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Effects of Environmental and Nutritional Factors on Production of the Polyketide Phytotoxin Coronatine by *Pseudomonas syringae* pv. Glycinea†

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***Pseudomonas syringae* pv. glycinea PG4180 produces the polyketide phytotoxin coronatine. The effects of environmental, nutritional, and host factors on growth and coronatine production by PG4180 were examined by varying the components of a defined basal medium which contained the following nutrients per liter: glucose (10 g), NH_4Cl (1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), KH_2PO_4 (4.1 g), $K_2HPO_4 \cdot 3H_2O$ (3.6 g), and $FeCl_3$ (2 μM). Bacterial growth was recorded as dry weight, and coronatine production was measured by high-performance liquid chromatography. Both growth and the quantity of coronatine synthesized were significantly affected by carbon source, nutrient levels (glucose, NH_4Cl, phosphate, Mg, and SO_4), amino acid supplements, and the presence of complex carbon and nitrogen sources. The yield of coronatine generally declined when conditions were varied from those in the basal medium. Coronatine production and growth were not affected when the pH was adjusted from 6.5 to 7.8. Increases in the osmolarity of the basal medium significantly decreased coronatine production without affecting growth. The addition of plant extracts, plant-derived secondary metabolites, or zinc did not affect growth or coronatine production, while the addition of millimolar levels of KNO_3 or micromolar levels of $FeCl_3$ significantly enhanced coronatine production. The yield of coronatine was maximized after a 7-day incubation at 18°C and 280 rpm. The results of the present study were used to formulate a medium which allowed for enhanced coronatine production in nearly all strains of *P. syringae* tested. A rapid method for extracting coronatine from small volumes of culture supernatant was also developed.**

Coronatine is a chlorosis-inducing phytotoxin produced by several members of the *Pseudomonas syringae* group of pathovars, including pathovars glycinea, atropurpurea, morsprunorum, and tomato (21, 25). The active toxin acts as a virulence factor in pathovars glycinea, tomato, and atropurpurea (1, 9, 33, 34), possibly by enhancing ethylene biosynthesis in planta (7, 18). The biosynthetic origin of coronatine is unusual because it consists of two moieties, coronafacic and coronamic acids, which are derived from separate biosynthetic pathways. The coronafacic acid portion, which is derived via polyketide biosynthesis, is coupled to coronamic acid, a cyclized derivative of isoleucine, by an amide bond (16, 22, 29).

Very few studies have examined the effect of environmental, nutritional, and host factors on the biosynthesis of phytotoxins by phytopathogenic pseudomonads (13). The effect of nutritional regimens on the production of the phytotoxin syringomycin was investigated and used to formulate a defined minimal medium which was optimal for syringomycin production (12). More recently, a *lacZ* fusion was used to demonstrate that the syringomycin locus *syrB* was induced by iron, phenolic glucosides, and specific sugars (26, 27). However, comparable studies have not been undertaken with other phytotoxins produced by *P. syringae*.

Since coronatine is derived from the products of two separate pathways (isoleucine and polyketide), it is possible that regulatory controls exist in both pathways and also for the biosynthetic step(s) which catalyzes amide bond formation between the pathway components. For example, Mitchell and Frey (24) speculated that coronatine synthesis is sensitive to feedback controls in the isoleucine pathway. The addition of valine to broth cultures of *P. syringae* pv. atropurpurea reduced coronatine production, possibly due to an inhibition of isoleucine biosynthesis (24). It is also possible that specific plant metabolites influence coronatine production in the host plant. Ma et al. (19) demonstrated that CorII, a complementation unit required for coronatine synthesis by *P. syringae* pv. tomato DC3000, was expressed at higher levels in the host plant than in vitro.

We are studying coronatine synthesis by *P. syringae* pv. glycinea PG4180, a model organism for studying the biosynthesis and regulation of this important phytotoxin (3). The genes encoding coronatine synthesis in PG4180 are encoded by p4180A, a 90-kb plasmid, and are clustered in a 32-kb contiguous region of DNA (2, 40). The present study was designed to conduct a systematic survey of the environmental, nutritional, and host factors which regulate coronatine synthesis in *P. syringae* pv. glycinea PG4180. One outcome of the survey was the development of a defined minimal medium which is optimized for coronatine production. A rapid analytical method for the quantitative analysis of coronatine production was also developed.

MATERIALS AND METHODS

Bacterial strains and media. *P. syringae* pv. glycinea PG4180 (21, 40) was used in studies designed to determine the effects of environmental, nutritional, and host factors on coronatine production. The quantity of coronatine produced in the optimized medium was determined for the following strains of *P. syringae*: pv. tomato PT23.2 (1), pv. tomato

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DC3000 (19), pv. atropurpurea 1304 (34), pv. morsprunorum 567 (21), pv. morsprunorum 3714 (21), pv. maculicola 438 (D. Cuppels, London, Ontario, Canada), pv. glycinea 4182 (21), pv. glycinea 5562 (International Collection of Microorganisms from Plants, Auckland, New Zealand), and pv. glycinea 7a (37). Coronatine production by *Xanthomonas campestris* pv. phormiicola 4297 and 4293 (23) was also investigated in the optimized medium.

