Colonization of tobacco seedling roots by fluorescent pseudomonad suppressive to black root rot caused by *Thielaviopsis basicola*

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Abstract Fluorescent pseudomonad strain RD : 1, isolated from soil containing decomposing rye residues, was introduced into the rhizosphere of tobacco seedlings by root bacterization. Bacterized and non-bacterized seedlings were planted in non-sterilized fallow soil and in soil containing decomposing rye residues artificially infested with the chlamydospores of *Thielaviopsis basicola*. Root bacterization with strain RD : 1 significantly increased shoot and root dry weights of tobacco seedlings after 45 days when planted in fallow soil and in soil containing decomposing rye residues, compared with non-bacterized controls. Strain RD : 1 established population densities ranging from 1.0×10^3 to 3.6×10^5 c.f.u. g^{-1} dry weight of roots on rhizosphere of tobacco seedlings following introduction. It also significantly reduced the severity of black root rot disease in both soils. Populations of fluorescent and non-fluorescent pseudomonads and total aerobic bacteria associated with the seedling rhizospheres were significantly reduced by root bacterization with RD : 1. Strain RD : 1 also inhibited vegetative growth of *T. basicola in vitro*. The growth-promoting and disease-suppressing effects of RD : 1 make it potentially useful in biocontrol of *T. basicola*.

Keywords *Thielaviopsis basicola*; fluorescent pseudomonad; black root rot, bacterization; biocontrol; tobacco

Introduction

Black root rot of tobacco, caused by the soil-borne fungus *Thielaviopsis basicolas* (Berk. & Br.) Ferraris, affects a wide variety of crop plants (Lucas, 1975). The fungus is capable of survival in soil even in the absence of host plants (Lucas, 1975). Soils that appear to be naturally conducive or suppressive to *T. basicola* have been described, but the specific factors involved are mostly undefined (Hsi, 1978; Stutz, Defago and Kern, 1986).

Black root rot is one of the major diseases of tobacco in Canada, particularly in southwestern Ontario, and may also occur on legumes and other crops. Effective control of black root rot in tobacco is generally obtained by the use of resistant cultivars. Disease severity may be reduced by crop rotation with non-host crops (Smith 1960; Hsi, 1978); however, various investigators have shown that non-host plants, such as wheat, corn, rye or sorghum, have little influence on population densities of *T. basicola* in field soils (Bateman, 1963; Hsi, 1978; Reddy and Patrick, 1989). Reddy and Patrick (1988, 1989) have suggested that the disease-suppressing effects of rye occur during its growth and during its decomposition and are associated with microbial antagonism and antibiotic production with rye as a substrate. Antagonistic root-colonizing rhizobacteria might therefore have a role in suppression of black root rot

In recent years there have been many reports on the role of fluorescent pseudomonads that promote plant growth and control soil-borne pathogens (Broadbent *et al.*, 1977; Burr, Schroth and Suslow, 1978; Howell and Stipanovic, 1979, 1980; Kloepper, Schroth and Miller, 1980; Schroth and Hancock, 1981; Suslow, 1982; Cook and Baker, 1983; Weller, 1983, 1988; Schroth, Loper and Hildebrand, 1984; Kloepper *et al.*, 1986; Stutz *et al.*, 1986; Weller and Cook, 1986; Kloepper *et al.*, 1988; Sivamani and Gnanamanickam, 1988; Reddy and Rahe, 1989a). These pseudomonads are often referred to as 'plant growth-promoting rhizobacteria' (PGPR) (Kloeper *et al.*, 1980). One mechanism of action postulated for PGPR is suppression or displacement of deleterious micro-organisms or inhibition of pathogens that are associated with the plant root–soil ecosystem (Kloepper and Schroth, 1981; Suslow and Schroth, 1982; Weller, 1983; Burr and Caesar, 1984; Schippers, Bakker and Bakker, 1987; Reddy and Rahe, 1989b). Whether similar mechanisms are involved in the control of black root rot of tobacco and other plants has not been fully explored. Keel *et al.* (1989) reported significant reduction of black root rot of tobacco by a strain (CHAO) of *Pseudomonas fluorescens* under gnotobiotic conditions. In earlier studies (Reddy and Patrick, 1989) fluorescent pseudomonads that were antagonistic to *T. basicola* were isolated from soil. The purpose of the study described here was to test the disease-suppressive and plant growth-promoting properties of one of the fluorescent

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pseudomonad strains, RD: 1, on tobacco seedlings under greenhouse conditions.

