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FINAL REPORT

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U.S. Agency for International Development

BIOCONTROL OF BEAN ASHY STEM BLIGHT BY IMPROVED RHIZOBIUM BIOTECHNOLOGY

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EXECUTIVE SUMMARY

The Science Advisor Office of the Unites States Agency for International Development (USAID) sponsored a research project entitled "Biocontrol of Bean Ashy Stem Blight by Improved Rhizobium Biotechnology." The project was a collaborative effort between the Soil Microbiology Laboratory of the Secretaría de Estado de Agricultura (SEA) of the Dominican Republic and the BNF Laboratory of the University of Puerto Rico at Mayagüez. The overall goal of this project was to develop bacterial-based methods to control fungal diseases in common bean (*Phaseolus vulgaris*). The priority of our efforts was directed towards biocontrol of Ashy Stem Blight (*Macrophomina phaseolina*). In the Dominican Republic, the disease was detected only in the San Juan de la Maguana province. Two fungal isolates from there, were characterized and compared to one isolate from Isabela, Puerto Rico. A collection of 35 *Rhizobium phaseoli* strains were obtained and tested for fungal inhibition. Neither these nor other strains from our collection were capable of inhibiting the fungal pathogen to be considered potential biocontrol agents. However, other bacterial genera (*Pseudomonas* and *Bacillus*) produced strong in vitro inhibition in a new media compatible with both bacterial and fungal growth. In vivo screening highlighted the potential of some strains as plant growth promoting rhizobacteria (PGPR) inoculum. Greenhouse experiments demonstrated the value of *P. cepacia* strain UPR 5C as a biofungicide against *M. phaseolina*. Personnel from Colombia and the Dominican Republic, in particular, received training and in some cases M.S. degrees while working for the project. Although the results obtained during the present project indicate the potential of some bacterial strains as biofungicidal agents, further research is needed before the present technology could be commercially applied.

Research Objectives.

Ashy stem blight (*Macrophomina phaseolina*) has been detected as a serious problem in bean growing areas of the Dominican Republic and Puerto Rico. Since no highly resistant varieties are available, and fungicide control of soilborne fungi is expensive and a source of environmental problems, the development of biological control methods are justified.

The objectives of the project as originally approved were:

1) To identify and select *Rhizobium* strains that inhibit root rot producing fungi in vitro and in vivo; and

2) To evaluate the biological control effect of *Rhizobium* inoculation on bean genotypes susceptible to ashy stem blight.

The innovative aspect of the project was the use of rhizobia as biocontrol agents to protect against bean ashy stem blight (ASB). Since antagonism of *Rhizobium* was very weak, we added other bacterial genera as potential antagonists towards *M. phaseolina* and other soilborne plant pathogenic fungi.

Methods and Results.

i) Dominican Republic (D.R.) component.

Field surveys were conducted during several growing seasons in Azua, Higuey, San Cristóbal, San José de Ocoa, Constanza and San Juan de la Maguana. During January 1990, plant pathologist Maximo Halpay conducted a survey at the Higuey Province. This area, and particularly the municipality of San Rafael de Yuma, is the bean growing region during the winter season. Approximately 80% of the fields planted with beans were surveyed, but the presence of ASB disease was not detected. Most of the plots were already at the final growth stage (close to harvest). Since there was a long drought, the area sown to beans was severely reduced. The major planting area has now been shifted to San Juan de la Maguana. During the winter period (January to March), ASB was surveyed in the San Juan de la Maguana region. The percentage of incidence was lower than the September-December growing season. The soil microbiologist and plant pathologist team from the Dominican Republic project component identified two sites (Arroyo Loro and Jinova, San Juan de la Maguana Province) with a high incidence of ASB disease. Two isolates of *Macrophomina phaseolina* were obtained and characterized. They were coded RDMp1 (Jinova) and RDMp2 (Arroyo Loro). Together with the isolates from Puerto Rico (PRMp1, obtained from Fortuna and PRMp2 from Isabela) they were tested for pathogenicity in several landraces of *Phaseolus vulgaris* (Pompadour

type) of the Dominican Republic. Published results (Echávez-Badel and Perdomo, 1991 and Echávez-Badel and Sánchez, 1992) indicate that isolate PRMp2 was more virulent. When tested in the DR landraces, DRMp2 was the most pathogenic isolate, and in contrast to literature reports, it is also pathogenic in soils with high moisture.

At the different bean farmer fields visited, healthy looking plants were selected, excavated, and checked for nodulation. The samples were taken to the Soil Microbiology Laboratory (CESDA, San Cristóbal) for rhizobia strain isolation. During some periods (June, July and August, 1989) in the Constanza region, the vast majority of plants observed lacked nodules. During this period, there was a severe drought, and most fields were irrigated. The lack of nodulation can be explained by several factors:

1) The high natural fertility of the soils in the region (high soil N),
2) The frequent application of fungicides and insecticides, characteristic of this area of high inputs agriculture,
3) Lack of inoculation with rhizobia, and
4) Biological factors (such as antagonism and predation) affecting nodulation.

As a result of the field trips to the different regions of the country, more than 41 strains of *R. phaseoli* were obtained. However, because of electric power failures, at least six strains have been lost. The isolated strains have been coded RD, and characterized according to the CIAT methodology (growth, pH and colony morphology). Strain efficiency was determined under greenhouse conditions. This information will provide insight into the symbiotic potential of native *R. phaseoli*. Since in the future inoculation might be introduced, it is important to have a previous assessment of the nodulation before foreign commercial strains are introduced.

