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# Nutritional factors regulating growth and accumulation of phenazine 1-carboxylic acid by *Pseudomonas fluorescens* 2-79

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**Summary.** *Pseudomonas fluorescens* strain 2-79 (NRRL B-15132) is a biocontrol agent of take-all of wheat caused by the fungus *Gaeumannomyces graminis* var. *tritici*. Strain 2-79 produces the antibiotic phenazine 1-carboxylic acid, which acts as the primary mechanism of disease suppression. As a first step toward designing efficient methods of mass producing and formulating this biocontrol agent, the regulation of growth and antibiotic production by growth factors (including purines, pyrimidines, vitamins) and minerals (supplying $B^{3+}$, $Ca^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Mo^{6+}$, $Zn^{2+}$) was examined in defined liquid culture. Additions of boric acid and sulfates of iron and magnesium enhanced both cell and associated antibiotic accumulation. However, accumulation of the antibiotic alone improved with additions of zinc sulfate, ammonium molybdate, and cytosine, but worsened with addition of adenine. Interactive effects involving the sulfates of iron, magnesium, and zinc were observed, and optimal iron-magnesium and iron-zinc combinations were demonstrated with respect to biomass and antibiotic accumulation, respectively.

## Introduction

Root diseases such as take-all, *Pythium* root rot, and *Rhizoctonia* root rot are major yield-limiting factors in the production of wheat worldwide (Cook and Veseth 1991). In general, wheat varieties resistant to root diseases are not available and cultural control practices often are not economically feasible. Chemical methods of control face a number of barriers including prohibitive expense (e.g., use of fumigants and benomyl), unreliability in certain regions (e.g., triadimenol and metalaxyl), and public concern over environmental safety. Biological control methods using fluorescent pseudomonads, which have been implicated in the natural biological control known as take-all decline, have great potential as an alternative method of disease control (Weller 1988). When applied with the seed, the introduced pseudomonads colonize the roots and limit primary infection and/or subsequent secondary spread of the target pathogens. In spite of success in control of wheat root pathogens in field trials (Weller 1988), bacterial biocontrol products have not been registered for use on wheat. One impediment to commercialization is the lack of liquid culture technology for mass producing inexpensive and storable biocontrol agents.

*Pseudomonas fluorescens* 2-79, originally isolated from a wheat field in spontaneous take-all decline (Weller and Cook 1983), produces the antibiotic phenazine 1-carboxylic acid (PCA). This antibiotic is active against several soil-borne fungal pathogens including *Gaeumannomyces graminis* var. *tritici* (which causes take-all), *Pythium* spp., and *Rhizoctonia solani* (Weller and Cook 1984; Gurusiddaiah et al. 1986; Brisbane et al. 1987). When applied as a seed treatment, strain 2-79 protects wheat against take-all, and production of PCA has conclusively been shown to be the primary mechanism responsible for disease suppression in the rhizosphere (Bull et al. 1991; Thomashow and Weller 1988; Thomashow et al. 1990).

One objective of liquid culture process development for strain 2-79 is to maximize viable cell accumulation. In addition, however, the degree of PCA accumulation will require optimization to maximize the storage survival and biocontrol efficacy of the cells harvested. On the one hand, phenazine compounds are known to have toxic effects that may play a role in reducing cell viability or seedling emergence rates when applied in microbial seed coatings. On the other hand, cells harvested from cultures actively producing PCA may be more tolerant of PCA toxicity and/or able to enter into PCA metabolism more rapidly once introduced into the rhizosphere. In theory, controlled (non-toxic) levels of PCA in cell formulations may help protect seeds against fungal inva-

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sion during the first hours after planting (i.e., while the seed coat imbibes moisture, and cells gradually reactivate).

In this report, strain 2-79 was tested on defined liquid media to explore the regulation of cell and PCA accumulation by growth factors and minerals. The immediate benefit of knowledge gained from this research was a nutritional basis for controlling the amount of PCA yielded while maintaining a high level of cell production. This knowledge will allow us to design future experiments to investigate the relationship(s) between liquid culture phenazine productivity and the biocontrol efficacy of the stored cell harvest. As a side benefit, the knowledge gained may also be relevant to developing the nutritional environment of seed formulations in order to improve the rhizosphere competence of the bacterium.