The basal medium used in the present study was a modified version of that described by Hoitink and Sinden (14) and contained the following nutrients (per liter): 1.0 g of NH_4Cl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 4.1 g of KH_2PO_4, 3.6 g of $K_2HPO_4 \cdot 3H_2O$, 10 g of D-glucose, and 2 μM $FeCl_3$. The pH of the medium was adjusted to 6.8 with 10 N NaOH before autoclaving. Glucose and $FeCl_3$ were always autoclaved separately. The concentrations of individual nutrients in the basal medium were varied as follows: D-glucose, 1/5, 1/2, 1, 2, and 5 times the basal amount; NH_4Cl, 1/5, 1/2, 1, 2, and 5 times; PO_4, 1/3, 2/3, 1, 2, and 3 times; Mg, 1/25, 1/5, 1, 5, and 25 times; SO_4, 1/25, 1/5, 1, 5, and 25 times; and $FeCl_3$, 0, 1, 5, 20, 60, and 200 μM. Magnesium and sulfate levels were varied separately by replacing the $MgSO_4$ in the basal medium with an equimolar amount of $MgCl_2 \cdot 6H_2O$ and K_2SO_4. Zinc chloride was amended to the medium at 0, 1, 10, 100, 1,000, and 10,000 nM.

Two treatments with very low phosphate levels of 7.2 and 0.72 mM were prepared by adding 1/5 and 1/50 the normal (1×) amounts of phosphate salts, respectively. The buffering capacity of the medium was maintained by adding PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer at 29 and 36 mM for the 7.2 and 0.72 mM phosphate levels, respectively.

The effect of various carbon sources was investigated by replacing the glucose in the basal medium with xylose, fructose, sucrose, inositol, and glycerol. Molarities were adjusted to keep the amount of carbon per liter constant between treatments.

The effect of the following amino acids was investigated by adding them to the basal medium at 3 mM: serine, cysteine, *S*-methylcysteine, phenylalanine, alanine, valine, leucine, isoleucine, asparagine, aspartate, lysine, homoserine, threonine, methionine, homocysteine, ornithine, arginine, glutamate, and glutamine. The effect of nitrate was studied by adding 3 mM KNO_3 to the basal medium. The effect of complex carbon and nitrogen sources was investigated by adding 0.25, 0.025, and 0.0025 g of yeast extract per liter to the basal medium.

Several plant-derived secondary metabolites were added to the medium to determine whether these specifically induced coronatine synthesis. Quercetin, ferulic acid, chlorogenic acid, caffeic acid, naringenin, vanillic acid, flavone, and coniferyl alcohol were each added separately to the basal medium at 1 and 100 μM. In addition, soybean (*Glycine max*) or broad bean (*Vicia faba*) leaf tissue was macerated and extracted into Tris buffer or methanol, and 10- or 100-μl aliquots were added to the basal medium.

The effects of osmolarity, pH, duration of incubation, and temperature on coronatine production were also investigated. The osmolarity of the basal medium (calculated to be 201 mosM) was increased from 0 to 120% with NaCl or KCl and from 0 to 320% with sorbitol. The pH of the basal medium was adjusted to 4.8, 5.8, 6.5, 6.7, 6.8, 6.9, 7.1, 7.8, and 8.8 with 10 M NaOH or concentrated HCl before autoclaving. The pH did not change significantly after sterilization. The effect of incubation time on coronatine biosynthesis was studied by analyzing cultures for coronatine production every 24 h over a 10-day period starting 2 days after inoculation. Data were also taken 13 days after inoculation to assess the stability of coronatine in the growth medium. Temperature effects were investigated by incubating PG4180 broth cultures at 14, 18, 24, and 30°C.

An optimal medium for coronatine production was developed by changing the levels of glucose, NH_4Cl, and $FeCl_3$ in the basal medium and by adding KNO_3. Coronatine production by PG4180 was examined in the following variations of the basal medium: *C* + *N*, Fe + NO_3, *C* + Fe, *N* + Fe, *C* + NO_3, *N* + NO_3, *C* + *N* + Fe, *C* + *N* + NO_3, *C* + Fe + NO_3, *N* + Fe + NO_3, and *C* + *N* + Fe + NO_3, where *C* is the addition of 10 g of glucose per liter for a final concentration of 20 g/liter; *N* is the addition of 1 g of NH_4Cl per liter for a final concentration of 2 g/liter; Fe is the substitution of 2 μM $FeCl_3$ in the basal medium with 20 μM $FeCl_3$; and NO_3 is the addition of 0.3 g of KNO_3 per liter to the basal medium. Modifications resulting in coronatine yields 80% or higher than that observed in the basal medium were investigated further. PG4180 was cultured in these modified media in two additional experiments consisting of two replications.

Growth conditions. Bacterial strains were grown overnight in mannitol-glutamate medium (17) containing 0.25 g of yeast extract per liter at 24°C. After 24 h, 1 ml of the culture (A_{600} of 0.5; approximately 4×10^8 CFU/ml) was pelleted in a microcentrifuge at 14,000 rpm and resuspended in an equivalent volume of isotonic saline (0.85% NaCl). Each 10-ml aliquot of medium was then inoculated with 20 μl of the bacterial suspension. Culture volumes of 10 ml were agitated in 20-ml test tubes at a 45° incline; two duplicate tubes made up one sample (total volume of 20 ml). At least two replicate samples were used in all experiments. Cultures were incubated at 18°C for 10 days at 280 rpm on a rotary shaker.

