Influence of growth factors on the suppression of soil-borne plant pathogens by select Pseudomonads

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Abstract

Rhizobacteria were evaluated for their ability to inhibit the growth of damping-off pathogens such as *Pythium ultimum*, *Rhizoctonia solani* and common root rot pathogen, *Cochliobolus sativus*. The level of antagonism by Pseudomonads 63-28, U-14 and Ral-3 was examined on solid growth media under varying conditions of temperature, pH, and sources of C, N, and amino acids. *In vitro* antibiosis of the pathogens was greatly influenced by environmental and nutritional conditions. Generally, antagonism was greatest at 25-30°C and decreased at 40°C. The influence of pH appears to be rhizobacteria and pathogen dependent. For example, optimum inhibition of *P. ultimum* growth by 63-28 was between pH 4.6-6.0, whereas, optimum inhibition of *R. solani* growth by U-14 was at 7.6-9.1. Growth inhibition of *C. sativus* by Ral-3 was pH independent. Mannitol or trehalose strongly enhanced the antifungal activity of 63-28, whereas lactose had a negative effect. Antagonism of 63-28, U-14 and Ral-3 was enhanced by nitrogen sources tested except $NaNO_2$ on U-14. The influence of the amino acids did not effect Ral-3, but, antibiosis by 63-28 was increased by amino acids phenylalanine, arginine, or histidine. U-14's activity was increased with the addition of proline, serine, or arginine. The results from this study clearly show that growth factors have a significant impact on microbial antagonism.

Introduction

Biological control of soil-borne plant pathogens and growth promotion using plant growth-promoting rhizobacteria (PGPR) is timely because of public pressure to reduce the environmental impact of synthetic fungicides. In recent years, there have been many reports on the role of Pseudomonads that promote plant growth and suppress diseases caused by soil-borne plant pathogens. One of the mechanisms of action postulated for these bacteria is their ability to inhibit deleterious microorganisms, or pathogens of the plant-root-soil ecosystem. Also, the disease suppressing effects are associated with microbial antagonism. Many abiotic factors influence the level of antagonism. Research was started to find out whether pH, temperature, sources of C, N, and amino acids have a role on antagonistic microorganisms. For this purpose we tested the influence of these factors to enhance the antagonism of selected rhizobacteria. We report here some of the results obtained.

Materials and Methods

Bacterial and fungal isolates

Three rhizobacterial strains 63-28 (*Pseudomonas fluorescens*), U-14 (*Pseudomonas fluorescens*) and Ral-3 (*Pseudomonas cepacia*) were selected from many of Esso Ag Biologicals culture collection. Strain 63-28 has a commercial potential to suppress damping-off of cucumber

seedlings caused by *P. ultimum*, strain U-14 enhanced the healthy stand of canola grown in *Rhizoctonia* infested fields and Ral-3 significantly suppressed the common root rot of wheat caused by *C. sativus*. Bacterial cultures were stored at -80 ^{0}C in tryptic soy broth (TSB) amended with 20% glycerol prior to use. For use in experiments, rhizobacteria were streaked onto Pseudomonas agar F (PAF) and checked for purity after incubation for 24 h at 30 ^{0}C. Purified single colonies were grown on PAF plates for experimental use.

Three soil-borne fungal pathogens, *P. ultimum*, *R. solani* and *C. sativus* were selected for this study to test the effectiveness of antagonism by the rhizobacterial strains. *P. ultimum* was maintained on hemp seed in sterile water or 2% water agar. *R. solani* and *C. sativus* were maintained on potato dextrose agar.

Influence of growth parameters on antibiosis of Pseudomonads

The antagonistic activities of strain 63-28 on *P. ultimum*, U-14 on *R. solani* and Ral-3 on *C. sativus* was examined on M9 medium ($Na_2HPO_4.7H_2O$, 3 g; KH_2PO_4, 1.5 g; NaCl, 0.25 g; NH_4Cl, 0.5 g; Bacto-Agar, 7.5 g; distilled H_2O, 500 mL) by a dual culture method under varying growth parameters listed in Table 1. The M9 medium was autoclaved at 121 ^{0}C for 20 minutes and then supplemented with the following after filter sterilization: 1 mL of 1M $MgSO_4$, 5 mL of 20% glucose, 0.05 mL of 1M $CaCl_2$ and 0.25 mL of 1% vitamin B1. The pH of the medium was adjusted by varying the weights of the acid (KH_2PO_4) and base (Na_2HPO_4) to reach the levels indicated in the Table 1. To determine the effect of various temperatures the plates containing dual cultures on M9 medium were incubated at 5, 15, 25, 30, 35 and 40^{0}C. The 17 carbon sources used in this study were added to M9 medium at a final concentration of 0.2% w/v. The 8 nitrogen and 15 amino acid sources were added to the M9 medium at a final concentration of 0.1% w/v.