Materials and methods

Pathogen source and inoculum production

The isolate of *T. basicola* used in this study was obtained from naturally infested soil collected near Delhi, Ontario using the carrot disc method described by Yarwood (1946) and was maintained on V-8 agar or stored in sterilized sandy loam soil at 25°C. The inoculum (chlamydospores) of *T. basicola* was produced according to the procedure described by Reddy and Patrick (1989).

Bacterial source and bacterization method

The fluorescent pseudomonad strain RD: 1 was originally obtained from soil with decomposing rye residues, as described in earlier studies (Reddy and Patrick, 1989). The strain was purified by repeated streaking on King's medium B (KB) (King, Ward and Raney, 1954) and stored at −80°C in tryptic soy broth (TSB) and 10% glycerol. The strain was marked with resistance to rifampicin (100 μg ml^{-1}) in KB according to the procedure of Reddy and Rahe (1989a). Thirty-day-old tobacco seedlings (cv. Coker 319), which is highly susceptible to black root rot, were used for the root bacterization experiments. Fresh cultures of marked RD: 1 were grown in sterile 500 ml flasks containing 300 ml sterilized TSB on a continuously reciprocating shaker at 24–25°C for 72 h. Following centrifugation for 30 min at 2000*g*, the bacterial pellet was washed with sterile distilled water and suspended in 50 ml 0.1M $MgSO_4$ buffer. The optical density of the suspension was adjusted to 0.5 at 780 nm; this density equalled 1.0×10^7 c.f.u. ml^{-1}.

Chlamydospores of *T. basicola* were added to soils with or without decomposing rye residues and mixed thoroughly. This procedure resulted in an initial concentration of log 6.8 chlamydospores g^{-1} dry weight in each soil sample. The appropriate treated soils were placed in plastic pots (15 cm diameter). Each pot received 850 g of the appropriate soil. The treatments were fallow soil with and without *T. basicola* and soil containing decomposing rye residues with and without *T. basicola*. The roots of 30-day-old tobacco seedlings were bacterized with strain RD: 1 by dipping the roots into the bacterial suspension prepared as described above. The seedlings were then planted (four seedlings per pot) into the appropriate treated soils. Each treatment was replicated four times and treatments were arranged in a randomized complete block design on a plant growth bench in the greenhouse maintained at 23 25°C. The soil moisture was maintained in the range of 60% of field capacity throughout the experiment by adding sterile distilled water periodically on a weight basis to the pots to replace moisture lost (Johnson and Curl, 1972). Tobacco roots treated with an identical amount of sterile 0.1 M $MgSO_4$ solution as above served as non-bacterized controls. The experiments were run for 45 days and were repeated twice.

Soils

Experiments were conducted in sandy loam soil (fallow soil) collected from tobacco fields from two locations around Delhi, Ontario. In all cases, composite samples were collected from the upper 15 cm profile, bulked and stored moist at 18–22°C in covered containers until use. Two soil treatments were used: (1) soil without decomposing rye residues (fallow soil); (2) soil with decomposing rye residues. The latter was prepared by incorporating growing rye plants, collected from fields near Delhi, into the soil, as follows. Rye plants at a height of ~20–25 cm were cut into small pieces (2 cm long), and either used immediately or stored in autoclavable bags at 4°C until use. The sections were incorporated into the soil at a ratio of 1 : 4 (w/w). The soils were placed in clay pots (30 cm diameter), using ~2 kg rye and soil mix per pot). Enough tap water was added to each pot to attain 60% moisture content. Soil without rye material was placed in similar pots and treated as controls. The pots were placed outdoors for a 30-day decomposition period before being used in the experiments. The outdoor temperatures during this period fluctuated between 10°C at night and 20°C during the day.

