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Isolation of Enterobacteria, *Azotobacter* sp. and *Pseudomonas* sp., Producers of Indole-3-Acetic Acid and Siderophores, from Colombian Rice Rhizosphere

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ABSTRACT. Ethyl acetate extracts from superimposed liquid concentrated cell cultures of *Azotobacter chroococcum, Azotobacter vinelandii, Pseudomonas aeruginosa, Pseudomonas putida, Serratia* sp. and *Klebsiella pneumoniae* strains, obtained from rhizosphere of rice cultivated in the Tolima region, Colombia S. A., have shown to be producers of extra cellular indole-3-acetic acid (IAA) at concentrations from 3.5 mg/mL to 32.2 mg/l. *A. vinelandii,* and *K. pneumoniae* yielded the highest concentrations. *Pseudomonas* sp. was found *in vitro* to antagonize the *Phytophthora infestans*, mainly by production of siderophores under low presence of iron. Colonization of hyphae and production of antibiotics were additional activities observed.
Key words: indole-3-acetic acid, siderophores, *Azotobacter chroococcum, Azotobacter vinelandii, Pseudomonas aeruginosa, Pseudomonas putida, Serratia* sp., *Klebsiella pneumoniae*.

RESUMEN. Extractos concentrados de acetato de etilo de cultivos de cepas de *Azotobacter chroococcum, Azotobacter vinelandii, Pseudomonas aeruginosa, Pseudomonas putida, Serratia* sp. and *Klebsiella pneumoniae* obtenidas de la rizósfera del arroz cultivado en la región de Tolima, Colombia S.A., han producido ácido indol-3-acético (IAA) a concentraciones de 3.5 mg/ml to 32.2 mg/l. *A. vinelandii* y *K. pneumoniae* produjo las más altas concentraciones. *Pseudomonas* sp. Se encontró que *in vitro* antagoniza a *Phytophthora infestans*, principalmente por la producción de sideróforos bajo bajas concentraciones de fierro. La colonización de hifas y producción de antibióticos fueron actividades adicionales observadas.
Palabras clave: ácido indol-3-acético, sideróforos, *Azotobacter chroococcum, Azotobacter vinelandii, Pseudomonas aeruginosa, Pseudomonas putida, Serratia* sp, *Klebsiella pneumoniae*.

INTRODUCTION

Some microorganisms of soil, like *Azospirillum* sp.,[18] *Enterobacter* sp., *Azotobacter* sp., *Pseudomonas* sp.,[9,16] *Klebsiella* sp., *Alcaligenes faecalis, Azoarcus* sp., *Serratia* sp., cyanobacteria and sulfur oxidizing bacteria have shown to encourage plant growth,[1,6] by promoting the outbreak of secondary roots, acting as protectors against phytopathogenic microorganisms via plant hormones release and siderophores.[2,8,13,3,5] In this work some members of Enterobacteriaceae, *Azotobacter* sp. and *Pseudomonas* sp. were isolated from the identified Colombian rice rhizosphere, with production *in vitro* of indole-3-acetic acid and siderophores evaluated by colorimetry, TLC, densitometry and bioassays.

MATERIALS AND METHODS

Sampling. Microorganisms were collected from rhizosphere of twenty rice plants, chosen using the zigzag technique in a large plantation (approximately 10^5 m^2) in Espinal region of Tolima, Colombia, S.A., after two months of planted.

Microorganisms isolation and identification. Samples of 10 g of rhizosphere (roots and soil) were shaken with 90 ml of culture broth; tripticase soy was used for *Pseudomonas* sp, EMB for Enterobacteria and Asbhy and Asbhy-benzoate solutions for *Azotobacter* sp. An aliquot of 1 ml from each broth was added to a selective medium to purify the bacteria. Identification of grown isolated colonies was based on morphologic, biochemical and culturing characteristics.

Strains and media conditions. *Serratia* sp. and *Klebsiella pneumoniae* were grown in agar EMB (Oxoid), *Pseudomonas fluorecens* and *Pseudomonas putida* in King B (BBL) and *Azotobacter chrocoocum* and *Azotobacter vinelandii* in agar Ashby and agar Ashby-benzoate, respectively.