A preliminary field experiment was conducted at the Arroyo Loro Experiment Station of the Secretaría de Estado de Agricultura (SEA). Bean seeds were inoculated with nine rhizobial strains, and a tenth control received no inoculum. Statistical analysis of the number of diseased plants, showed significant differences among treatments. The best strain (better biocontrol) was *R. phaseoli* strain RD 013, followed by RD 004, Kim-5 and RD 028. The yield differences between treatments were not significant at harvest. Further field experiments are necessary to determine the factors that affect the biofungal activity of the bacterial strains before a commercial application is recommended.

ii) Puerto Rico component

Characterization of Macrophomina phaseolina isolates. Isolate PRMp2 from Isabela (PR) and two isolates from the Dominican Republic (RDMp1 and RDMp2) were further characterized by PAGE and growth properties. Gels indicated that the isolate from Puerto Rico was different from the Dominican Republic Isolates. They differed in the number and amount of protein bands produced. Isolates from the Dominican Republic grew faster, produced more and darker biomass than the PRMp2 isolate.

In vitro screening. During the first year of the project, strains of *R. phaseoli* from several collections (NifTAL, CIAT, USDA, Nitragin Co.,) and new strains isolated from the Dominican Republic were screened for *M. macrophomina* in vitro inhibition. Three methods were tested:

a) Streak plate method.- Generally, significant differences in radial growth rates between control (fungus alone) and tested bacterial strains on YEM and TY agar cultures were obtained (figures 1A and 2A). The strains KIM-C and Act1 (actinomycete) showed highest inhibitory effect to *M. phaseolina* on both culture media (figures 1 and 2). No microsclerotia on TY agar were observed after 72 hours of incubation. The antagonistic activity of the actinomycete (Act1) could be due to antibiotics or mycolytic enzymes; it is widely known that *Streptomyces griseus* strains produce a variety of antibiotic compounds.

b) Double-layer plate method.- In the double-layer plate all the bacterial strains were found to inhibit the fungi growth on YEM medium (figure 3). More over, the microsclerotia production was not evident in the interaction of KIM-C (*Pseudomonas cepacia*) and *M. phaseolina*. Apparently, the nutrient composition of YEM was responsible for the poor microsclerotial production.

c) Spent culture method.- Results are summarized in figures 4 and 4A. Significant differences in fungal biomass between control and all bacterial strains, excluding *Azotobacter vinelandii* (Av), were obtained. The KIM-N (*Xanthomonas maltophilia*) strain showed the highest degree of inhibition towards *M. phaseolina*. In contrast, *Xanthomonas campestris* pv. *phaseoli* (Xc) has a stimulatory effect on the fungus growth (figure 4A). Apparently, YEM or TY broth could be supplemented by a substance secreted by *X. campestris*. Two hormonal substances have been identified in *X. campestris* pv. *phaseoli* (Zapata et al., 1992).

Since there is a strain x media interaction, YEM and TY media were used in all tests to evaluate inhibitory effects. The most effective and consistent antagonist towards *M. phaseolina* in the streak plate, double layer and spent culture methods was the KIM-C strain (figure 3A). KIM-C and KIM-N were isolated as contaminants from *R. phaseoli* strain KIM 5, and identified as *Pseudomonas*

cepacia (and codified as UPR 5C) and *Xanthomonas maltophilia* (codified UPR 6). Attempts to identify the antifungal compound produced by strain UPR 5C are being conducted.

Recent literature and our results indicate that *P. cepacia* strains are good candidates for biocontrol (Heddar et al., 1992). We have already isolated a strain from cultivated mushrooms (*Pleurotus* sp.), obtained two strains that degrade toxic compounds (G4 and MB2), two strains from Dr. A. Hirsch, and one from the ATCC collection (ATCC 25416). They will be tested for their inhibitory effect on some soilborne fungi.

Recently, a new method to test fungal inhibition was developed. *M. phaseolina* was suspended in yeas extract manitol-dextrose (YEMD) and grown for four hours. Then, four drops of 10 μl of bacterial suspension were added. The method was tested with only 10 strains, but it performs very well for the fast-growers, and further testing should be performed following this procedure.

Development of screening media. As discussed previously, strains of *R. phaseoli* from several collections and new strains isolated from the Dominican Republic were screened for *M. phaseolina* in vitro inhibition. Although some strains inhibited fungal growth, results varied according to medium and screening method. Therefore, we compared the growth of *Rhizobium* and *Macrophomina* in different media. A new medium, where both bacteria and fungi has optimal growth was developed. It is based on YEM with added dextrose (YEMD). When *Rhizobium* strains were retested in the new medium, no inhibition of the pathogenic fungi was detected. Furthermore, *M. phaseolina* produces abundant microsclerotia in YEMD. However, *P. cepacia* UPR 5C, *Bacillus cereus*, and *X. maltophilia* were still inhibiting *M. phaseolina* PRMp2 in petri dishes. The new medium was also utilized to screen several rhizobacteria isolated from tropical legumes. Approximately 40% of the strains showed biofungicide activity towards *M. phaseolina*.