Extraction technique. Protocols for isolating coronatine are based on the almost exclusive occurrence of this phytotoxin in the organic acid fraction of the culture supernatant. In the standard isolation procedure utilized in the present study, broth cultures (20 ml) were centrifuged at 20,000 × *g* for 15 min, and the supernatants were decanted and saved. Cell pellets were transferred to tared microcentrifuge tubes, dried at 50°C, and weighed. Supernatants were adjusted to pH 9 and extracted twice with 20-ml volumes of ethyl acetate to remove nonacidic hydrophobic compounds. The aqueous phase was then adjusted to pH 2 and extracted three times with 20-ml volumes of ethyl acetate to remove the organic acid fraction. The organic acid phase was then filtered through anhydrous Na_2SO_4 and dried by evaporation. Organic acids were recovered by rinsing the flask twice with 500 μl of 10% acetonitrile in 0.05% trifluoracetic acid. Samples were stored at 4°C prior to high-performance liquid chromatography (HPLC) analysis. Unless otherwise indicated, coronatine titers are divided by the dry weight of the bacterial culture to show coronatine yield as a function of growth. The Student-Newman-Keuls test was used to determine differences in means.

For rapid analysis of samples, an abbreviated extraction protocol was developed. In this procedure, 500 μl of culture supernatant was transferred to a microcentrifuge tube, and 30 μl of 3 N HCl was added to reduce the pH to below 3. The supernatant was washed three times with 0.5 ml of ethyl acetate, and the organic phase was transferred to a second microcentrifuge tube. The ethyl acetate was evaporated from the organic acids by blowing a stream of air into the microcentrifuge tube, which was incubated at 45°C. HPLC starting buffer (250 μl; 10% acetonitrile in 0.05% trifluoracetic acid) was added to resuspend the sample. Samples were

injected into the HPLC without dilution. This technique was used for the medium optimization and strain comparison studies.

HPLC analysis. Samples were analyzed with a Beckman System Gold HPLC system, consisting of dual 116 programmable solvent modules, a 166 programmable detector module, System Gold chromatography interface and software, and an ALTEX Ultrasphere-Octyl C8 column (4.6 by 150 mm). Samples were analyzed in 200-μl volumes in a 250-μl injection loop. Solvents used in the present study were HPLC-grade acetonitrile and an aqueous solution of 0.05% trifluoracetic acid (pH 2.9). The flow rate (2.0 ml/min) and detection wavelength (208 nm) remained constant during all chromatographic separations. Initial solvent concentrations were 10% acetonitrile in 0.05% trifluoracetic acid; this ratio was increased linearly to 50% acetonitrile for 5.5 min. Isocratic conditions (50% acetonitrile) were maintained for 2.5 min, and the system was then returned to initial conditions (10% acetonitrile) in 1 min, using a linear gradient.

A calibration curve for quantifying coronatine was constructed with System Gold chromatography software. The HPLC was calibrated by injecting a dilution series of known coronatine concentration and measuring the A_{208} (UV_{max} for coronatine). The programmed calibration curve made it possible to obtain quantitative information on coronatine yields from the peak areas produced during each chromatographic separation.

Authentic standards of coronafacoylvaline and coronafacoylisoleucine, supplied by Robin Mitchell, Auckland, New Zealand, were used in the qualitative identification of these compounds in selected organic acid extracts.

RESULTS AND DISCUSSION

Observations on physical growth conditions. An agitation speed of 280 rpm and a tube angle of 45° was required to prevent the aggregation and precipitation of bacterial cells. The amount of initial inoculum (5, 20, 50, or 100 μl of PG4180 cells at A_{600} = 0.5 optical density unit) did not affect the ultimate growth of the cultures as determined by measuring pellet dry weight. However, the volume of medium used did significantly affect coronatine production. Increasing the culture volume from 10 to 50 ml decreased coronatine production by 14% (not significant at $P = 0.01$; $n = 3$; least significant difference [LSD] = 15.8%; coefficient of variation [CV] = 27.7), while increasing the volume from 50 to 500 ml significantly decreased coronatine production by 83% ($P = 0.01$; $n = 3$; LSD = 15.8%; CV = 27.7).

Environmental effects on coronatine production. The effect of the incubation period on coronatine production and growth in modified HS medium is shown in Fig. 1. Coronatine synthesis increased until 7 days after inoculation and then remained relatively constant; dry weight increased until approximately 7 days after inoculation and then declined slightly. Coronatine yields were not significantly different when calculated at 10 and 13 days after inoculation, indicating that differential degradation of coronatine did not occur during this experiment. The induction of coronatine synthesis over time was very gradual, unlike the dramatic increase in secondary metabolite production exhibited by *Streptomyces* and *Bacillus* spp. during stationary phase (4). The rapid, marked increase in syringomycin production by *P. syringae* pv. syringae B301D (12) also differs from the gradual increase in coronatine synthesis and more closely resembles the pattern of secondary metabolite production typical of gram-positive bacteria.

