation in animal cells (15, 21). It also alters the genetic activity of *Saccharomyces cerevisiae* and *Neurospora crassa* (17). In contrast, EC does not exhibit significant toxicity in animals (12). Peak production of EC occurs earlier than peak production of PR toxin, and a decrease in the amount of EC is always associated with a rapid increase in the PR toxin level (8). Recently, we found that EC is the precursor of PR toxin and that EC is transformed to PR toxin by EC oxidase (1, 5).

Workers in several research groups have been involved with PR toxin research in the past 20 years. Variability in PR toxin production by *P. roqueforti* in the same medium is well known. Different workers have found that the maximal amount of PR toxin produced by this fungus in yeast extract-sucrose medium was between 82 and 770 mg/liter (8, 14, 16, 19, 20). The factors that affect PR toxin biosynthesis that have been studied are fungal strain, pH, and composition of the medium (6, 13, 14, 16). This study was designed to examine the effects of several factors, including strain, culture medium composition, pH, incubation temperature, length of incubation, agitation, and spore age of inoculum, on toxin production. Our results may supply some of the information necessary to optimize the production of EC and PR toxin in *P. roqueforti*.

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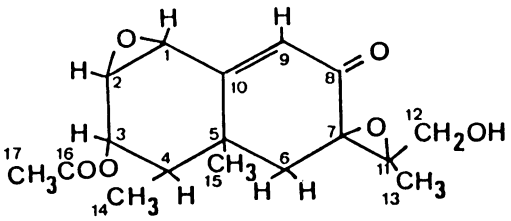
MATERIALS AND METHODS

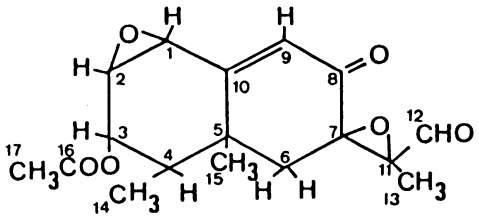
Strains. A total of 17 strains of *P. roqueforti* were obtained in the lyophilized state from the American Type Culture Collection (ATCC), Rockville, Md. After cultures had been established, fungal strains were maintained on potato dextrose agar slants and stored at 4°C. Spores of each stock culture were transferred to fresh potato dextrose agar slants and incubated at 24°C for 7 days before use. To provide uniform inocula, spore suspensions were prepared by adding 6 ml of sterile distilled water containing 0.01% sodium dodecyl sulfate to each cultured slant. The concentration of spores was adjusted to 10^7 to 10^8 spores per ml. A 0.5-ml aliquot of a spore suspension was then inoculated into each Roux bottle or flask, which contained either liquid or solid medium.

Media. *P. roqueforti* is usually grown in a culture medium composed of 2% yeast extract (Difco) and 15% sucrose (20). Recently, we noted that the ability of the fungus to produce PR toxin gradually decreased when it was repeatedly transferred on synthetic medium. In an investigation to determine a way to restore the ability of *P. roqueforti* to produce PR toxin, we discovered that addition of corn extract to the medium significantly increased the production of EC and PR toxin (5). Therefore, we formulated another medium containing 1% yeast extract, 7.5% sucrose, and various concentrations of corn extract. Corn extract was prepared as described below. A 1-kg portion of fresh corn grains was boiled in 1,200 ml of distilled water for 20 min, and the extract was filtered through three layers of cheesecloth. Then the filtrate (corn extract) was adjusted to a total volume of 1,000 ml with distilled water. Usually 150 ml of liquid medium was added to a 600-ml Roux bottle for each culture.

The fungi were cultured on various media in our investigation of EC and PR toxin production. Each 250-ml flask contained 15 g of a cereal or 15 g of a legume and 20 to 25 ml of distilled water. All of the flasks containing media were fitted with cotton plug stoppers and autoclaved at 121°C for 15 min. After cooling at room temperature, all of the bottles were inoculated with the same amount of *P. roqueforti* spores. The autoclaved cereals or legumes were weighed.



Eremofortin C



PR toxin

FIG. 1. Chemical structures of EC and PR toxin.