Previous results indicated that *P. cepacia* strain UPR 5C was the strongest inhibitor of *M. phaseolina* under in vitro conditions. Therefore it was tested against other soilborne phytopathogenic fungi isolated from diseased dry bean plants (table 1). UPR 5C was also a strong inhibitor of *Sclerotium rolfsii* and *Rhizoctonia solani* by the streak plate method.

Similar in vitro methods were utilized with the plant pathogenic fungi *Rhizoctonia solani* (*Thanatephorus cucumeris*) that causes web blight in beans. A smaller group of potential biocontrol bacteria were tested, and again *P. cepacia* UPR 5C was the strongest inhibitor. The next strongest inhibitors were *P. fluorescens* 23625 and *Xanthomonas maltophilia* UPR-6.

In vivo screening. To develop a fast screening method with plants, bean seeds were submerged 5 minutes in a bacterial suspension. The

set of 19 most inhibiting strains were used to compare in vitro with in vivo screening results. Treated seeds were planted in 125 ml erlenmeyers with 60 ml of sterile vermiculite. *M. phaseolina* inoculum was prepared by mixing 1 kg of sterile soil and 6 g of sclerotia. To evaluate disease severity, a 1 to 5 scale was used. The method was very effective, since controls with only *M. phaseolina* were 100% infected. Most of the 19 strains stimulated germination. Again, *P. cepacia* strain UPR 5C was the most effective, and a 100% germination was obtained. However, the best biocontrol bacteria was *Bacillus thuringiensis* UPRM 10001, followed by strain UPR 5C. The drawback of *B. thuringiensis* was its strongly reduction of plant emergence. Further experiments are required to confirm this result and identify mechanisms of strain activity.

Colonization study. Colonization of *Phaseolus vulgaris* roots by *P. cepacia* UPR 5C was studied by qualitative and quantitative methods. For qualitative studies, impressions on King's B media (supplemented with streptomycin and kanamycin) from roots of previously inoculated seeds were made. Ten days after planting, the roots system was completely colonized. For quantitative studies, 1 cm cuttings of top, medium and bottom segments of the bean roots were suspended, diluted and the number of bacteria determined. Up to 400 colonies/cm were obtained at the fardest point from inoculation.

Compatibility test. The compatibility between *R. phaseoli* CIAT 632 and *P. cepacia* UPR 5C was studied by liquid culture and streak methods. The rhizobial strain was not affected by co-inoculation with the pseudomonad.

Greenhouse experiments. In vivo experiments to determine the fungicide effect of *Pseudomonas cepacia* UPR 5C were carried out under greenhouse conditions. The experiment included 7 treatments in a completely randomized block design with 10 replications. Pots were filled with a pasteurized mixture of Promix and soil, and 3 seeds per pot were planted. Disease severity and percentage of plants established were measured. Results are shown in Table 2. These results indicate that *P. cepacia* inhibits *M. phaseolina* under greenhouse conditions. Therefore, it has potential as a biofungicide, but field experiments are needed before it is recommended for commercial applications.

P. cepacia pathogenicity test. *P. cepacia* has been reported as causing soft rot in onions, therefore, an experiment to test strain UPR 5C pathogenicity was carried out. The experiment involved five varieties of *Allium cepa* treated with the strain and its filter sterilized culture supernatants from different ages. Results showed that only live cells were slightly pathogenic to yellow onions. This implies that the use of this strain is restricted to fields where the yellow onion variety will not be grown.

Inhibition mechanisms. To further understand the inhibition mechanisms of *P. cepacia*, other experiments were performed. Since no fluorescence is produced by this species, siderophore production can be ruled out. Therefore, antibiotic and gas production were tested as potential mechanisms.

a) Antibiotic production. Results of treating cells with heat and chloroform showed that only life cells inhibit fungal growth, indicating the production of a heat-sensitive antifungal substance. Our results confirm those of Lambert et al. (1987), Janisiewicz and Roitman (1988) and Homma et al. (1989) who isolated and identified several antibiotics in different strains of *P. cepacia*.

b) Gas production. When *P. cepacia* was grown with *M. phaseolina* in divided petri dishes, it was able to reduce its growth by 81%. Although we did not collect and identify the gases produced, they probably included ammonia, since the growth media (TY) is rich in N. This findings are similar to those reported by Howell et al. (1988) and DePasquale and Montville (1990). Baligh et al. (1990) identified ammonia among the volatiles produced by another strain of *P. cepacia*.

Impact, Relevance and Technology Transfer.

The project enabled the scientists in the Dominican Republic to initiate work in an area of biological control of diseases not previously carried out in that country. It consolidated the development of the Soil Microbiology Laboratory of the SEA, and stimulated interdisciplinary activities between plant pathologist and microbiologists.

Results of the research conducted under this project have uncovered new strains of bacteria with strong antifungal activities under laboratory conditions. One strain gave promising results under greenhouse conditions, but field experiments and application technologies need to be developed before transferring the results to the farmer production practices.

Through participation in the project, the Research Department of SEA was encouraged to conduct research in a new aspect of integrated pest management. Like in many developing countries, support for research in the Dominican Republic is not a priority for the government, and linkages such as the one established in this project are very important for local scientists. The group is now looking for support from other sources to continue research. In summary, the San Cristobal Research Center enhanced its service to productive sectors, scientists were trained in graduate studies and technical subjects, and in general improve the institutional frame that enhances regional cooperation in agricultural technology.