Indole-3-acetic acid production. This was obtained from 3 days and 20°C cultures of Enterobacterias and *Pseudomonas* sp., in Tripticase soy supplemented with 0.2 % powdered soy (tryptophan source) and from *Azotobacter*

sp., in Ashby broth supplemented with 0.5% soy flour.

Colorimetric analysis. After centrifugation (100 rpm. 20 min), the liquid portion of an aliquot of each broth was mixed with Salkowski reagent (2:1) and the developed (30 min) color was measured by spectroscopy at 530 nm.[12] Concentrations were calculated from an adjusted calibration curve. In separate experiments, quantitative spectroscopic analysis was done, also at 530 nm, after isolation of IAA by TLC, scraping its colored spot and dissolution in methanol.

Thin layer chromatography. In a successful approach, the concentrated (4:1) aliquots (100 ml) of the liquid portion of centrifuged sample of each broth were brought to pH 3.0 and extracted three times with ethyl acetate. The organic phase was concentrated to dryness and then diluted with 0.5 ml methanol. Application of this solution on silica gel G plate (20 cm x 5 cm) was diluted as red band with mixture of chloroform-ethyl acetate-formic acid (5:3:2) and developed with Salkowiski reagent giving the correct Rf value (0.57).

Densitometry. This technique was used on TLC spots, to estimate the relative concentration.

Siderophores *In vitro* production. This was evaluated by *Pseudomonas* sp. antagonistic capacity against *Phytophthora infestans* strains. *Pseudomonas* sp. was grown in King B agar and King B agar modified with $FeCl_3$ (1.36 ppm), on circled lines close to the border of the dish. Eight days before the same agar dishes had been spread at their center points with *P. infestans*. The dishes were left at 28° C, during 8 additional days and inhibition zones were measured.

Hyphae colonization and antagonistic capability.[17] Supernatants resulted from cultures of *Pseudomonas* sp., left at 25°C during 5 days in King B broth with centrifugation (100 rpm, 20 min) were spread on several Petri plates, each containing a strain of *P. infestans* in an agar medium and incubated at 25°C during eight days. The inhibition zones were measured and calculated as percentages. The antagonistic effect of *Pseudomonas* sp. was estimated as follow: On a plate containing a strain of *P. infestans*, grown around and close to the border during eight days at 25°C in agar – potato medium, bacteria were in line inoculated near the fungus and incubated again at 25°C and during additional eight days. The inhibition zone after measured were calculated as percentages and compared to *P. infestans* grown in absence of bacteria.

RESULTS AND DISCUSSION

Bacteria in rhizosphere of Colombian rice. An evaluation of rice rhizosphere from Espinal, Tolima, Colombia, showed to included 69 bacteria; among them, 51% were *Pseudomonas* sp., mainly of species *P. putida, P. aeruginosa, P. fluorescens* and *P. citchori*; 26% were of genus *Azotobacter* sp., species, *A. vinelandii, A. chroococcum* and *A. nigrificans;* 21% were enterobacteria of genus

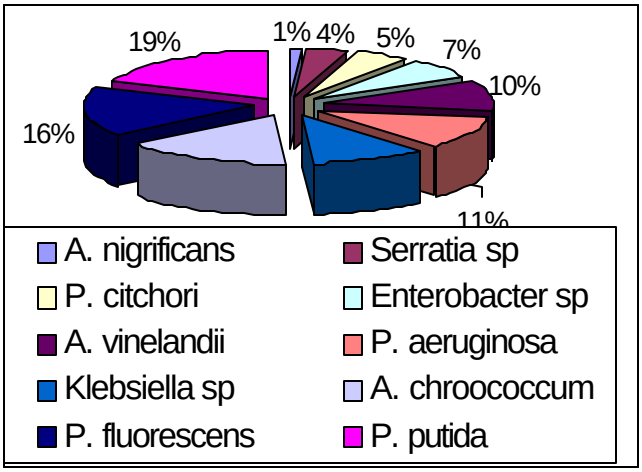

Fig. 1. Percentage distribution of the isolated microbial population.