Tests for antagonism on agar plates

Primary tests for antagonism *in vitro* against *T. basicola* were made as follows. Strain RD: 1 was streaked ~2.5 cm from the edge of a Petri plate (9 cm diameter) containing potato dextrose agar (PDA) (Difco, Detroit, MI), and allowed to grow for 24 h. A disc (5 mm diameter) taken from the edge of an actively growing colony of *T. basicola* on V-8 agar was then placed in the dish 2.5 cm from the edge on the opposite side. The plates were incubated in the dark for up to 15 days at 25°C. The zones of inhibition of vegetative fungal growth were estimated.

Enumeration of population densities of RD: 1 and indigenous bacteria from the tobacco seedling rhizosphere

Population densities of RD: 1 and indigenous bacterial populations associated with the rhizosphere of tobacco seedlings planted in the appropriate treatment soils described above, were enumerated 15, 30 and 45 days after planting. The method used in the enumeration of bacterial populations was the standard dilution plate technique (Johnson and Curl, 1972). The population of RD: 1 was determined on KB supplemented with rifampicin (100 μg ml^{-1}), cycloheximide (100 μg ml^{-1}) and benomyl (30 μg ml^{-1}). Total counts of root-colonizing aerobic bacteria were made on dilute tryptic soy agar, 1/10 strength (Difco tryptic soy broth, 3.0 g; Difco agar, 1.5 g; and deionized water, 1000 ml). Total counts of fluorescent pseudomonads were assessed by plating on to KB supplemented with novobiocin (45 μg ml^{-1}), chloramphenicol (100 μg ml^{-1}), cycloheximide (75 μg ml^{-1}) and benomyl (30 μg ml^{-1}). After incubation for 48 h, fluorescent colonies were differentiated from non-fluorescent ones under a u.v. light (366 nm).