Project Activities/Outputs

Collaboration, Travel, and Training:

Work is in progress to identify the toxins (antibiotics) produced by *P. cepacia* and *B. thuringiensis* in direct cooperation with the Department of Chemistry (Dr. Emilio Díaz). However, we have not yet been able to detect the presence of rhizobitoxine.

The following list includes all meetings and trips made during the project, although some funds were from other sources:

Person	Place	Date	Purpose
R. Echávez-Badel	Dom. Rep.	Jul 88	Signing agreement
R. Echávez-Badel	Colombia	Sep 88	APS-Caribbean Meeting
R. Echávez-Badel	Dom. Rep.	Oct 88	Disease/Nodul. Survey
R. Echávez-Badel	Colombia	Jul 89	APS-Caribbean Meeting
Eduardo Schröder	Dom. Rep.	Jan 90	Project site visit
Eduardo Schröder	TN, USA	May 90	Intl. N Fixation Cong.
R. Echávez-Badel	MI, USA	Aug 90	APS Annual Meeting
Eduardo Schröder	Guatemala	Nov 90	L.A. Rhizobium Meeting
R. Echávez-Badel	Costa Rica	May 91	APS-Caribbean Meeting
Eduardo Schröder	Canada	Aug 91	North Am. Symbiot Conf
R. Echávez-Badel	Dom. Rep.	Nov 91	Materials; Plan Review
R. Echávez-Badel	Venezuela	Sep 92	APS-Caribbean Meeting
Eduardo Schröder	Mexico	Dec 92	Intl. N Fixation Cong.

The Project sponsored the following graduate students (M.S.) during their studies:

Student name	Country	Completion year
Floilán Perdomo	Dominican Republic	1990
Alfonsina Sánchez	Dominican Republic	1991
Rodrigo Campo	Colombia	1992

The titles of their thesis are reported under publications. Besides these graduate students, several undergraduate and one graduate student were trained while performing special research projects on topics related to the grant. In a related project, Ms. Adriana Bueno, from the Dominican Republic, characterized some of the *R. phaseoli* strains from the Dominican Republic, and was trained in

BNF (M.S., Department of Biology, UPR, RUM). Also, Ms. Teresa Ruiz, a laboratory aid to the project in the Soil Microbiology Laboratory was sponsored for an internship training at the BNF Laboratory from September 1, 1989, to November 30, 1989.

PUBLICATIONS

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Project Productivity

Based on the first years results, the original project objectives were modified. The use of other bacterial species was introduced to accomplish the overall goal of using biological control methods to reduce the ASB disease severity in beans.

As mentioned in previous annual reports, the project in the Dominican Republic faced three serious problems: 1) transportation, 2) power failures and 3) adverse climate.

1) Transportation: Since the beginning of the project, government officials of the Department of Agriculture (including one of the Minister of Agriculture) promised official transportation, but the promise never materialized. This created serious problems for field work. Therefore, surveys and field experiments were done with the cooperation of other projects and by renting vehicles. Although AID policy is not to purchase vehicles, we believe that sometimes they are essential to carry out some projects.

2) Power failures: Approximately when the project started, very frequent and extended cuts in the electricity supply affected the Dominican Republic, and the Agricultural Center (CESDA), where the

Soil Microbiology is located (San Cristóbal). A large emergency diesel power plant is located at the Center, and it could easily have provided energy to the Soil Microbiology Laboratory. However, even though the Project offered to cover the expenses of making the appropriate cable connection, the administrative decision was never implemented.

3) Adverse climate: The hurricane Gilbert affected the Dominican Republic during the first year of the project, delaying the scheduled timetable. Therefore, the project was extended for 18 months.

In spite of the mentioned problems, we believe that the project was able to achieve almost all the proposed goals. Results from the last months of the project will be submitted for publication in the near future.

Budget: The allocated budget was used according to the original and modified work plans, and the complete balance statement will be submitted when the last expenditures are cleared by the finance office. A preliminary financial report is enclosed.

Future Work

As a result of the project, a bacterial strain has been selected as having potential as a biocontrol agent to protect legume seeds against soilborne disease producing fungi. Further research is needed, but it might be possible to patent this strain and license its use to a commercial company. As mentioned in the results, nodulation of beans was very poor. Therefore, a new proposal to study biological constrains has been submitted.

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Table 1. In vitro antagonistic effect of *Pseudomonas cepacia* UPRM 5C towards plant pathogenic fungi. (Means of four replications).

	Inhibition (%)	
Fungi	Streak plate	Double layer
Sclerotium rolfsii	100.00	100
Rhizoctonia solani	95.37	100
Macrophomina phaseolina	93.18	100
Fusarium equiseti	88.41	100
Pythium aphanidermatum	67.08	100
Fusarium solani	41.46	60
LSD (0.05)	3.39	-

Tabla 2: Effects of *P. cepacia* (UPR-5C) inoculation on ashy stem blight disease severity of common bean under greenhouse conditions. Year 1991.

Treatments [2]	Disease severity [1] 10 DAP	45 DAP	Established plants (%) 15 DAP
Mp	6.0 [3]	7.6 [4]	50.0 [3]
Rp	1.0	1.0	96.6
Rp + Pc	1.0	1.0	100.0
Rp + Mp	5.7	6.6	50.0
Mp + Pc	1.6	2.4	86.6
Rp + Mp + Pc	1.4	2.2	100.0
Plant alone (Control)	1.0	1.0	96.6
L.S.D. (0.05)	0.9	1.5	--

[1] CIAT scale: 1= No visible disease symptoms; 9=Aproximately 50% or more of the hypocotyl and stem tissues covered with lesions.