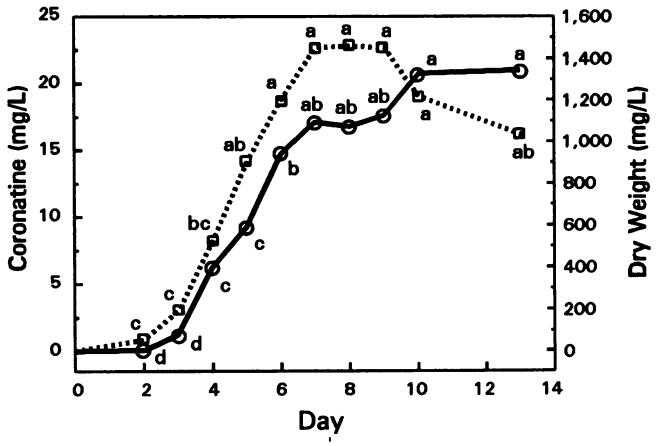

FIG. 1. Production of coronatine (solid line) and dry weight (dotted line) starting at 2 days and ending at 13 days after inoculation. Means are from one experiment of three replicates. Treatments accompanied by the same letter are not significantly different at $P = 0.01$, using the Student-Newman-Keuls test. Coronatine LSD = 4.3, CV = 14.6; dry weight LSD = 450, CV = 20.1.

Coronatine production was maximal at 18°C, significantly less at 24°C, and minimal at 30°C (Fig. 2). Except for slight decreases at 18 and 24°C, bacterial growth was relatively unaffected by the range of temperatures tested. Optimal temperatures for phytotoxin production in *P. syringae* often correlate with optimal temperatures for symptom development in the field (6, 10). Reduced toxin production has also been reported in other plant-pathogen interactions when incubation temperatures were raised to 30°C (20, 25, 28, 36). The reduced toxin yields observed at higher temperatures could be caused by decreased synthesis, or reduced activity, of the enzymes involved in toxin production. Nuske and Fritsche (28) demonstrated that the decrease in phaseolotoxin production at elevated temperatures was due to a decreased synthesis of the producing enzymes and not to

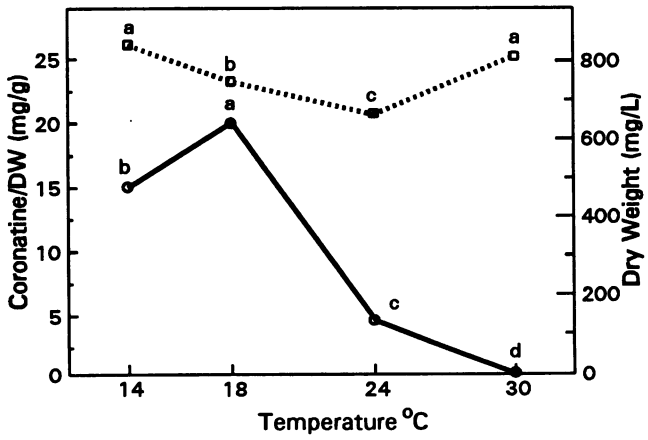

FIG. 2. Coronatine synthesis (solid line) and growth (dotted line) at different temperatures. Means are from two experiments of three replicates each. Treatments accompanied by the same letter are not significantly different at $P = 0.01$, using the Student-Newman-Keuls test. Coronatine LSD = 3.1, CV = 18.4; dry weight LSD = 61, CV = 4.6.

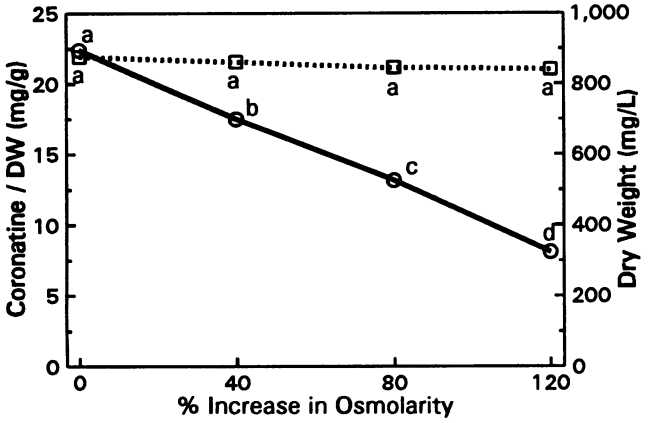

FIG. 3. Effects of osmolarity on coronatine production (solid line) and growth (dotted line). Osmolarity was varied with NaCl. Means are from two experiments of three replicates each plus one experiment of two replicates. Treatments accompanied by the same letter are not significantly different at $P = 0.01$, using SAS General Linear Models. Coronatine LSD = 3.9, CV = 17.2; dry weight LSD = 58, CV = 4.6.

reduced enzyme activity. The cause of decreased coronatine production at elevated temperatures is currently unclear.

No significant effect on growth or coronatine production was observed when the pH of the basal medium was adjusted from 6.5 to 7.8. At pH 5.8, growth was reduced by approximately 65%, but coronatine per unit of dry weight was not affected. At the lower limit (4.8) of the pH range tested, there was no growth; at the upper limit (8.8), the solubility of the medium was unstable and precipitation occurred.