Fig 2. Samples of the attained solutions of indole-3-acetic acid.

Klebsiella sp., *Serratia* sp., *Enterobacter* sp. These results, seen in Fig. 1, have some similarities with those found elsewhere for rhizosphere of maize.[3] The aforesaid bacteria have been recognized to possess plant growing promotion properties.[4]

Production of indole-3-acetic acid. All forty treated strains (15 Enterobacteria species, 19 *Azotobacter* sp. and 6 *Pseudomonas* sp.) in a culture medium containing soy flour as tryptophan source, not used before, produced IAA, as detected by the Salkowski reagent under colorimetry,[12] in the range 3.5 mg/l to 32.2 mg/l. Fig. 2 shows four samples of the attained solutions of this compound. The highest concentrations of IAA was obtained from *A. chroococcum*, 1-7 (32.2 mg/l – 16.1 mg/l; *A. vinelandii* (32.2 mg/l – 21.2 mg/l); *P. putida*, 1-3 (28.7 mg/l – 14.8 mg/l); *P. aeruginosa* (21.2 mg/L) and *K. pneumoniae* (15.2 mg/l). Table 1 contains the IAA concentrations found in the superimposed liquid and in ethyl acetate extract, derived

Table 1. Indole-3-acetic acid concentration found in superimposed liquid and in ethyl acetate extract derived from the cultures of the most productive species of some genus.

Strains	Superimposed liquid, by colorimetry	Ethyl acetate extract		
		Colorimetric	Colorimetric, (after preparative TLC)	Densitometry (after TLC)
Enterobacter sp. (Non specified)	10.2	6.5	5.8	8.9
K. pneumoniae	15.2	10.6	9.4	12.6
P. putida 1	28.7	3.6	3.1	69.4
P. putida 2	21.2	2.8	2.3	49.2
Ps. aeruginosa	21.2	3.3	3.0	40.5
A. chroococcum 1	32.2	19.3	16.8	26.7
A. chroococcum 2	29.5	17.8	15.6	23.0
A. chroococcum 3	25.6	15.1	12.2	20.2
A. chroococcum 4	25.6	15.0	12.1	19.8
A. chroococcum 5	21.7	12.6	11.2	17.5
A. chroococcum 6	17.0	10.1	9.0	13.6
A. chroococcum 7	16.1	9.8	8.8	12.7
A. vinelandii 1	32.2	19.9	17.4	26.7
A. vinelandii 2	28.7	17.7	15.5	24.6
A. vinelandii 3	21.2	13.1	11.2	17.6

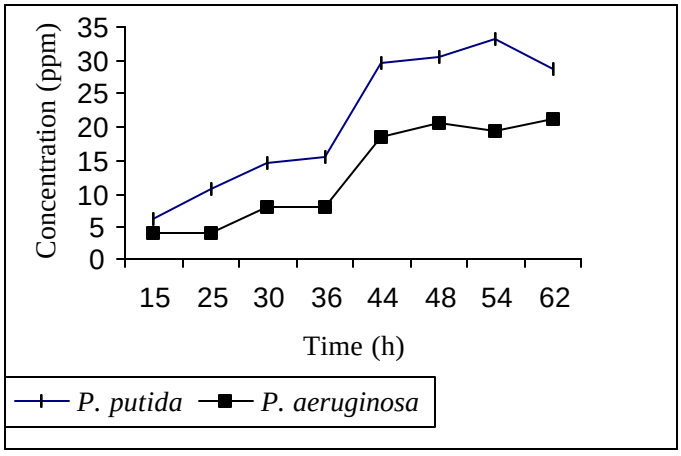

Fig. 3. *Pseudomonas* species AIA production curves.