[2] Mp= *Macrophomina phaseolina*; Rp= *Rhizobium phaseoli*; Pc= *Pseudomonas cepacia*; DAP= Days after planting.

[3] Average of 10 replications; [4] Average of 5 replications

FIG.1-IN VITRO BACTERIAL STRAINS EVALUATED AS BIOCONTROL OF Macrophomina phaseolina.STREAK PLATE METHOD.YEM MEDIA

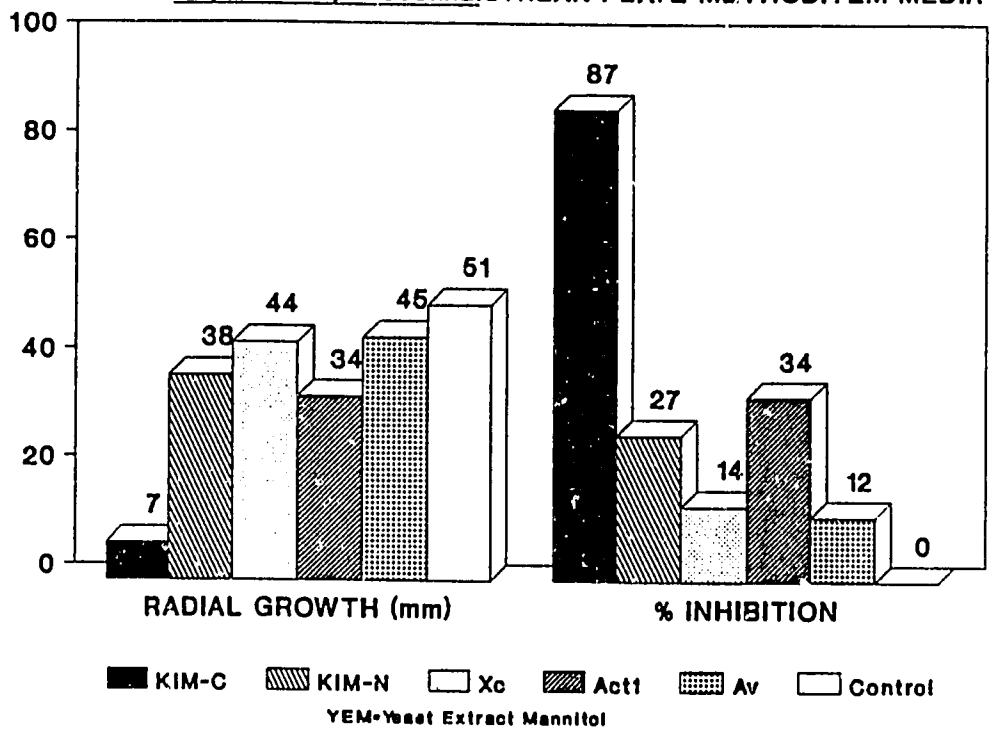
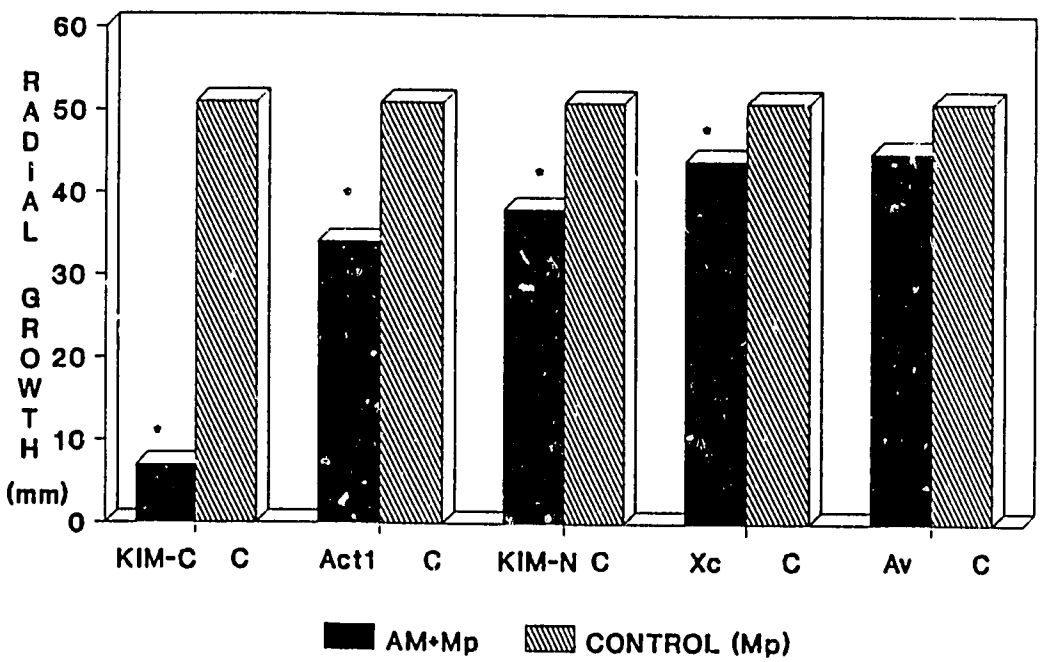