When NaCl was used to increase the osmolarity of the medium from 0 to 120% above the basal level, there was a linear decline in coronatine production (Fig. 3). Dry weight was not changed by the range of osmolarities tested (Fig. 3). The decrease in coronatine production with increasing osmolarity was reproduced when KCl was used to adjust osmolarity in a separate experiment. Also, decreases in coronatine production were observed with the addition of 27 mM $MgCl_2$ (a 40% increase in osmolarity), 222 mM glucose (a 110% increase in osmolarity), or 91.8 mM potassium phosphate (a 107% increase in osmolarity).

Sorbitol, a sugar which does not support growth of PG4180, was also used to adjust the osmolarity. However, sorbitol had repressive effects on coronatine production which could not be explained by a change in osmolarity. When sorbitol was added to media at concentrations above 1 mM, coronatine production was reduced by approximately 95%.

Nutritional effects on coronatine production. Coronatine production per unit of dry weight was significantly reduced by high glucose levels (fivefold; Fig. 4A) and high phosphate levels (threefold; Fig. 4C), but not by high ammonium levels (Fig. 4B). Coronatine production per unit of dry weight was not significantly affected when glucose or ammonium was added to the medium at 1/5 or 1/2 the basal level, respectively (Fig. 4A and B); however, coronatine synthesis was significantly reduced when the phosphate level was reduced to 1/2 or 1/3 the basal level (Fig. 4C). Coronatine production was further reduced when PIPES was used as a replacement buffer and the concentration of phosphate was adjusted to 1/5 and 1/50 the basal level.

One explanation for the reduction in coronatine biosynthesis at high concentrations of glucose and phosphate is that carbon catabolite and phosphate repression might function in the regulation of coronatine. However, results from the osmolarity experiments indicate that the reduction in coronatine production at high glucose and phosphate levels may be caused by the resulting increase in medium osmolarity. The asterisks in Fig. 4 indicate the expected levels of coronatine production based on changes in medium osmolarity. The reduction in coronatine synthesis observed at the five- and threefold levels of glucose and phosphate, respectively, can therefore be explained by an increase in the osmolarity of the medium.

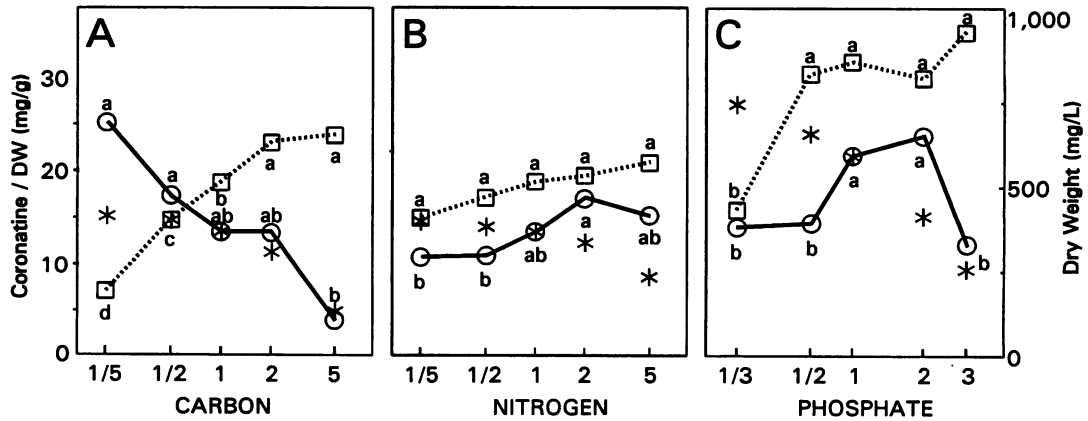

FIG. 4. Effects of glucose (A), ammonium chloride (B), and phosphate (C) on growth and coronatine production by *P. syringae* pv. glycinea PG4180. Solid line, coronatine biosynthesis; dotted line, dry weight. Carbon and nitrogen means are from three experiments of two replicates each; phosphate means are from two experiments of two replicates each plus one experiment of three replicates each. Treatments accompanied by the same letter are not significantly different at $P = 0.01$, using the Student-Newman-Keuls test (carbon and nitrogen data) or SAS General Linear Models (phosphate data). Carbon data, coronatine LSD = 9.8, CV = 38.7; dry weight LSD = 55, CV = 6.7. Nitrogen data, coronatine LSD = 3.7, CV = 16.0; dry weight LSD = 131, CV = 15.3. Phosphate data, coronatine LSD = 4.1, CV = 15.2; dry weight LSD = 173, CV = 14.1. Asterisks indicate expected coronatine production based on calculated changes in the osmolarity of the medium and the osmolarity response data in Fig. 3.

TABLE 1. Effects of various carbon sources on growth and coronatine yields of *P. syringae* pv. glycinea PG4180

Carbon source	Coronatine yield (mg/g, dry wt)[a]	Dry wt (mg/liter)[a]
Glucose	11.1 a	700 b
Sucrose	7.0 b	1,024 a
Glycerol	4.7 c	668 b
Inositol	4.2 c	257 c
Fructose	3.5 c	743 b
Xylose	0.4 d	325 c
LSD	2.16	95
CV	32.7	11.9

[a] Means for three experiments of three replicates each; treatments within a column followed by the same letter are not significantly different at $P = 0.01$ (Student-Newman-Keuls test).