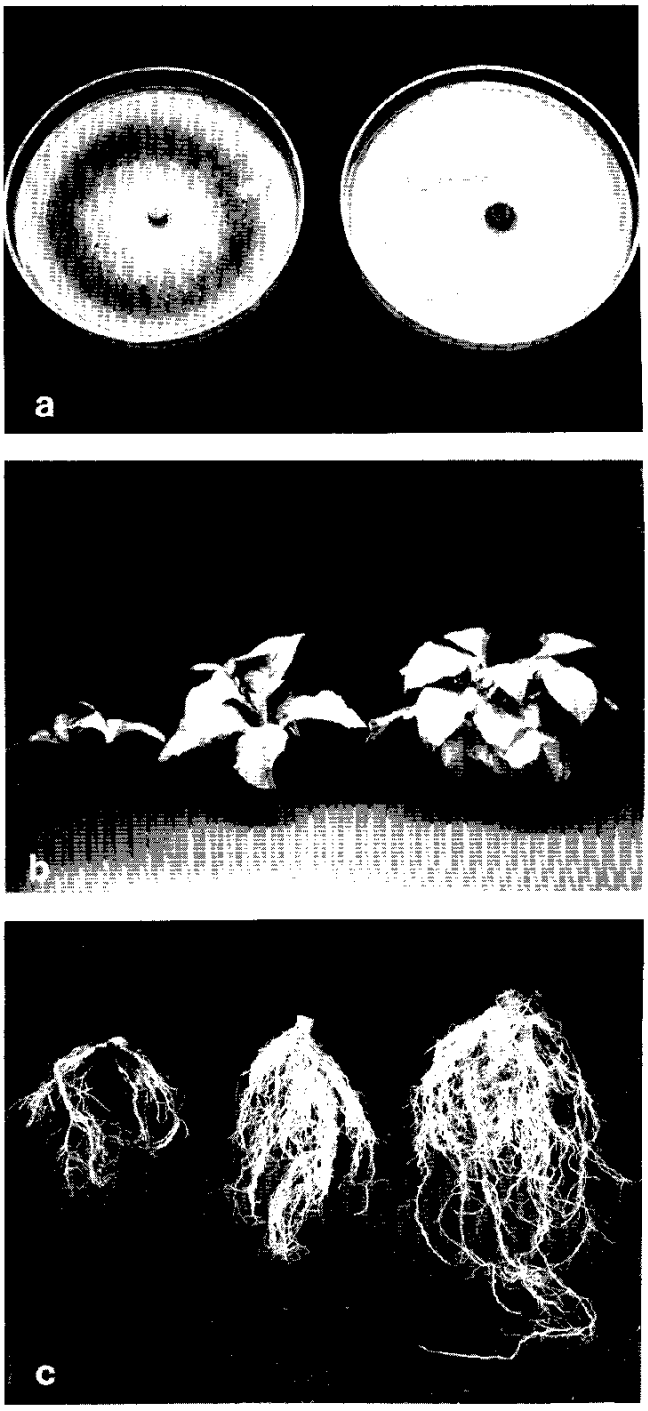

Figure 1. a, Inhibition of vegetative growth of *Thielaviopsis basicola* by fluorescent pseudomonad RD :1. Plate on left without RD : 1 and on right with RD : 1 on potato dextrose agar. b, Effect of root bacterization with RD : 1 on growth of tobacco seedlings 45 days after transplanting. Left to right: tobacco seedlings inoculated with *T. basicola*; not inoculated; seedlings bacterized with RD : 1 and inoculated with *T. basicola*. c, Roots of plants of tobacco seedlings as shown above

To sample the bacteria from tobacco seedlings, plants were carefully dug from the soils and roots were shaken to remove excess soil. The entire root system with soil adhering after gentle shaking was detached from the shoot and designated as rhizosphere. The roots were cut into segments (~ 5 mm) using a surface-sterilized surgical blade for easy washing. The segments were placed in 250 ml flasks containing 10 ml sterile 0.1 M $MgSO_4$ and washed for 30 min by gentle magnetic stirring. The resulting suspensions were serially diluted in a tenfold series. Aliquots of 0.1 ml of the appropriate dilution were spread with a glass rod on well-dried plates in triplicate on the various media described above. Plates were incuated at 25°C. Colonies of RD : 1 were counted after 72 h. Total pseudomonads and aerobic bacteria were counted 3–5 days after incubation. The data of the results were presented as c.f.u. g^{-1} dry weight of roots. The relative proportions of bacteria on roots of tobacco in fallow soil and soil containing decomposing rye residues were estimated, as well as that of the RD : 1. The experiments were repeated once.

Assessment of growth of tobacco seedlings in response to root bacterization

The response of the seedlings to bacterization was determined by measuring the growth of shoots and roots and by assessing severity of black rot caused by *T. basicola* 45 days after transplanting in the appropriate treatments. Individual seedlings were carefully removed from the pots in each treatment and shaken to remove excess soil. Roots and shoots were separated, washed, air dried and weighed. The roots were first scored for disease severity on a 0–5 scale (0, healthy; 5, severe infection). The disease index was calculated by averaging the rate of infection in four replications involving 20 plants per replication per treatment. The experiments were repeated once.

The data of all experiments were transformed and were analysed by analysis of variance (ANOVA) and Student Newman–Keuls' test at the 5% level of significance.

Results

Effect of root bacterization on black root rot severity and on plant growth

Strain RD : 1 significantly reduced the vegetative growth of *T. basicola* in dual culture tests (*Figure 1a*). There was also an increase in shoot and root growth of tobacco seedlings (*Figure 1b*) and a reduction in severity of black root rot induced by *T. basicola*, by root bacterization with strain RD : 1 (*Figure 1c*). The roots of tobacco seedlings bacterized with isolate RD : 1 had significantly less ($p = 0.05$) root rot than the non-bacterized controls (*Table 1*). Root bacterization significantly increased shoot and root weights of tobacco seedlings over those of the non-bacterized controls (*Table 1*). Root-rot severity decreased and growth of the plants increased significantly in soil containing decomposing rye residues, compared with fallow soil, for bacterized as well as non-bacterized seedlings (*Table 1*).