## Materials and methods

### *Stock cultures*

Lyophilized *P. fluorescens* 2-79 was obtained from the Agricultural Research Service Patent Culture Collection (National Center for Agricultural Utilization Research, USDA Peoria, Ill., USA) where it is deposited under the number NRRL B-15132. Stock cultures were prepared for storage at −20° C in 2-ml cryovials by mixing equal volumes of 80% glycerol and 24-h culture broth [from single colony inoculum, 50 ml NBY medium (Vidaver 1967), 125-ml flask, 150 rpm]. On a weekly basis, NBY slants were inoculated from glycerol stocks, incubated for 48 h, and then refrigerated for preculture inoculations. All incubations were carried out at 25° C with an initial pH of 6–7 (Gurusiddaiah et al. 1986).

### *Defined medium*

All formulations tested were modifications of a defined medium, which contained the following ingredients per liter: 1 g each of buffers $K_2HPO_4$ and $KH_2PO_4$, 5 g Difco (Detroit, Michigan, USA) vitamin-free casamino acids, 0.05 g tryptophan, 0.2 g cysteine, 0.01 g each of Sigma (St. Louis, Missouri, USA) purines and pyrimidines (adenine, cytosine, guanine, uracil, thymine), 0.5 mg each of vitamins (thiamine, riboflavin, calcium pantothenate, niacin, pyridoxamine, thioctic acid), and 0.05 mg each of vitamins (folic acid, biotin, and $B_{12}$), 0.1 g $MgSO_4 \cdot 7H_2O$, 0.01 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, and 50 g glucose (Zabriskie et al. 1980). Separate stock solutions of buffers, amino acids, purines and pyrimidines, Mg and Na salts, and glucose were prepared and autoclaved at 121° C. The purines and pyrimidines were boiled in 100 ml water with 10 ml concentrated HCl until dissolved, then neutralized to pH 7.0 prior to autoclaving, and diluted to 200 ml. Vitamin and fresh daily iron stock solutions were filter-sterilized using Nalgene (Fisher Scientific, Springfield, New Jersey, USA) 0.22 μm disposable units. Sterile stock solutions were combined promptly to provide a complete medium that was free of precipitates and had a pH of 6.5–7.0.

### *Experimental designs*

*Growth factor dependence.* Biomass and PCA production were examined as a function of the presence or absence of vitamins and purines/pyrimidines. Four combinations of the two-ingredient groups were tested in a $2^2$ factorial design that addressed all of the main and interactive effects. In a follow-up experiment, eight combinations of the five purines and pyrimidines were tested in a $2^5$ regular fractional factorial design that could identify all main effects, but only adenine-thymine interactive effects (Webb 1971).

*Mineral dependence.* The following minerals were studied at the micromolar concentrations given in brackets [corresponding to levels 1, 2, 3, respectively]: $FeSO_2 \cdot 7H_2O$ [36, 180, 360]; $ZnSO_4 \cdot 7H_2O$ [7, 35, 70]; $CoCl_2 \cdot 6H_2O$ [6, 7]; $MnCl_2 \cdot 4H_2O$ [25.3]; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ [0.81, 4.05, 8.1]; $CuSO_4 \cdot 5H_2O$ [6.4]; $CaCl_2 \cdot 2H_2O$ [37.4]; $H_3BO_3$ [19.4, 97, 194]; $MgSO_4 \cdot 7H_2O$ [406, 2028, 4057]. All cultures were provided with 134 μm EDTA as a chelating agent. Initially, the responses of biomass and PCA accumulations were examined in the absence and presence of minerals at concentration level 1. Sixteen combinations of the nine ingredients were analyzed using a $2^9$ regular fractional factorial design that addressed all main effects and Fe-Zn, Fe-Co, Fe-Mn, Fe-Mo, Fe-Cu, and Fe-Ca source interactions (Webb 1971). Test cultures were run at high and low initial cell concentrations.