The final moisture contents of the cereals and legumes were as follows: buckwheat, 80%; barley, 75%; wheat, 60%; oats, 75%; indica rice, 65%; japonica rice, 60%; glutinous rice, 65%; millet, 60%; Job's tears, 65%; corn, 75%; adzuki beans, 50%; mung beans, 60%; kidney beans, 120%; soy beans, 110%; common beans, 80%; soy beans (black seed coat), 85%; peanuts, 65%; and peas, 90%.

Effects of growth conditions on the production of EC and PR toxin. To determine the optimum pH for the production of EC and PR toxin by *P. roqueforti*, the fungi were grown at different pH values by using the following buffer systems: hydrochloric acid-potassium chloride, pH 2.0; citrate-phosphate, pH 3.0 to 6.0; sodium barbital-hydrochloric acid, pH 7.0 to 8.0.

The effect of temperature on the production of EC and PR toxin was studied by measuring the yields of the two compounds at 16, 20, 24, 28, and 32°C.

Cultures of *P. roqueforti* have been incubated previously under stationary conditions for the production of PR toxin (20). In order to investigate whether the production of EC and PR toxin can be enhanced by shaking, the yields of these compounds from cultures that were incubated with and without shaking at 120 rpm were compared.

The influence of spore age on the production of EC and PR toxin was studied by using as inocula spores obtained from 3- to 48-day-old mycelia.

When we compared the production of EC and PR toxin under various growth conditions, we monitored the production of both compounds in the culture medium during the growth of the fungus. We chose as the incubation time the time that the production of EC and PR toxin reached maximum levels.

Preparation of EC and PR toxin standards. EC and PR toxin were isolated from the culture medium by chloroform extraction and were further purified by using previously described methods (7, 20). Purified EC and PR toxin were stored in brown vials at −20°C and were dissolved in chloroform immediately before analysis.

TABLE 1. Production of EC and PR toxin by different *P. roqueforti* strains in YES and YESC media[a]

Strain	Culture medium	Maximal concn of EC (μg/ml)[b]	Maximal concn of PR toxin (μg/ml)[b]	Incubation temp (°C)
ATCC 6987	YES[c]	30	15	24
	YESC[d]	181	638	24
ATCC 6989	YES	19	28	20
	YESC	160	936	20
ATCC 9295	YES	82	73	20
	YESC	179	481	20
ATCC 10422	YES	0	0	20 and 24
	YESC	0	0	20 and 24
ATCC 34907	YES	0	0	20 and 24
	YESC	236	162	20
ATCC 34908	YES	0	0	20 and 24
	YESC	47	36	20
ATCC 36201	YES	0	0	20 and 24
	YESC	0	0	20 and 24
ATCC 42294	YES	0	0	20 and 24
	YESC	0	0	20 and 24
ATCC 46838	YES	0	0	20 and 24
	YESC	34	121	24
ATCC 48778	YES	0	0	20 and 24
	YESC	248	625	20
ATCC 48779	YES	0	0	20 and 24
	YESC	258	549	24
ATCC 48780	YES	0	0	20 and 24
	YESC	89	137	24
ATCC 48936	YES	44	28	24
	YESC	1,640	550	24
ATCC 52322	YES	0	0	20 and 24
	YESC	203	84	24
ATCC 56758	YES	0	0	20 and 24
	YESC	15	7	20
ATCC 58626	YES	0	0	20 and 24
	YESC	0	0	20 and 24
ATCC 58628	YES	0	0	20 and 24
	YESC	724	521	24

[a] The media were inoculated with spore suspensions of various strains and incubated under stationary conditions in the dark at 20 and 24°C.

[b] The maximal concentration was the highest yield of EC or PR toxin that was reached in a time-course study.

[c] YES medium contained 2% yeast extract and 15% sucrose.

[d] YESC medium contained 1% yeast extract, 7.5% sucrose, and 20% corn extract.

Quantitative analysis of EC and PR toxin. EC and PR toxin were analyzed by high-performance liquid chromatography, using chloroform as the solvent at a flow rate of 2 ml/min, as described by Moreau et al. (9). High-performance liquid

TABLE 2. Effect of corn extract on the production of EC and PR toxin by *P. roqueforti* ATCC 48936

Concn of corn extract added (%)	Maximal concn (μg/ml) of [a]:	
	EC	PR toxin
0[b]	51 (1.0)[c]	38 (1.0)
6.7[d]	1,244 (24.4)	437 (11.5)
20.0[d]	1,650 (32.4)	550 (14.5)
60.0[d]	1,750 (34.3)	607 (16.0)
100.0[d]	1,705 (33.4)	590 (15.5)

[a] See Table 1, footnote *b*.