Plugs of mycelium (5 mm dia.) were cut from the edge of an actively growing fungal colony with a No. 2 cork borer, and one plug was placed in the centre of each M9 media plate (100 x 15 mm). Two parallel 3.5 cm long streaks of bacteria were then made 2 cm apart on opposite sides of the fungal mycelium plug. Plates were incubated in the dark for 2-9 days at room temperature except for varying temperatures after which time colony radius was measured and compared to that of colonies emerging from plugs not challenged by bacterial isolates. All tests with each bacterial isolate and pathogen combination were carried out in triplicate and the data were analyzed using one way ANOVA and T-test at p=0.05, with results represented as a percent inhibition towards the control.

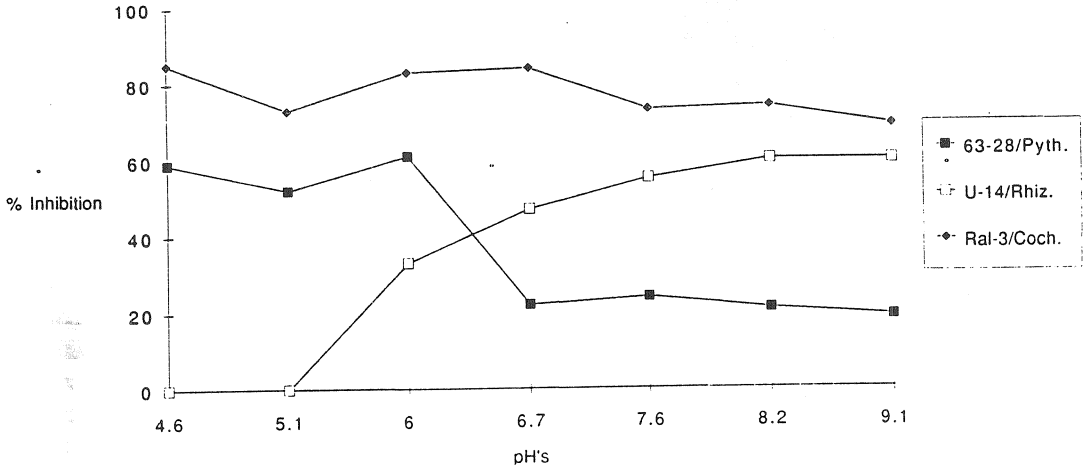
Table 1. Growth factors used

Temperatures	pH's	Carbon sources		Nitrogen sources	Amino Acids	
5	4.6	Acetate	Peptone	NH4Cl	Ala	Phe
15	5.1	Arabinose	Ribose	NH4NO3	Arg	Pro
25	6	Citrate	Sorbitol	(NH4)2SO4	Asp	Ser
30	6.7	Galactose	Succinate	Ca(NO3)2	Glu	Thr
35	7.6	Glucose	Sucrose	KNO3	Gly	Try
40	8.2	Inositol	Trehalose	NaNO3	His	Val
	9.1	Lactose	TSB	NaNO2	Leu	
		Maltose	Xylose	UREA	Lys	
		Mannitol			Met	

Results

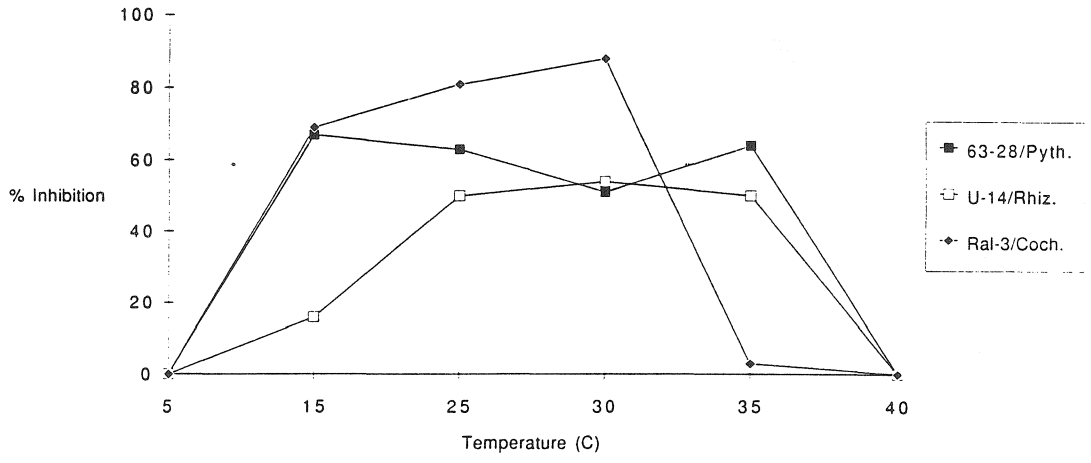
The antagonistic activity of the three strains had very different responses under the pH conditions tested are shown in Figure 1. The influence of pH appears to be rhizobacteria and pathogen dependent. The optimum inhibition of *P. ultimum* growth by 63-28 was between pH 4.6-6.0, where as, the optimum inhibition of of *R. solani* growth by U-14 was at 7.6-9.1. Growth inhibition of *C. sativus* by Ral-3 was pH independent. These results indicate that antagonism of bacterial strains against fungal pathogens tested is affected by the pH of the medium.