Significant mineral effects identified in the first experiments were further studied using a $3^3$ partial factorial design that tested all interactions (Webb 1971). Fourteen combinations of three minerals at levels 1, 2, and 3 were examined. The influence of $Zn^{2+}$, $Fe^{2+}$ and $Mo^{6+}$ source combinations on PCA accumulation were analyzed in cultures supplied with high initial cell concentrations, whereas the influence of $Fe^{2+}$, $BO_3^{3+}$, and $Mg^{2+}$ source interactions on both growth and antibiotic accumulation were tested in cultures given low initial cell concentrations. The six minerals not varied in the $3^3$ designs were included at level 1.

### *Test cultures*

Precultures of 50 ml NBY with 50 g glucose/l in 125-ml erlenmeyer flasks were inoculated from slant cultures and incubated at 150 rpm in a New Brunswick (Edison, New Jersey, USA) Psychotherm shaker with 2.6 cm eccentricity. For each medium composition tested, duplicate test cultures were inoculated with washed cells from 48-h precultures. Cells were washed by centrifuging precultures for 5 min at 7000 rpm and resuspending the cell pellets to approx. 2 g dry cells/l in 1 g/l of potassium phosphate buffer. Test cultures were inoculated at low initial cell concentrations (approx. 0.04 g dry weight/l) by adding 1 ml of this washed cell suspension. Other test cultures were inoculated at high initial cell concentrations (approx. 1.2–1.6 g dry weight/l) by pelleting washed cells and resuspending them in the test medium. All test cultures were incubated under the same conditions as precultures. Daily samples were drawn over a period of 1 week to monitor growth and PCA accumulation.

### *Analyses*

*Biomass.* Dry weight accumulation was determined by filtering and drying known volumes of culture on preweighed, dry cellulose nitrate filters. Filter disks were dried at 100° C in a vacuum oven for 1.5–2.5 h to constant weight ± 0.0005 g. Dry weight concentrations (b) were linearly correlated with culture optical density (A), i.e. $b = kA$, where $k = 0.408$ g/l with a correlation coefficient of $r^2 = 0.992$. Samples were diluted as needed to give readings of 0.05–0.50 absorbance units such that Beer's law was linear with cell concentration. Absorbance contributions from fluorescent pigments were negligible.

*Phenazine 1-carboxylic acid (PCA).* Whole culture samples (2.5 ml) were adjusted to pH 4.0 with 1 M HCl and extracted twice with 1.25 ml methylene chloride. The organic layer was transferred to an HPLC vial and allowed to evaporate overnight in a fume hood at room temperature. The PCA residue was dissolved

in 2.5 ml of 5% $NaHCO_2$. HPLC analyses of PCA concentration were carried out using an Alltech (Deerfield, Illionis, USA) Econosphere C18 column (250 mm length, 5-μm particles) eluted at 1 ml/min with (by volume) 65% acetonitrile, 34.9% water, and 0.1% glacial acetic acid at 25° C. A Spectroflow 757 detector (ABI Analytical, Kratos Division, Ramsey, New Jersey, USA) was used to monitor chromatograms at 250 nm.