[b] The culture medium contained 2% yeast extract and 15% sucrose.

[c] The numbers in parentheses are the fold increases in EC or PR toxin production.

[d] The culture media contained 1% yeast extract, 7.5% sucrose, and various concentrations of corn extract.

TABLE 3. Production of EC and PR toxin on solid media containing various stored cereals and legumes by *P. roqueforti* ATCC 48936[a]

Stored cereal or legume	EC		PR toxin	
	Maximal concn (μg/g)[b]	Incubation period (days)	Maximal concn (μg/g)[b]	Incubation period (days)
Stored cereals				
Buckwheat (*Fagopyrum vulgare*)	694	13	297	14
Barley (*Hordeum vulgare* L.)	694	13	182	14
Wheat (*Triticum sativum* L.)	87	7	149	8
Oats (*Avena sativum* L.)	313	13	74	14
Indica rice (*Oryza sativa* L.)	1,368	13	361	14
Japonica rice (*Oryza sativa* L.)	262	13	136	14
Glutinous rice (*Oryza sativa* L.)	222	13	292	14
Millet (*Setaria italica* Beauv.)	80	10	19	13
Job's tears (*Coix lacryma-jobi* L.)	153	7	161	8
Corn (*Zea mays* L.)	478	13	362	15
Legumes				
Adzuki beans (*Phaseolus angularis* W. F. Wight)	17	6	22	6
Mung beans (*Vigna radiata* (L.) Wilczek)	29	6	4	6
Kidney beans (*Phaseolus calcaratus* Roxb.)	4	6	2	6
Soy beans (black seed coat) (*Glycine max* L.)	21	6	20	6
Common beans (*Phaseolus coccineus* L.)	8	6	1	6
Soy beans (*Glycine max* (L.) Merrill)	6	6	2	6
Peanuts (*Arachis hypogaea* L.)	13	6	2	6
Peas (*Pisum sativum* L.)	5	6	3	6

[a] The assay conditions are described in Table 1, footnote *a*.
[b] Amount of EC or PR toxin produced per gram of stored cereal or legume before addition of water.

chromatography analyses were carried out with a Waters model ALC/GPC 204 high-performance liquid chromatography system equipped with a model U6K universal injector, a model M-6000A pump, a μPorasil column (30 cm by 3.9 mm [inside diameter]), and a model 440 absorbance detector equipped with a 254-nm filter. The retention times of PR toxin (2 min) and EC (6 min) were verified with PR toxin and EC standards. The amounts of EC and PR toxin in a sample were determined by measuring the peak area of each compound in the chromatogram against a known standard.

RESULTS

Table 1 shows the production of EC and PR toxin by 17 strains of *P. roqueforti* grown in yeast extract-sucrose (YES) and yeast extract-sucrose-corn extract (YESC) media under stationary culture conditions in the dark at 20 and 24°C. A total of 13 strains produced the two compounds in YESC medium, but only four strains (strains ATCC 6987, ATCC 6989, ATCC 9295, and ATCC 48936) produced EC and PR toxin in both media. The dry weights of the mycelia of fungi grown under the same cultural conditions were not significantly different (range, 2.0 to 3.5 mg). Therefore, the differences in EC and PR toxin production were due to differences among strains. Differences in fungal growth contributed little, if any, to the differences in the production of the two compounds. EC and PR toxin production by the four strains was much higher on YESC medium than on YES medium. Because strain ATCC 48936 produced the highest levels of EC and PR toxin in YESC medium, this strain was selected for further studies.