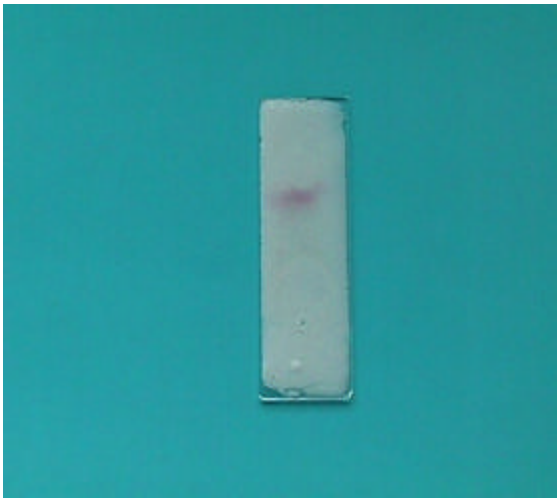

Fig. 4. TLC of indole-3-acetic acid detected by Salkowiski reagent

Table 2. AIA production by isolated strains.

Time (h)	Strains					
	A. chroococcum	*A. vinelandii*	*S. rubidae*	*K. pneumoniae*	*P. putida*	*P. aeruginosa*
	AIA production (ppm)					
12			0.87	0.87		
15					6.41	3.97
18			2.96	2.48		
24	2.72	4.75	4.75	3.97		
25					10.87	3.97
30			6.41	6.12	14.75	8.21
36	10.17	12.72			15.62	8.21
38			6.99	6.99		
42			9.17	8.21		
44					29.50	18.46
48	14.75	16.07	13.92	13.11	30.37	20.59
54			16.07	18.46	33.21	19.50
60	20.59	22.34				
62			25.62	28.67	28.67	21.16
72	25.62	32.22				
84	29.50	34.25				
94	30.37	33.21				

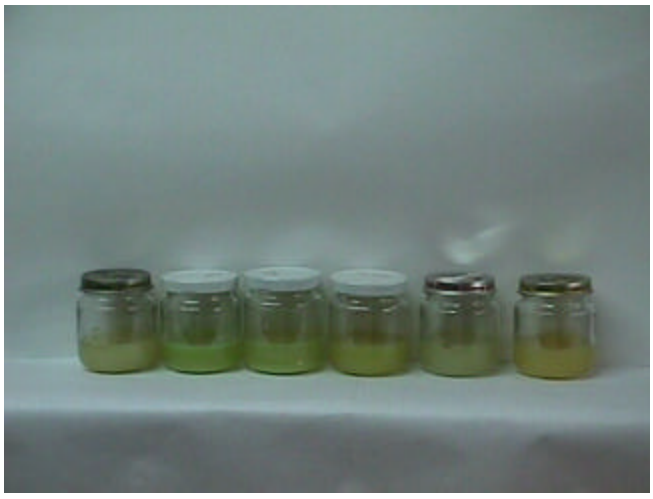

Fig. 5. Siderophores production by different species of *Pseudomonas*

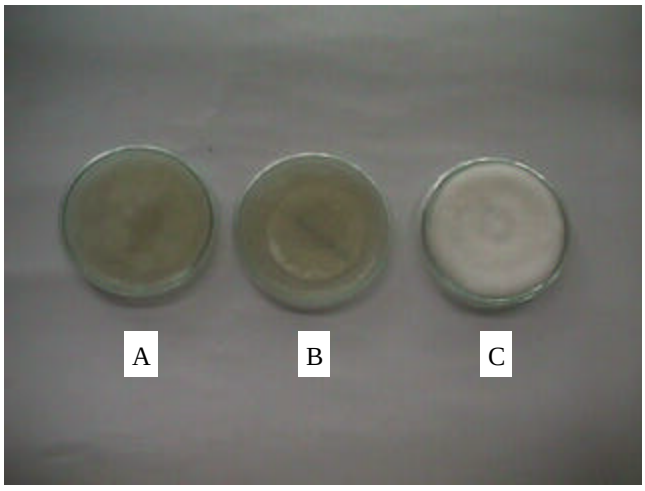

Fig. 6. Colonization and inhibition of *Phytophthora infestans* (C) growth by *P. aeruginosa* (A) and *P. putida* 3 (B)