PCA for HPLC standards was produced by culturing *P. fluorescens* strain 2-79 in the defined medium with 15.75 g amino acids/l and 15 g glucose/l as respective nitrogen and carbon sources. Two-liter antibiotic fermentations were carried out in B. Braun (Melsungen, FRG) Biostat ER2 fermentors with 150 rpm and 215 ml/min stirring and air-flow rates, respectively (oxygen transfer coefficient approx. 0.03 $min^{-1}$). The PCA was extracted from 120-h cultures using the procedure of Chang and Blackwood (1969). The PCA crystals recovered from the final chloroform extraction were dissolved in methylene chloride and adsorbed onto silica (32–63 6A from ICN Biochemicals, Cleveland, Ohio, USA) for further purification as described by Gurusiddaiah et al. (1986). The crystals isolated were confirmed to be PCA via proton nuclear magnetic resonance (NMR) and electron impact (EI) mass spectroscopy, and their purity was indicated by HPLC chromatography. A single peak was detected (350 nm) at 5.6±0.2 min in chromatograms generated using a Dupont (Wilmington, Delaware, USA) Zorbax silica column (4.6 mm ID × 25 cm) eluted at 1.5 ml/min with 90:10 chloroform:acetone at 25° C. Similarly, a single peak at 4.6±0.2 min was observed in chromatograms generated via the PCA assay procedure above. Our purified PCA had a molar extinction coefficient of $9.47 \times 10^4$ l/mol per centimeter [250 nm, 5% (w/v) $NaHCO_3$].

*Statistics.* The relationship of medium composition to growth and PCA accumulation was determined from normal analysis of variance with SAS (SAS Institute, Cary, N. C.). Our significance criterion was $P<0.05$ with borderline cases falling in the range $0.05<P<0.1$. Values representing PCA and biomass accumulation in the graphs provided are averages from all cultures at the ingredient level being tested (Webb 1971).

## Results

### *Growth factors*

As shown in Table 1, vitamins had no significant influence on the accumulation of biomass and PCA. However, the inclusion of purines and pyrimidines increased PCA production without any net effect on growth. When individual purines and pyrimidines were examined, adenine and cytosine were respectively singled out as having significant negative and positive effects on PCA accumulation. Since both compounds were ≥99.9% pure (Sigma, St. Louis, Missouri, USA), trace element contamination was not responsible for their observed effects. Although thymine, guanine, and uracil appeared to enhance PCA accumulation, they did not cause significant changes. Furthermore, there was no significant adenine-thymine interactive effect on the accumulation of biomass and PCA to coincide with A-T pairing in DNA.

**Table 1.** Significance ($P$)[a] of growth factor presence (+) versus absence (−): effects on biomass and phenazine 1-carboxylic acid (PCA) accumulation

| Factorial design | Growth factor | Biomass (g/l) | | | PCA (g/l) | | |
|---|---|---|---|---|---|---|---|
| | | + | − | $P$ | + | − | $P$ |
| $2^2$ | Purines/ pyrimidines | 2.55 | 2.68 | 0.31 | 0.025 | 0.007 | 0.04 |
| | Vitamins | 2.75 | 2.48 | 0.08 | 0.014 | 0.018 | 0.61 |
| $2^5$ | Adenine | 2.50 | 2.45 | 0.54 | 0.113 | 0.198 | 0.05 |
| | Thymine | 2.49 | 2.46 | 0.71 | 0.175 | 0.136 | 0.34 |
| | Cytosine | 2.44 | 2.51 | 0.39 | 0.196 | 0.116 | 0.05 |
| | Guanine | 2.42 | 2.53 | 0.19 | 0.173 | 0.138 | 0.39 |
| | Uracil | 2.53 | 2.43 | 0.23 | 0.156 | 0.156 | 0.99 |

[a] Effects were significant if $P \leq 0.05$

**Table 2.** Significance ($P$)[a] of mineral presence (+)[b] versus absence (−): effects on biomass and PCA accumulation in $2^9$ partial factorial experiments inoculated at low or high initial cell concentrations