Enumeration of RD : 1 and of indigenous bacterial populations from the rhizosphere of tobacco seedlings

The population density of RD : 1 on the tobacco seedling rhizosphere declined 15 days after planting. Approximately 10^4 c.f.u. g^{-1} dry weight (d. wt) of roots were

Table 1. Stimulation of tobacco seedling growth and suppression of black root rot caused by *Thielaviopsis basicola* by fluorescent pseudomonad strain RD : 1

Soil	Treatment	TB[a]	Shoot dry weight[b] (g)	Percentage change compared with control	Root dry weight[b] (g)	Percentage change compared with control	Disease index[c]
Fallow	RD : 1[d]	−	4.50b[f]	14.2	2.10b[f]	12.9	0c
		+	5.23a	32.7	2.50a	34.4	2.6b
	Control[e]	−	3.94c	0	1.86c	0	0c
		+	2.10d	−46.7	0.95d	−48.9	4.1a
Containing decomposing rye residues	RD : 1[d]	−	5.80b[f]	38.1	3.14b[f]	30.8	0c
		+	6.60a	57.2	3.64a	51.7	1.3b
	Control[e]	−	4.20c	0	2.40c	0	0c
		+	2.65d	−36.9	1.64d	−31.7	3.2a

[a]TB, chlamydospores of *Thielaviopsis basicola*; +, soil infested with TB; −, soil uninfested with TB. [b]45 days after planting. [c]Numerical index of severity of black root rot caused by *T. basicola* on tobacco cv. Coker; roots were assessed on a 0–5 scale, 45 days after planting; 0, no disease; 5, roots severely infected. [d]30-day-old tobacco seedling roots were bacterized before planting with strain RD : 1, a fluorescent pseudomonad marked with resistance to rifampicin. [e]Non-bacterized control seedlings. [f]Mean of combined data from two experiments each having four replications per treatment; means within a column for each soil followed by the same letter do not differ significantly according to Student Newman–Keul's test ($p = 0.05$)

Table 2. Population densities of RD : 1 and of associated indigenous bacteria on roots of tobacco seedlings planted in fallow soil

Time after planting (days)	Treatments[a]	TB[b]	Population densities of bacteria (c.f.u. g^{-1} dry weight of roots)[c] RD : 1 ($\times 10^4$)[d]	FP ($\times 10^5$)[e]	Total P ($\times 10^5$)[f]	Total aerobic ($\times 10^7$)[g]
15	RD : 1	−	16.3b	6.6d	11.2d	1.9c
		+	26.6a	9.4b	16.9b	2.4c
	Control	−	0c	7.9c	13.8c	5.8b
		+	0c	13.4a	20.3a	9.4a
30	RD : 1		4.8b	2.5d	4.9d	0.8d
		+	13.2a	5.7c	9.4c	1.4c
	Control	−	0c	9.8b	21.8b	19.2b
		+	0c	11.3a	39.4a	26.6a
45	RD : 1	−	0.1b	0.1d	0.2d	0.1c
		+	0.6a	0.5c	0.9c	0.2c
	Control	−	0c	16.4b	33.4b	21.6b
		+	0c	27.8a	56.9a	46.5a

[a]RD : 1, 30-day-old tobacco seedling roots were bacterized before planting with a strain of antagonistic fluorescent pseudomonad marked with resistance to rifampicin; Control, non-bacterized control seedlings. [b]Chlamydospores of *Thielaviopsis basicola* (TB): +, soil infested with TB; −, soil uninfested with TB. [c]Mean of combined data from two experiments each having four replications per treatment, and five plants per replication. Means within a column for each time period followed by the same letter do not differ significantly according to Student Newman–Keul's test ($p = 0.05$). [d]Populations of marked isolate RD : 1. [e]Populations of fluorescent pseudomonads. [f]Total populations of pseudomonads. [g]Total populations of aerobic bacteria