Figure 1. Effect of pH on antibiosis



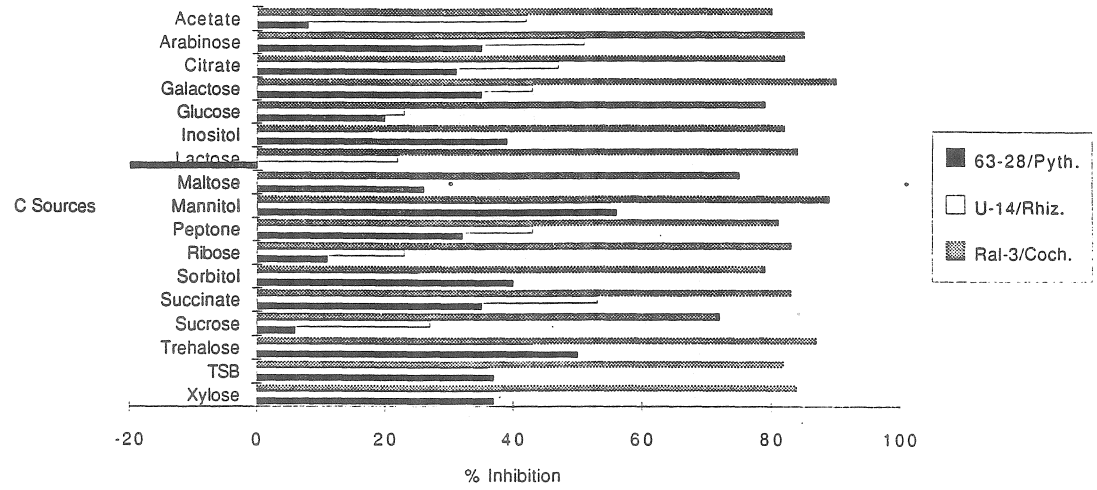
As shown in Fig. 2, the general trend of antagonism of bacterial strains was greatest at 25-30^{0}C and decreased at 40^{0}C. At 5^{0}C, neither bacteria nor fungi grew.

Figure 2. Effect of temperature on antibiosis



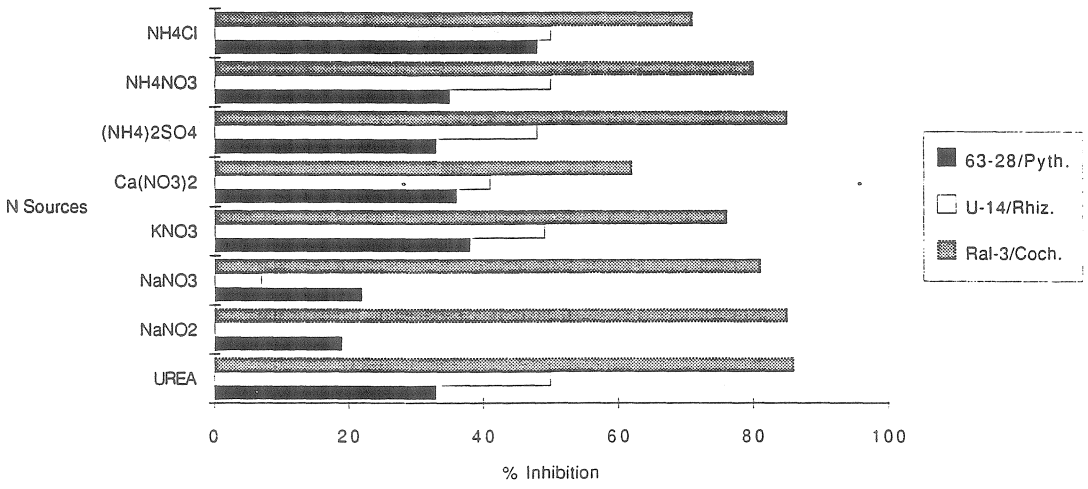
There was a high degree of variation in the level of antagonism under different carbon sources in M9 medium (Fig. 3). Mannitol was most effective to enhance the level of antagonistic activity of the bacteria against fungi tested. Trehalose (50%) and sorbitol (40%) for 63-28 against *P. ultimum*, succinate (53%) and arabinose (51%) for U-14 against *R. solani* and galactose (90%) and trehalose (87%) for Ral-3 against *C. sativus* were most effective. Lactose had a negative effect for 63-28 against *P. ultimum*.