FIG. 1A IN VITRO ANTAGONIST MICROORGANISMS (AM) EVALUATED TO CONTROL Macrophomina phaseolina (Mp).STREAK PLATE METHOD.YEM



• SIGNIFICANTLY DIFFERENT TO CONTROL (P=0.05)

FIG.2-IN VITRO BACTERIAL STRAINS EVALUATED AS BIOCONTROL OF Macrophomina phaseolina.STREAK PLATE METHOD.TY MEDIA

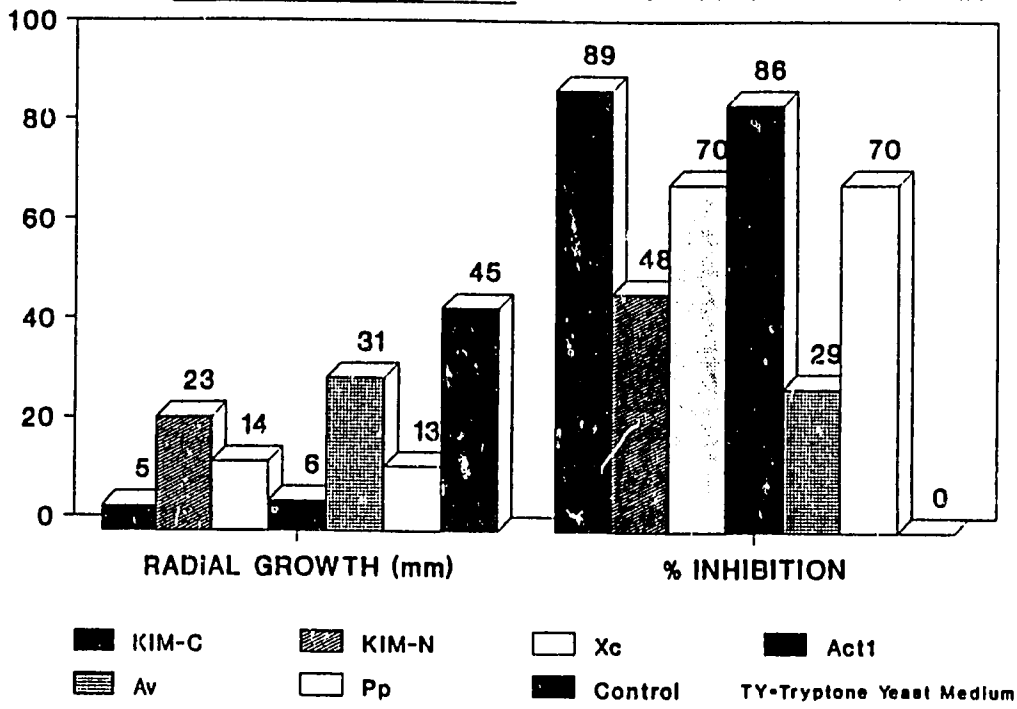
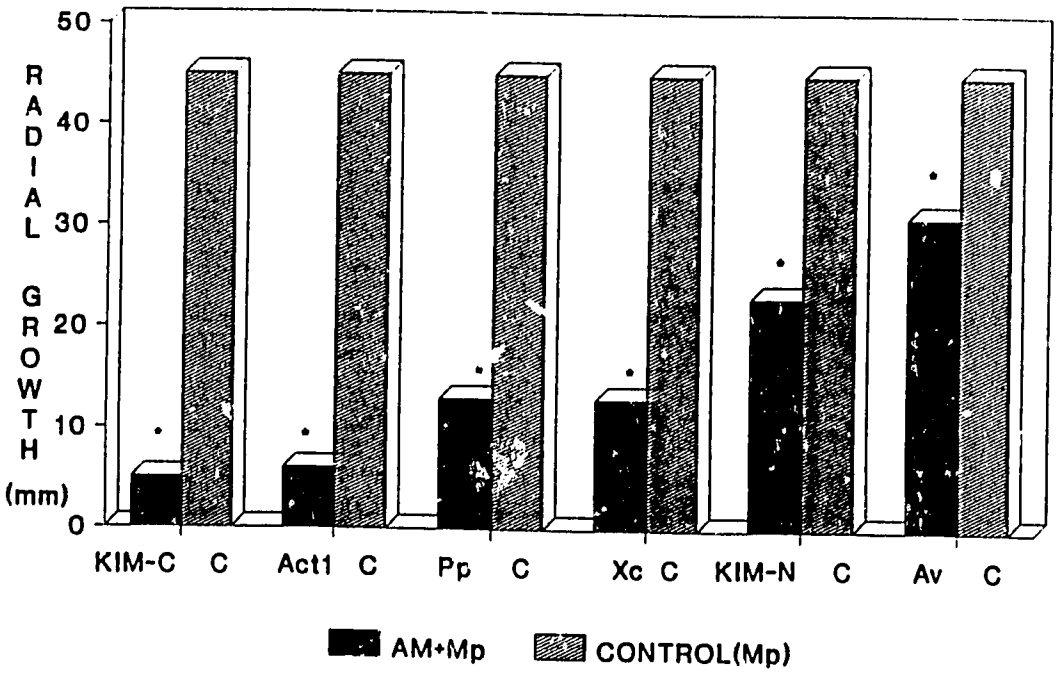