| Initial cells (g/l) | Mineral | Biomass (g/l) | | | PCA (g/l) | | |
|---|---|---|---|---|---|---|---|
| | | + | − | $P$ | + | − | $P$ |
| 0.04 | $CaCl_2$ | 1.93 | 2.08 | 0.0003 | 0.013 | 0.007 | 0.615 |
| | $CoCl_2$ | 2.01 | 1.99 | 0.441 | 0.007 | 0.013 | 0.074 |
| | $CuSO_4$ | 2.09 | 1.90 | 0.027 | 0.007 | 0.014 | 0.103 |
| | $FeSO_4$ | 2.38 | 1.67 | 0.0001 | 0.021 | 0.0002 | 0.0005 |
| | $H_3BO_3$ | 2.19 | 1.81 | 0.0001 | 0.020 | 0.0003 | 0.0004 |
| | $MgSO_4$ | 2.14 | 1.86 | 0.0001 | 0.020 | 0.0000 | 0.0003 |
| | $MnCl_2$ | 2.02 | 1.98 | 0.040 | 0.010 | 0.010 | 0.432 |
| | $(NH_4)_6Mo_7O_{24}$ | 2.00 | 2.00 | 0.179 | 0.011 | 0.009 | 0.368 |
| | $ZnSO_4$ | 2.06 | 1.93 | 0.154 | 0.013 | 0.007 | 0.594 |
| 1.2–1.6 | $CaCl_2$ | 3.31 | 3.45 | 0.168 | 0.009 | 0.013 | 0.060 |
| | $CoCl_2$ | 3.45 | 3.31 | 0.217 | 0.013 | 0.008 | 0.060 |
| | $CuSO_4$ | 3.32 | 3.43 | 0.310 | 0.010 | 0.012 | 0.238 |
| | $FeSO_4$ | 3.20 | 3.54 | 0.003 | 0.022 | 0.0003 | 0.0001 |
| | $H_3BO_3$ | 3.58 | 3.16 | 0.0004 | 0.008 | 0.013 | 0.075 |
| | $MgSO_4$ | 3.47 | 3.27 | 0.042 | 0.008 | 0.013 | 0.078 |
| | $MnCl_2$ | 3.32 | 3.43 | 0.296 | 0.011 | 0.011 | 0.096 |
| | $(NH_4)_6Mo_7O_{24}$ | 3.38 | 3.37 | 0.952 | 0.013 | 0.008 | 0.009 |
| | $ZnSO_4$ | 3.38 | 3.37 | 0.884 | 0.016 | 0.005 | 0.002 |

[a] Effects were significant if $P \leq 0.05$

[b] Minerals were added at concentration level 1

*Minerals*

Biomass and PCA accumulations in cultures inoculated to low cell concentrations were significantly increased by $FeSO_4$, $MgSO_4$, and $H_3BO_3$ additions, although growth was reduced by $CaCl_2$ addition (Table 2). The accumulations of PCA and biomass were significantly correlated with a linear $r^2$ value of 0.72 (i.e., minerals that enhanced growth were also the key to subsequent PCA accumulation). Presence-absence testing of the nine minerals was repeated in cultures inoculated to high cell concentrations. Under these conditions, $FeSO_4$, $H_3BO_3$, and $MgSO_4$ again influenced biomass accumulation (Table 2). However, in contrast to the low cell inoculum experiment, the significance levels were lower (i.e., higher $P$ values), and the addition of 36 μM $FeSO_4$ had a negative influence. With respect to antibiotic production, $FeSO_4$ was essential, while $ZnSO_4$ and $(NH_4)_6Mo_7O_{24}$ brought improvements (Table 2). Significant interactions of iron with zinc and molybdenum sources were observed. Average PCA accumulations were increased from 0.010 to 0.031 g/l ($P=0.0021$) and from 0.016 to 0.029 g/l ($P=0.0087$) by adding $ZnSO_4$ and $(NH_4)_6Mo_7O_{24}$, respectively, to cultures containing $FeSO_4$. Other minerals supplying $Co^{2+}$, $Mn^{2+}$, and $Cu^{2+}$ had small or no effects on culture yields, but it is possible that trace amounts provided as impurities may have precluded any benefit of further additions.

The dependence of growth and PCA accumulation on combinations of $FeSO_4$, $MgSO_4$, and $H_3BO_3$ (at three concentration levels each) were further characterized in cultures inoculated to low cell densities. Although borate had only a marginal effect ($P=0.070$) over the concentration range tested, biomass accumulation was enhanced by the combination of 36 μM $FeSO_4$ with 406 μM $MgSO_4$. Since the addition of higher levels (Fig. 1A) or the exclusion (Table 2) of either mineral led to reduced biomass accumulation, the existence of an optimal Fe-$MgSO_4$ combination was indicated with respect to growth. Similarly, additions of 36 μM $FeSO_4$, 406 μM $MgSO_4$, and 19 μM $H_3BO_3$ maximized PCA accumulation (Fig. 1B, Table 2).