Figure 3. Effect of carbon sources on antibiosis



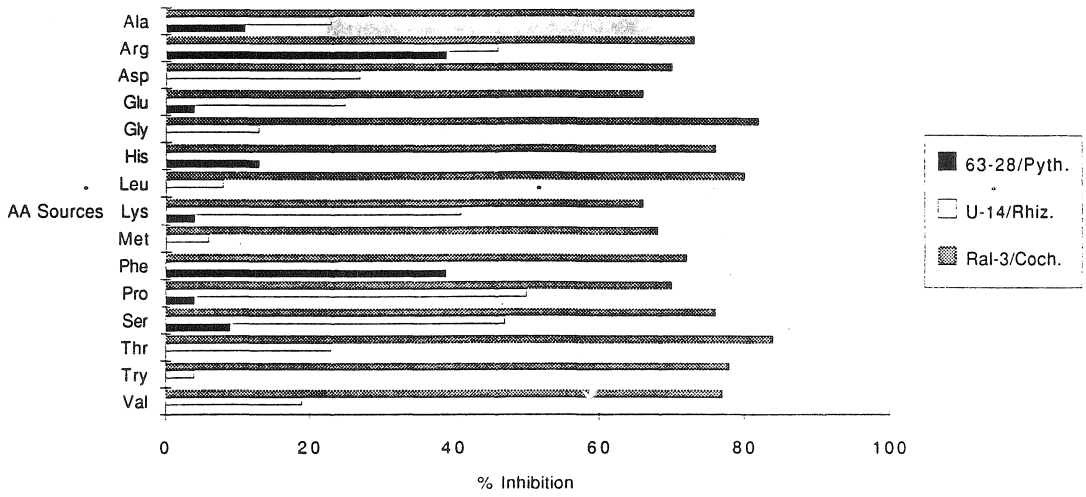
The antagonistic activities of three rhizobacteria influenced by various nitrogen sources in M9 medium was shown in Fig. 4. Inhibition of *P. ultimum* by strain 63-28 was greatest with NH_4Cl (48%), KNO_3 (38%) and $Ca(NO_3)_2$ (36%). Inhibition of *R. solani* by U-14 was significantly better with NH_4Cl, NH_4NO_3, and Urea. Strain Ral-3 showed maximum antagonistic activity against *C. sativus* with Urea (86%), $NaNO_2$ (85%) and $(NH_4)_2SO_4$ (85%).

Figure 4. Effect of nitrogen sources on antibiosis



The level of antagonistic activity of the rhizobacteria against fungi under the influence of various amino acids was shown in Fig. 5. The amino acids phenylalanine (39%), arginine (39%) and histidine (13%) enhanced the activity of 63-28 against *P. ultimum*. Proline (50%), arginine (46%) and serine (47%) increased strain U-14 activity against *R. solani*. Inhibition of *C. sativus* by Ral-3 was common for all the amino acids tested.

Figure 5. Effect of amino acids on antibiosis



Conclusion

The environmental and nutritional growth factors played a very significant role on the level of antagonism induced by the three bacterial strains tested against the three soil-borne pathogens. Antifungal activity of strain U-14 against *R. solani* was greatly affected by low pH ranges and where as the activity of Ral-3 on *C. sativus* was not changed irrespective of the pH's. Temperature also played a significant role. At lower temperatures the growth of bacteria was very slow and the fungi did not grew. There was no data to conclude whether bacteria is able to produce antifungal compounds at lower temperatures. As expected the optimum temperatures (25 -30^{0}C) provided a rapid growth to both the bacteria and fungi. Likewise these temperatures enhanced the antagonistic activity of bacteria. Our study shows that the two strains of *P. fluorescens* (63-28 and U-14) and one strain of *P. cepacia* (Ral-3) were able to inhibit the growth of fungi on a variety of carbon, nitrogen and amino acid sources. Understanding of the basic environmental and nutritional factors on the growth of bacteria and their potential antagonistic activity towards the soil-borne plant pathogens is a prerequisite for any microbial agent for a selection as a potential candidate for biological control of fungal pathogens. Our earlier studies indicated that there was a direct positive correlation between *in vitro* antibiosis and *in vivo* suppression of diseases under field conditions for the strains studied here. The results reported here raise many questions, and further work is planned to address some of these.