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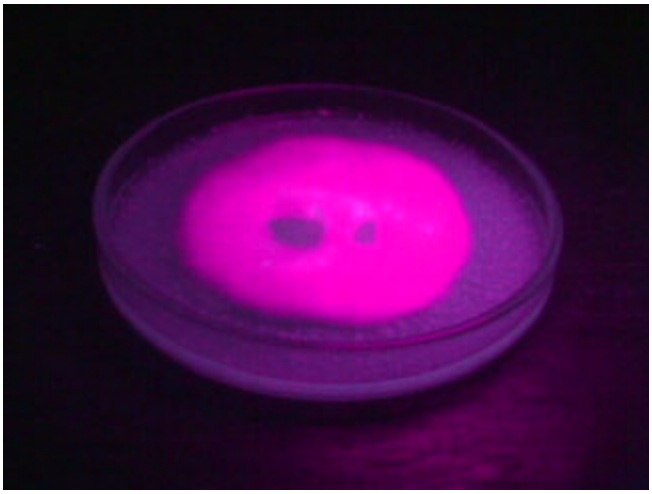

Fig. 7. Inhibition of *Phytophthora infestans* by *P. putida* 2. observed under UV light.

from cultures of some genus most productive species. Highest IAA concentrations were obtained at 75 h for *A. chroococum* and *A. vinelandii*; at 62 h for *P. putida*; at 48 h for *P. aeruginosa* and 60 h for *K. pneumoniae*, as estimated by production curves (concentration versus time, table 2 and fig. 3). Results for *A. chroococum* and *A. vinelandii* agree with those of Lee et al. (1970).[11] IAA was isolated by TLC and detected as a red spot by the Salkowiski reagent (R_f: 0,57) as seen in Fig. 4. This technique also indicated the presence of some IAA derivative compounds, whose R_f values agree with those of indole-3-acetamide[11] and indole-3-lactic acid .[7]

Siderophores production. Production of colored siderophores by *Pseudomonas* sp. is known.[15] Siderophores production by the *Pseudomonas* species, isolated from King B liquid medium free of $FeCl_3$ can be seen in Fig. 5. When a medium of King B agar was supplemented to 1.36 ppm of iron as $FeCl_3$, siderophores production had no relevant changes; however, inhibition percentage of *P. infestans* growth by *Pseudomonas* sp. decreased substantially (from 94% to 75% with *P. putida* 2, from 100% to 57% with *P. putida* 3, from 95% to 68% with *P. putida* 1, from 98% to 78% with *P. aeruginosa* and from 74% to 66% with *P. fluorescens).* These important variations may be partially due to some involvement of pigments in inhibition processes[10,14] however, in the case of *P. fluorescens* 1, the appearance of antibiotics could be an important factor.

Colonization of hyphae. Strains of *P. aeruginosa, P. putida* 3 and *P. fluorescens* 1, grown in King B broth and spread on hyphae of *P. infestans*, revealed good colonization and fungicide properties upon them, and therefore antagonistic[17] capabilities (Fig. 6). Fungicide property was corroborated by the lack of growth of treated hyphae, while remnants of these were taken from the edge of the inhibition zone and incubated in PDA agar. Other *Pseudomonas* sp. showed fungistatic effects since only growth inhibition was observed which might be attributed to the accompanying siderophores of spread *Pseudomonas* sp. broths (Fig. 7). PDA agar cultures could not yield these pigments because of high concentration of iron in the medium.

The most abundant bacteria in rice rhizosphere, grown in the Espinal region, Tolima, Colombia, S.A., belonged to genus *Pseudomonas* sp. (51%), *Azotobacter* sp. (26%) and Enterobacteria (21%). All these microorganisms produce IAA when tryptophan is present in the medium. An adequate method to evaluate the relative abundance of the IAA was based upon TLC, followed by spectrophotometric analysis.

P. aeruginosa, P. putida 3 and *P. fluorescens*, in absence of $FeCl_3$, show biological control and antagonistic activity against *P. infestans* and siderophores production. A technique to measure these antagonistic effects, based on inhibition zone width is provided. Antagonistic activities decreased when $FeCl_3$ was present in the medium, suggesting that the siderophores play an important role.

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