When YES medium was supplemented with 6.7, 20, 60, and 100% corn extract and inoculated with strain ATCC 48936 under stationary culture conditions in the dark at 24°C, the peak yields of EC and PR toxin were increased 24-, 32-, 34-, 33-fold and 12-, 15-, 16-, and 16-fold, respectively, compared with the peak yields obtained in YES medium (Table 2). These results indicated that corn extract enhanced the ability of *P. roqueforti* to produce EC and PR toxin in culture media. Moreover, corn extract at a concentration of 60% produced the greatest stimulatory effect on the production of EC and PR toxin. On the other hand, the peak amounts produced were 80 to 1,368 μg/g for EC and 19 to 362 μg/g for PR toxin with the 10 cereals tested (Table 3); therefore, cereals appear to be good substrates for the biosynthesis of EC and PR toxin by *P. roqueforti*. Nevertheless, only small quantities of both compounds were detected when *P. roqueforti* was grown in medium contain-

TABLE 4. Effect of growth temperature on the production of EC and PR toxin by *P. roqueforti* ATCC 48936[a]

Temp (°C)	EC		PR toxin		Dry wt of mycelium (g/Roux bottle)
	Maximal concn (μg/ml)	Incubation period (days)	Maximal concn (μg/ml)	Incubation period (days)	
16	660	24	54	26	3.2
20	968	20	454	24	3.3
24	1,699	12	552	13	3.0
28	738	7	231	8	2.4
32	29	9	21	10	2.3

[a] In each time-course study we monitored the production of EC and PR toxin in YESC culture medium during the growth of the fungus. We then chose as the incubation time the time that the production of EC or PR toxin reached the maximum, and the dry weight of mycelium was measured simultaneously.

TABLE 5. Effect of pH on the production of EC and PR toxin by *P. roqueforti* ATCC 48936

Culture medium[a]	pH		Maximal concn (μg/ml) of[b]:		Dry wt of mycelium (g/Roux bottle)
	Initial	Final	EC	PR toxin	
YESC + 50 mM buffer	2.0	2.1	53	23	2.4
	3.0	3.6	1,227	774	2.7
	4.0	4.5	2,100	1,427	3.0
	5.0	5.4	753	573	3.0
	6.0	5.5	820	520	3.2
	7.0	5.8	733	510	3.1
	8.0	6.2	67	60	3.6
YESC only	6.3	5.5	1933	552	2.9

[a] YESC medium contained 1% yeast extract, 7.5% sucrose, and 20% corn extract. The following buffer systems were used: hydrochloric acid-potassium chloride, pH 2.0; citrate-phosphate, pH 3.0 to 6.0; and sodium barbital-hydrochloric acid, pH 7.0 to 8.0.
[b] See Table 1, footnote *b*.

ing any one of the eight legumes tested despite good visible growth (Table 3).

P. roqueforti has traditionally been incubated as stationary cultures for the production of EC and PR toxin. In a time-course study, the peak amounts of EC and PR toxin in stationary cultures were found to be six times and two times, respectively, the amounts produced in shaken cultures (Fig. 2). Since EC is transformed into PR toxin by EC oxidase in the culture medium (1, 5), we compared the efficiency of transformation of EC to PR toxin in shaken and stationary cultures. When the maximal amount of PR toxin was divided by the maximal amount of EC in the culture medium, we found that the level of transformation of EC to PR toxin was close to 100% when the fungus was grown in a shaker at 120 rpm, whereas the level of transformation was only 35% in stationary cultures. The highest levels of EC and PR toxin were obtained when *P. roqueforti* was grown at 24°C (Table 4), and the optimal incubation time for the production of both compounds at 24°C was 12 to 14 days. At 28°C, the maximal amounts of EC and PR toxin were produced in 7 to 8 and 8 to 9 days, respectively. Moreover, at 28°C of both compounds in the culture medium were degraded quickly (within ca. 2 to 3 days). Although the fungus could grow at 32°C, the production of EC and PR toxin was significantly decreased. However, at 20°C the optimal yield was reached after 19 to 21 days for EC and after 23 to 25 days for PR toxin. Decreasing the incubation temperature to 16°C resulted in an increase in the time needed to reach maximal yields (24 to 26 days for EC and 26 to 28 days for PR toxin).

The effect of pH on EC and PR toxin production was studied at pH values ranging from 2.0 to 8.0. Table 5 shows that the highest yield of the two compounds was obtained when the initial pH of the medium was 4.0. At pH values above 4.0, the amounts of EC and PR toxin gradually decreased, and sharp decreases were observed at the extremes of pH 2.0 and 8.0. Furthermore, the production of PR toxin in the medium with an initial pH of 4.0 was 2.6 times greater than the production in the unbuffered medium. In contrast, the yields of EC were similar in both media.