Table 3. Population densities of RD : 1 and associated indigenous bacteria on roots of tobacco seedlings planted in soil containing decomposing rye residues

Time after planting (days)	Treatments[a]	TB[b]	Population densities of bacteria (c.f.u. g^{-1} dry weight of roots)[c] RD : 1 ($\times 10^4$)[d]	FP ($\times 10^5$)[e]	Total P ($\times 10^5$)[f]	Total aerobic ($\times 10^7$)[g]
15	RD : 1		36.1b	5.9b	18.6d	3.9d
		+	94.3a	14.2a	35.5b	5.7c
	Control	−	0c	6.8b	25.8c	8.6b
		+	0c	15.4a	63.6a	14.3a
30	RD : 1	−	13.2b	3.7d	11.4d	1.9d
		+	36.7a	9.7c	21.7c	3.9c
	Control	−	0c	16.4b	63.8b	36.5b
		+	0c	24.5a	94.9a	56.4a
45	RD : 1	−	3.7b	1.5d	4.0d	1.0c
		+	9.8a	2.7c	6.8c	1.4c
	Control	−	0c	26.7b	76.8b	64.7b
		+	0c	51.8a	99.4a	86.3a

[a–g]As in *Table 2*

Table 4. Proportion of RD : 1 in total fluorescent pseudomonads, pseudomonads and aerobic bacterial populations recovered from roots of tobacco seedlings which were preinoculated with the marked strain RD : 1 and planted in fallow soil and in soil containing decomposing rye residues

Soil	Time after planting (days)	Percentage of RD : 1 populations (c.f.u. g^{-1} dry weight of roots) in:					
		Total FP[a]		Total P[b]		Total aerobic bacteria	
		(+TB)[c]	(−TB)[d]	(+TB)	(−TB)	(+TB)	(−TB)
Fallow	15	28.3	24.7	15.7	14.6	1.1	0.9
	30	23.2	19.2	14.0	9.8	0.9	0.6
	45	12.0	10.0	6.7	5.0	0.3	0.1
Containing decomposing rye residues	15	66.4	61.2	26.6	19.4	1.7	0.9
	30	37.8	35.7	16.9	11.6	1.0	0.7
	45	36.3	24.7	14.4	9.3	0.7	0.4

[a]FP, fluorescent pseudomonads; [b]P, pseudomonads; [c]soil infested with chlamydospores of *Thielaviopsis basicola* (TB); [d]soil without *T. basicola*

recovered in fallow soil and soil containing decomposing rye residues, with or without incorporation of *T. basicola* (*Tables 2* and *3*). The ranges of population densities of strain RD : 1 recovered from the tobacco seedling rhizosphere in appropriate treatments were as follows: 16.3×10^4 c.f.u. g^{-1} d.wt of root without *T. basicola* and 26.6×10^4 c.f.u. g^{-1} d.wt of root with *T. basicola* for fallow and 36.6×10^4 c.f.u. g^{-1} d.wt of root and 94.3×10^4 c.f.u. g^{-1} d.wt of root, respectively, for soil containing decomposing rye. The population density of RD : 1 on roots of tobacco plants was higher in soils containing decomposing rye residues compared with fallow soil (*Tables 2* and *3*). In addition, the population of the RD : 1 strain on roots of plants was higher in soil infested with *T. basicola*. The higher population of the RD : 1 strain on the diseased roots was associated with less-severe black root rot. Populations of fluorescent pseudomonads, other pseudomonads and total aerobic bacteria were significantly reduced by root bacterization with RD : 1 in both soils (*Tables 2* and *3*).

The percentage of RD : 1 compared with the other bacterial populations on the tobacco roots was greater in soil containing rye residues than in fallow soil (*Table 4*).