Moreover, the high level (135 mM) of phosphate required for a reduction in coronatine synthesis is not consistent with levels reported previously for phosphate repression (5, 12, 32). Therefore, the reduction in coronatine production at 135 mM phosphate is more likely caused by high osmolarity in the growth medium than by phosphate repression. The relationship between coronatine synthesis and osmolarity resembles the downregulation of hypersensitivity and pathogenicity (*hrp*) loci in *X. campestris* pv. vesicatoria and *P. syringae* pv. phaseolicola by increased osmotic conditions in the growth medium (31, 35).

Sucrose, glycerol, inositol, fructose, and xylose, chosen because of their presence in planta (8), were used to replace glucose as the sole carbon source in the basal medium. All five significantly reduced coronatine production (Table 1), with xylose resulting in the least coronatine being produced. Bacterial growth was significantly decreased with inositol and xylose, but was significantly higher when sucrose was used as a carbon source (Table 1). A stimulatory or repressive effect by various carbon sources has been demonstrated previously for other factors which control host specificity and virulence in *P. syringae*. Transcription of *avrB* in *P. syringae* pv. glycinea was highest in media containing sucrose or fructose and lower in media containing glucose or glycerol (15). The *syrB* gene in some strains of *P. syringae* pv. syringae was also induced by sucrose and fructose, but was not activated by glucose or xylose (26). Expression of *hrpAB*, *hrpC*, *hrpD*, *hrpE*, and *hrpF* in *P. syringae* pv. phaseolicola was relatively high in media containing sucrose or fructose as the sole carbon source (31). In the present study, glucose was the preferred carbon source for maximum production of coronatine. The use of sucrose or fructose as a carbon source resulted in significantly lower levels of coronatine (Table 1). Consequently, it is not clear whether transcriptional activation of coronatine synthesis genes has any similarity to the transcription of *avrB*, *syrB*, and various *hrp* loci.

The supplementation of the basal medium with 3 mM KNO_3 significantly increased coronatine titers (Table 2). However, when asparagine, glutamate, isoleucine, leucine, and valine were added to HS media at 3 mM, coronatine production was significantly reduced (Table 2). The addition of isoleucine and valine resulted in increases in the heights of peaks corresponding to coronafacoylisoleucine and coronafacoylvaline, presumably because of the coupling of these amino acids with coronafacic acid (24). However, unlike the results obtained with isoleucine, the addition of exogenous valine caused a dramatic decline in the yield of all coronafacoyl compounds, suggesting that a mechanism other than competition between valine and coronamic acid is responsible for the reduced synthesis. A reduction in coronatine synthesis was also observed when leucine, methionine, asparagine, glutamate, threonine, homocysteine, and *S*-methylcysteine were added exogenously (Table 2). With the exception of leucine and methionine, no additional peaks were observed by HPLC when these amino acids were added, suggesting that the reduction of coronatine titers by these compounds is probably not due to competition with coronamic acid. Although the addition of leucine and methionine did result in the appearance of new peaks, the identity of the compounds eluting in the additional peaks was not determined.

TABLE 2. Effects of various amino acids and KNO_3 on coronatine production and growth of *P. syringae* pv. glycinea PG4180[a]

Data set	Supplement	Coronatine yield (mg/g, dry wt)[b]	Dry wt (mg/liter)[b]
1	KNO_3	27.4 a	820 d
	NH_4Cl	17.3 b	1,063 b
	Glutamine	16.9 bc	950 b
	Aspartate	16.2 bc	868 cd
	Lysine	13.4 bcd	1,183 a
	Asparagine	13.2 cd	933 c
	Glutamate	12.2 d	899 cd
	Isoleucine	8.2 e	879 cd
	Leucine	4.9 f	838 d
	Valine	0.1 g	1,076 b
	LSD	3.0	69
	CV	11.3	3.5
2	NH_4Cl	19.3 a	963 bc
	Alanine	18.1 ab	911 bc
	Serine	17.6 ab	889 c
	Ornithine	13.2 b	998 bc
	Threonine	8.5 c	1,380 a
	Homocysteine	6.6 cd	1,033 b
	S-methylcysteine	4.5 cd	913 bc
	Methionine	1.9 d	918 bc
	Cysteine	—[c]	—
	Homoserine	—	—
	LSD	4.4	99
	CV	19.0	4.7

[a] Amino acids and KNO_3 were added to the basal medium at 3 mM.

[b] Means for two experiments of two replicates each; treatments within a column followed by the same letter are not significantly different at $P = 0.01$ (Student-Newman-Keuls test).

[c] —, treatment was toxic to *P. syringae* pv. glycinea PG4180 culture and resulted in no growth.