Spores from 5- to 7-day-old subcultures were usually used as the inocula for production of EC and PR toxin. However, our results indicate that the highest yields of EC and PR toxin by *P. roqueforti* were not significantly different with spores obtained from mycelia that were 3 up to 48 days old.

DISCUSSION

In the past 17 years, several papers have been published on PR toxin research (8, 14, 16, 19, 20). On the basis of these reports, we found that PR toxin production by a given strain of *P. roqueforti* gradually decreases in the same medium. Table 1 shows that the amounts of PR toxin produced by *P.*

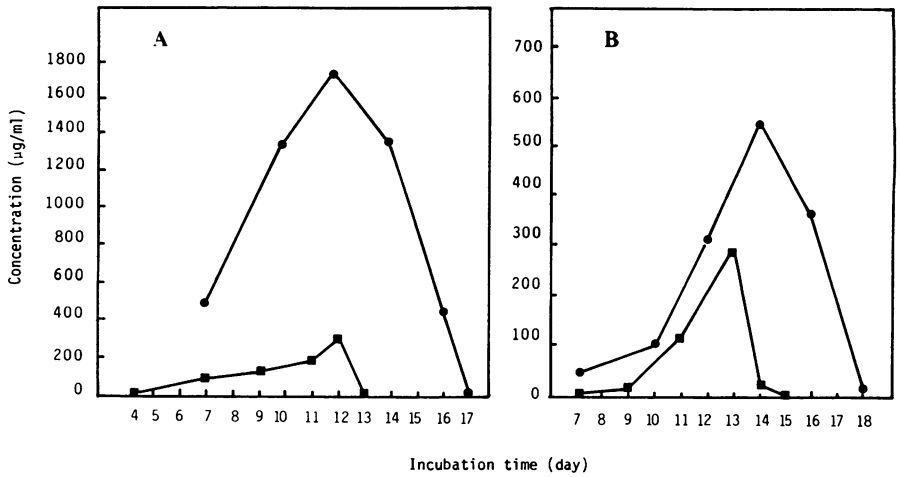


FIG. 2. Effect of agitation on the production of EC (A) and PR toxin (B) by *P. roqueforti* ATCC 48936. Symbols: ●, stationary cultures; ■, cultures shaken at 120 rpm.

roqueforti ATCC 6987, ATCC 6989, and ATCC 9295 were about 10% of the amounts obtained by Wei and Liu (16). These results indicate that the ability of *P. roqueforti* to produce PR toxin decreases with repeated transfers on synthetic media or with storage in the freeze-dried state for several years. When the medium was supplemented with 20% corn extract (YESC medium), not only was the PR toxin-producing ability restored, but the yields of PR toxin increased two- to threefold compared with the yields obtained by Wei and Liu (16). Since EC has been demonstrated to be the precursor of PR toxin and EC can be transformed to PR toxin by EC oxidase (1, 5), we also investigated the effects of corn extract on the production of EC and the activity of EC oxidase. The yield of EC correlated well with PR toxin production in the culture medium (Tables 1 and 2). The activity of EC oxidase was also significantly enhanced in YESC culture medium (unpublished data).

P. roqueforti is usually grown as stationary cultures for studies of toxin production. When the fungus was incubated in a shaker at 120 rpm, it still produced EC and PR toxin, but the yields of both compounds were significantly decreased (Fig. 2). These results indicate that different aeration conditions could influence the ability of *P. roqueforti* to produce these two compounds. The transformation of EC into PR toxin by EC oxidase requires oxygen for catalytic action (1, 5). With agitation the concentration of oxygen in a culture is obviously higher than the concentration of oxygen under stationary conditions. Thus, in shaking cultures the biochemical reaction that catalyzes EC into PR toxin is facilitated despite the fact that the yield of EC is lower than the yield in stationary cultures.

In conclusion, we extensively investigated the factors that affect the production of EC and PR toxin by *P. roqueforti*. Our results provide information for maximizing the yield of these secondary metabolites for basic research on elucidation of the metabolic pathway of biosynthesis and biodegradation of these heterocyclic eremophilane compounds.

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