Discussion

The results presented here showed that a fluorescent pseudomonad strain RD : 1 isolated from soil containing decomposing rye residues, when introduced into the rhizosphere of tobacco seedlings by root bacterization, increased growth of the plants and reduced severity of black root rot caused by *T. basicola* under greenhouse conditions. Bacterial strain RD : 1, was detected on the roots and in the rhizosphere of tobacco plants 45 days after planting. The population density of RD : 1 in the rhizosphere appeared to be stimulated when rye was used as a green-manure cover crop. The population density levels of RD : 1 on roots of tobacco were comparable to the levels of PGPR reported on roots of potato (Burr *et al.*, 1978; Kloepper *et al.*, 1980), radishes (Kloepper and Schroth, 1978), sugar beet (Suslow and Schroth, 1982) and tobacco (Keel *et al.*, 1989) following seed bacterization. Root colonization is always considered to be a major factor in successful inoculation of crop plants by beneficial rhizobacteria (Suslow, 1982).

The RD : 1 suppressed the activity of *T. basicola* on tobacco roots in both fallow soil and soil containing decomposing rye residues; however, this activity was significantly higher in the latter. This phenomenon was associated with the higher population density of RD : 1 in soil containing decomposing rye residues compared with fallow soil. The fact that RD : 1 was stimulated by rye, and that rye is widely used as a green-manure cover crop in southwestern Ontario and elsewhere, increases the potential usefulness of this beneficial rhizobacterium. The magnitude of suppression of black root rot by RD : 1 in both the soils was demonstrated by its qualitative ability to inhibit mycelial growth of *T. basicola in vitro* (*Figure 1*). Antibiosis of bacteria to soil-borne pathogenic fungi *in vitro* has often been demonstrated; however, only a few reports relate such activities to antagonism in field soil (Howell and Stipanovic, 1980; Kloepper and Schroth, 1981).

The growth observed under the influence of root bacterization with RD : 1 is similar to that described in the published reports on stimulation of plant growth by fluorescent pseudomonads in various crops (Burr *et al.*, 1978; Kloepper and Schroth, 1978; Suslow *et al.*, 1979; Howell and Stipanovic, 1979, 1980; Schroth and Hancock, 1981; Scher and Baker, 1982; Weller, 1983; Burr and Caesar, 1984; Schroth *et al.*, 1984; Kloepper *et al.*, 1986, 1988; Weller and Cook, 1986; Whipps and Lynch, 1986; Keel *et al.*, 1989; Reddy and Rahe, 1989a,b). The studies also revealed that rhizosphere populations of some of the indigenous soil bacteria associated with tobacco seedling roots were significantly inhibited by RD : 1. The relative degree of suppression of indigenous soil bacteria caused by RD : 1 was correlated with its qualitative ability to inhibit mycelial growth of *T. basicola* in dual culture. These results partially support the hypothesis that the mechanism of growth enhancement evaluated in this study was indirect and resulted from effects of the introduced bacteria on indigenous root zone microflora. Kloepper and Schroth (1981) reported that inoculation of potato seed pieces with PGPR resulted in generally decreased populations of indigenous microflora in the root-zone soil. Suslow (1982) observed both a reduction in the total population density

of root-colonizing fungi and a shift in the populations of particular components of the fungal microflora following inoculation of sugar-beet seed with PGPR.

Investigations with rhizobacteria suggest that there are a large number of relatively weak pathogens that damage roots and reduce plant growth (Suslow *et al.*, 1979). Parasitic and non-parasitic bacteria and fungi colonizing plant roots can cause disease or reduced plant vigour (Woltz, 1978; Salt, 1979). Others have speculated that reduction of parasitic and non-parasitic rhizosphere microflora by fumigation or chemical seed treatments contributes in part to the generally associated enhancement of plant growth (Merriman *et al.*, 1974; Bowen, 1979, 1980; Salt, 1979). The capacity of RD:1 to modify significantly the indigenous microflora reveals the possibilities offered by root bacterization for affecting the rhizosphere microflora and plant growth and health. The selection of potential strain RD:1 and elucidation of the mode of action in a tobacco crop management system pose immense and exciting challenges.

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