The dependence of PCA accumulation on combinations of $FeSO_4$, $(NH_4)_6Mo_7O_{24}$, and $ZnSO_4$ (at three concentration levels each) was observed in cultures inoculated at high cell concentrations. These results confirmed the significance of $FeSO_4$, $ZnSO_4$, and their interaction, and suggested 36 μM $FeSO_4$ and ≥70 μM $ZnSO_4$ as an opimal combination for PCA accumulation (Fig. 2). Although the presence of molybdenum was shown to be beneficial (Table 2), augmenting the addition of $(NH_4)_6Mo_7O_{24}$ from 1 μM to 10 μM provided no further improvement in antibiotic accumulation. None of the three minerals significantly affected biomass accumulation in this experiment (data not shown).

Although we did not explicitly test for sulfur requirement, our results indicated that the sulfates of Mg, Fe, and Zn played key roles in regulating the metabolism of strain 2-79. Since the interaction of trace $FeSO_4$ (36 μM) and $ZnSO_4$ (70 μM) optimized PCA accumulation in both the presence and absence of the macronutrient

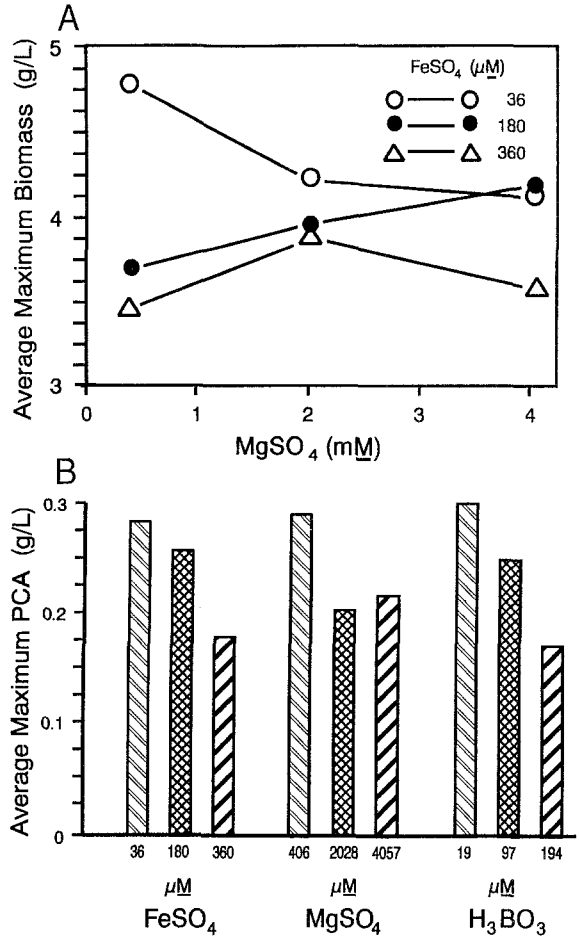


**Fig. 1A, B.** Effects of $FeSO_4$, $MgSO_4$, and $H_3BO_3$ on accumulation of biomass and phenazine 1-carboxylic acid (PCA) as demonstrated by a $3^3$ partial factorial experiment. **A** Biomass production was significantly influenced by $FeSO_4$ ($P=0.0001$) and its interaction with $MgSO_4$ ($P=0.0037$): ○, 0.36 μM; ●, 180 μM; △, 360 μM $FeSO_4$. **B** PCA accumulation was optimized by choice of $FeSO_4$ ($P=0.0039$), $MgSO_4$ ($P=0.0002$), and $H_3BO_3$ ($P=0.0085$) addition levels