Our previous observations had indicated that PG4180 produces substantially less coronatine in media containing yeast extract. When 0.25, 0.025, or 0.0025 g of yeast extract per liter was added to the basal medium in the present study, coronatine production was reduced by 34, 17, and 7%, respectively (34% was significant at $P = 0.05$; $n = 4$; LSD = 17%; CV = 11.6). Several investigators have reported the reduced expression of *P. syringae* virulence factors when complex sources of carbon and nitrogen are added to the growth medium. For example, expression of the *hrp* genes in *P. syringae* pv. syringae 61 (39), *P. syringae* pv. phaseolicola (11), *X. campestris* pv. vesicatoria (35), and *Erwinia amylo-*

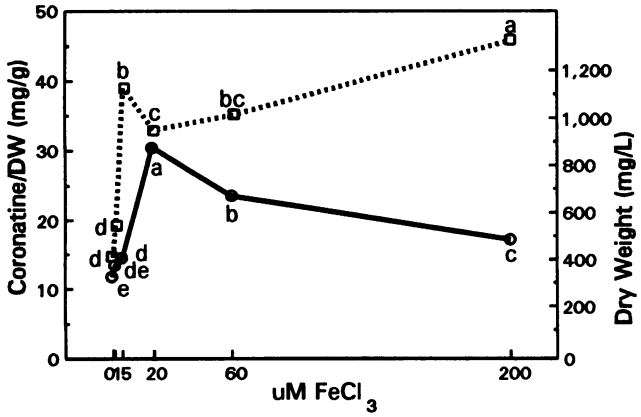

FIG. 5. Effects of varying the iron concentration ($FeCl_3$) in the basal medium. Solid line, coronatine production; dotted line, dry weight. Means from two experiments of three replicates each are shown. Treatments accompanied by the same letter are not significantly different at $P = 0.01$, using the Student-Newman-Keuls test. Coronatine LSD = 3.0, CV = 8.5; dry weight LSD = 106, CV = 8.7.

vora (38) and *avrB* in *P. syringae* pv. glycinea (15) was reduced in rich broth containing complex carbon and nitrogen sources and much higher in nutritionally simple media. Since coronatine production is also reduced in rich media, it may be possible that pathogenicity and virulence genes are globally regulated in *P. syringae*.

With one exception, variations in magnesium or sulfate levels did not significantly affect coronatine production or growth. When the sulfate level was reduced to 4% of that contained in the basal medium, coronatine yields and culture growth were significantly reduced to 36 and 52% of that produced in the basal medium, respectively ($P = 0.01$; n = 4; coronatine LSD = 13.7%, CV = 6.9; dry weight LSD = 16.6%, CV = 8.4).

The effects of iron and zinc, which are not included in the original HS recipe (14), were also investigated in the present study. The addition of iron to the original HS medium had substantial effects on coronatine production and growth (Fig. 5). The growth curve exhibited three phases: a sharp increase in growth when $FeCl_3$ was added at 0 to 5 μM, a decrease in growth from 5 to 20 μM $FeCl_3$, and a gradual increase in growth from 20 to 200 μM (Fig. 5). Production of coronatine as a function of $FeCl_3$ concentration increased sharply from 0 to 20 μM and then declined from 20 to 200 μM. The addition of zinc sulfate to the basal medium had no effect on coronatine synthesis or growth.

The induction of coronatine synthesis by iron resembles the effect of iron on syringomycin production in *P. syringae* pv. syringae B301D (12). Syringomycin production was low at 0 μM $FeCl_3$, but increased dramatically when iron was amended to the medium at 0.5 to 2 μM. No further increase in syringomycin production was observed when the iron concentration was increased to 100 μM. Our results also suggest that the addition of iron is largely responsible for the increased coronatine synthesis observed in the optimized medium. For example, the three medium formulations which resulted in enhanced coronatine production each contained $FeCl_3$ at 20 μM (see below and Table 3).

Development of an optimal medium for coronatine production. The results of the previous experiments were used to design a medium for maximal coronatine production. Since higher concentrations of iron, glucose, ammonium chloride, and KNO_3 increased coronatine production, different combinations of increased iron (20 μM $FeCl_3$), increased glucose (twice the basal amount), increased ammonium chloride (twice the basal amount), and KNO_3 (3 mM) were tested. The following three treatments resulted in coronatine yields at least 80% higher than that obtained in the basal medium: (i) increased glucose plus added $FeCl_3$; (ii) KNO_3 plus additional $FeCl_3$; (iii) increased glucose, added KNO_3, and additional $FeCl_3$. When these three treatments were replicated, it was found that increased glucose, KNO_3, and additional $FeCl_3$ gave the highest yield of coronatine (Table 3). The optimized medium, which contains 20 g of glucose, 1 g of NH_4Cl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 4.1 g of KH_2PO_4, 3.6 g of $K_2HPO_4 \cdot 3H_2O$, and 0.3 g of KNO_3 per liter and 20 μM $FeCl_3$, was named HSC medium (HS optimized for coronatine production).

TABLE 3. Coronatine production by *P. syringae* pv. glycinea PG4180 in various modifications of HS[a] broth

Medium composition[b]	Coronatine yield (mg/liter)[c]
HS	14.6 b
HS + Fe + NO_3	33.3 a
HS + *C* + Fe	34.1 a
HS + *C* + Fe + NO_3	34.4 a
LSD	6.2
CV	8.6

[a] The HS broth utilized in this experiment was that originally described by Hoitink and Sinden (14) and contained 10 g of glucose, 1 g of NH_4Cl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 4.1 g of KH_2PO_4, and 3.6 g of $K_2HPO_4 \cdot 3H_2O$ per liter.