FIG.2A IN VITRO ANTAGONIST MICROORGANISMS (AM) EVALUATED TO CONTROL Macrophomina phaseolina (Mp). STREAK PLATE METHOD.TY



* SIGNIFICANTLY DIFFERENT TO CONTROL (P=0.05)

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FIG.3-IN VITRO BACTERIA STRAINS EVALUATED AS BIOCONTROL OF Macrophomina phaseolina.DOUBLE LAYER METHOD. YEM MEDIA

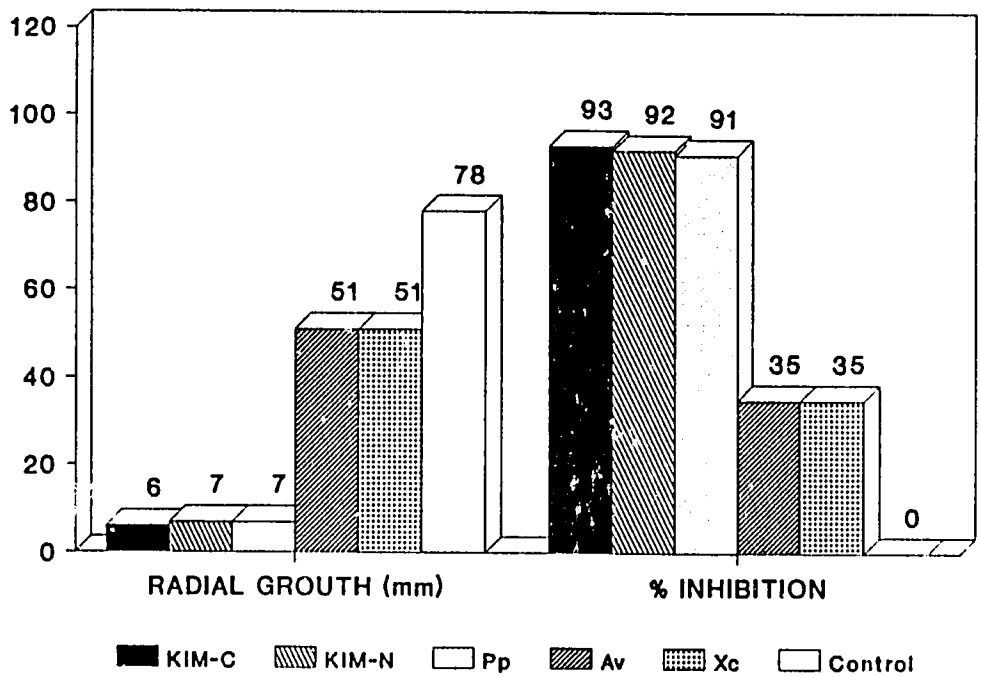
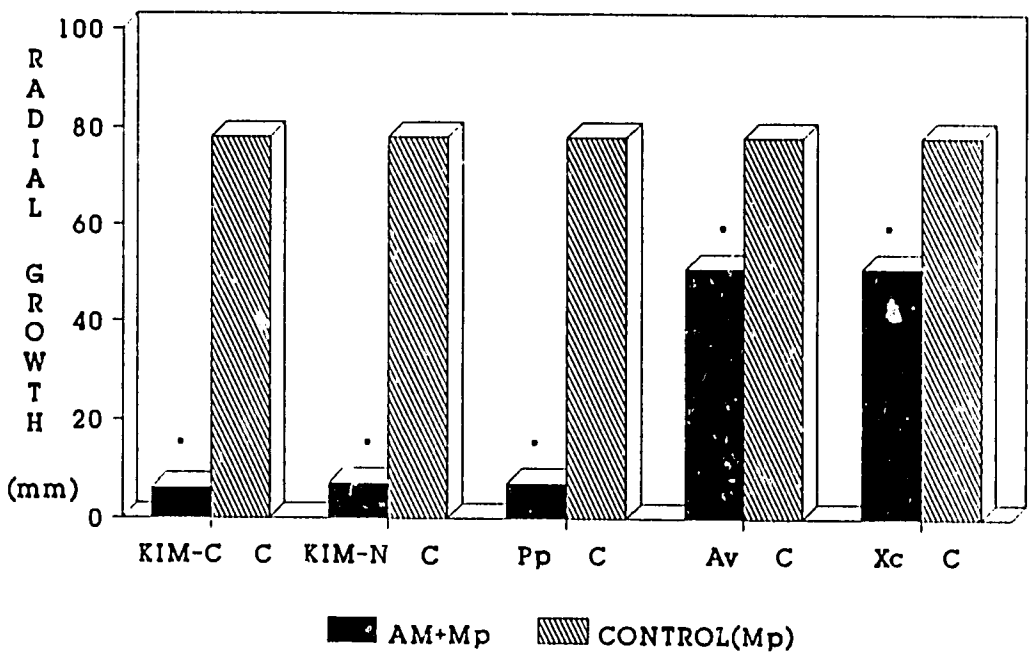
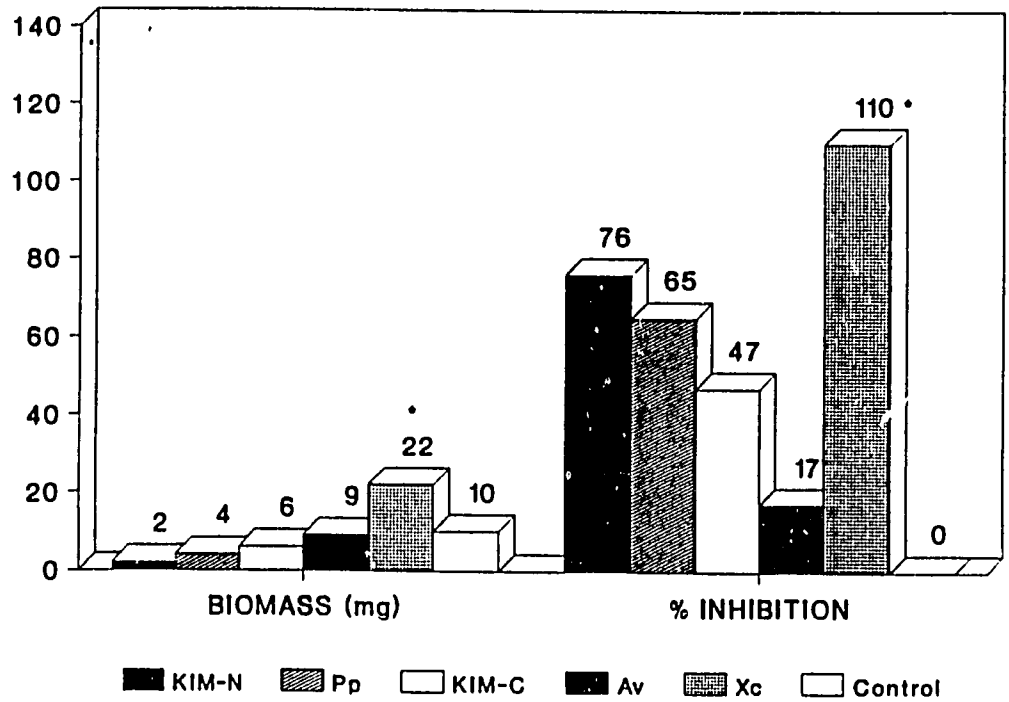