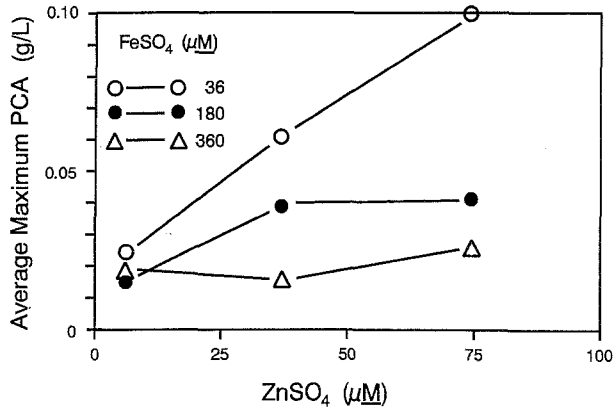


**Fig. 2.** Significant effects of $FeSO_4$ ($P=0.01$), $ZnSO_4$ ($P=0.025$), and their interaction ($P=0.044$) on PCA accumulation as demonstrated by a $3^3$ partial factorial experiment in which cultures were inoculated to provide high initial cell concentrations: symbols as in Fig. 1

$MgSO_4$ (406 μM), sulfur did not appear to play a significant role in regulating secondary metabolism. However, the experiments demonstrating growth promotion by $MgSO_4$ do not prove whether Mg, S, or both were responsible. Note also that mineral level "optima" reported here should not be interpreted as absolute quantities since we have tested only four levels of each mineral and have always referred to concentrations "added," exclusive of impurities and factors influencing "availability" (i.e., siderophore(s), EDTA, cell/glass surface chemistries, etc.).

## Discussion

Despite intensive study of *P. fluorescens* 2-79 in the rhizosphere, this is the first report on the nutritional requirements of both cell growth and PCA production in liquid culture. The two processes had different growth factor and mineral needs and were subject to manipulation via the medium composition. Both growth and antibiotic production increased with additions of $MgSO_4$, $H_3BO_3$, and $FeSO_4$. Since PCA accumulations in cultures inoculated to high cell concentrations were independent of $H_3BO_3$ and $MgSO_4$, these two minerals are probably best interpreted as being required for growth and, thus, indirectly beneficial to PCA accumulation. The optimal 36 μM iron requirement of strain 2-79 was essential for and typical of bacterial growth and secondary metabolism (Weinberg 1977). Unexpectedly, however, both $ZnSO_4$ and $(NH_4)_6Mo_7O_{24}$ interacted with iron to maximize PCA accumulation without affecting growth. Excepting actinomycetes, the requirement of an iron-zinc combination for optimal bacterial secondary metabolism is unusual (Weinberg 1977, 1986). Like other *Pseudomonas* spp., strain 2-79 grew well without added growth factors (Palleroni 1985); PCA accumulation by 2-79, on the other hand, was specifically enhanced by cytosine and deterred by adenine.

The existence of mineral-level optima suggests one reason for the inconsistency of *P. fluorescens* 2-79 in its performance against take-all from field to field (Weller 1988). Soils, even of the same structural class can vary considerably in the amounts of trace minerals. Although variable root colonization accounts for some of the inconsistency (Weller 1988), we speculate that trace mineral effects may also cause variability in PCA production, the primary mechanism of take-all suppression (Bull et al. 1991). Consequently, seed formulation techniques aimed at controlling the mineral and purine-pyrimidine environment of encapsulated cells may be of interest in future research endeavors to improve the field performance consistency of strain 2-79.

Most importantly, our defined media studies have shown that levels of $ZnSO_4$, $(NH_4)_6Mo_7O_{24}$, cytosine, and adenine can be manipulated to control the amount of PCA accumulated during liquid culture cell production. Given this new knowledge, media can now be designed to test the influence of nutritionally regulated PCA productivity on the capacity of the cell harvest to endure storage and protect wheat seedlings against take-all. Currently, the relationship between liquid culture PCA productivity and the biocontrol efficacy of the stored cell harvest is poorly understood. Yet, the elucidation of this relationship is essential for designing a liquid culture media for mass producing strain 2-79 as an effective biocontrol agent.

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