[b] *C* = 10 g of glucose per liter added to HS medium for a final concentration of 20 g/liter; Fe = 20 μM $FeCl_3$; and NO_3 = 0.3 g of KNO_3 per liter.

[c] Means for two experiments of two replicates each; treatments followed by the same letter are not significantly different at $P = 0.01$ (Student-Newman-Keuls test).

Several different strains were compared for their ability to produce coronatine in the original HS medium (14) and in HSC medium (Fig. 6). All pathovars of *P. syringae* tested produced significantly more coronatine in HSC medium than in the original HS medium, with the exception of *P. syringae* pv. maculicola 438 (Fig. 6) and *P. syringae* pv. tomato DC3000 (data not shown). The most notable results occurred with *P. syringae* pv. glycinea 7a, which produced barely detectable levels of coronatine in the regular HS medium but produced approximately 16 mg/liter in HSC. *X. campestris* pv. phormiicola 4297 and 4293 produced coronafacic acid, coronafacoylvaline, and coronafacoylisoleucine in both HS and HSC; however, coronatine was not produced by these two strains in either medium. *X. campestris* pv. phormiicola, the only xanthomonad known to produce coronafacoyl compounds, was shown previously to synthesize coronafacic acid, coronafacoylvaline, and coronafacoylisoleucine, but not coronatine (23).

High-speed extraction protocol for analysis of coronafacoyl compounds. The high titers of coronatine produced by PG4180 in HSC medium facilitated the development of a rapid extraction protocol. This protocol is many times faster than the traditional organic acid extraction (10 samples can be processed in 30 min rather than in 2.5 h), uses 98% less ethyl acetate, and results in coronatine yields equivalent to the traditional extraction method.

Effects of phenolic compounds and host extracts on coronatine synthesis. Plant phenolic compounds have been shown

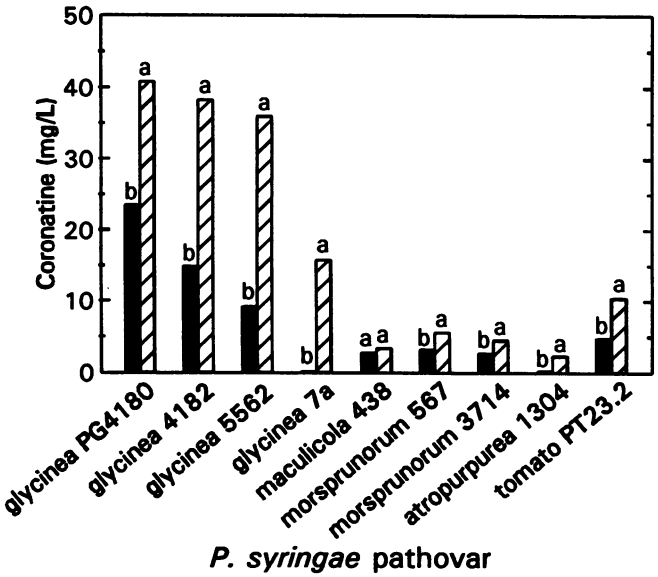

FIG. 6. Coronatine production by various strains in the original HS medium (shaded bars) and in HS medium optimized for coronatine production (HSC; hatched bars). (See text for composition of media.) The vertical axis represents the mean coronatine yield from three experiments of two replications each. When coronatine production was not significantly different for a given strain in HS or HSC, both shaded and hatched bars are accompanied by the same letter ($P = 0.05$, Student-Newman-Keuls test). LSD and CV values, respectively, from the individual tests are as follows: *P. syringae* pv. glycinea PG4180, 4.3, 7; pv. glycinea 4182, 4.6, 9; pv. glycinea 5562, 6.0, 15; pv. glycinea 7a, 2.1, 14; pv. maculicola 438, 7.4, 130; pv. morsprunorum 567, 2.1, 26; pv. morsprunorum 3714, 0.4, 5; pv. atropurpurea 1304, 1.3, 57; and pv. tomato PT23.2, 5.6, 42.

to induce genes involved in pathogen virulence (27, 30). To determine whether specific plant-derived secondary metabolites could induce coronatine synthesis, several phenolic compounds were added to the growth medium. None of the phenolic compounds tested caused a significant change in coronatine biosynthesis. Extracts from both healthy and diseased soybean plants had no significant effect on coronatine biosynthesis when added to the basal medium.

Ma et al. (19) reported previously the plant inducibility of a transcriptional unit required for coronatine production in *P. syringae* pv. tomato DC3000. Although this transcriptional unit, which was designated CorII, is also contained in PG4180 (41), plant inducibility of coronatine synthesis in PG4180 was not demonstrated in the present study. Moreover, strain DC3000 was one of two strains which did not show increased coronatine production in HSC medium. These observations suggest that the signals for induction of coronatine synthesis may differ in PG4180 and DC3000.

The present study has identified various environmental and nutritional factors which influence production of coronatine by *P. syringae* pv. glycinea PG4180. Future research will focus on using promoterless reporter genes to study the effects of environmental, nutritional, and host factors on the regulation of coronatine synthesis. The use of gene fusions will enable the identification of nutritional and environmental factors which regulate coronatine production at the transcriptional level. The results of the present study and data collected from the use of gene fusions in future studies will facilitate a detailed analysis of how coronatine production is controlled by the external environment.

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