FIG. 3A IN VITRO ANTAGONIST MICROORGANISMS (AM) EVALUATED TO CONTROL Macrophomina phaseolina (Mp).DOUBLE LAYER METHOD.YEM



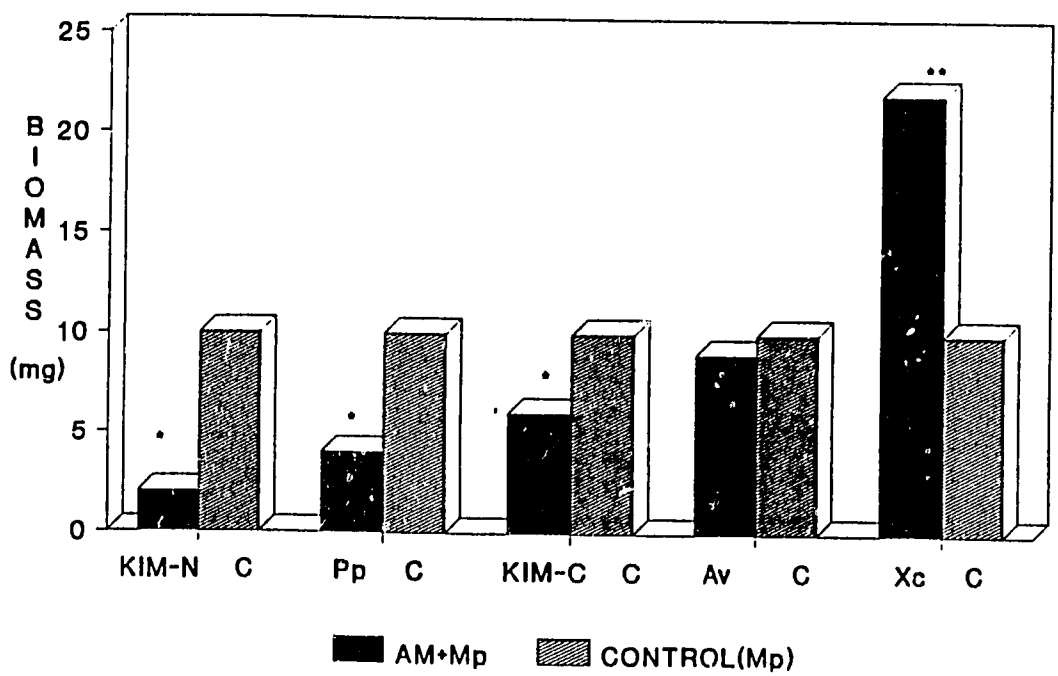
* SIGNIFICANTLY DIFFERENT TO CONTROL (P=0.05)

FIG.4 IN VITRO BACTERIA STRAINS EVALUATED AS BIOCONTROL OF Macrophomina phaseolina. SPENT CULTURE. TY MEDIUM



•ESTIMULATORY EFFECT

FIG. 4A IN VITRO ANTAGONIST MICROORGANISMS (AM) EVALUATED TO CONTROL Macrophomina phaseolina (Mp). SPENT CULTURE. TY



• SIGNIFICANTLY DIFFERENT TO CONTROL (P=0.05)
••STIMULATORY EFFECT