INDUCED SYSTEMIC RESISTANCE BY RHIZOBACTERIA AGAINST TOBACCO BLUE MOLD DISEASE IS SALICYLIC ACID INDEPENDENT AND NOT ASSOCIATED WITH ACTIVATION OF DEFENSE-ASSOCIATED GENES

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ABSTRACT

Five strains of plant growth-promoting rhizobacteria (PGPR) *Serratia marcescens* 90-166, *Bacillus pumilus* SE34, *Pseudomonas fluorescens* 89B-61, *Bacillus pumilus* T4, and *Bacillus pasteurii* C-9 which previously showed induced systemic resistance (ISR) activity on some crops against several pathogens were evaluated for their potential to induced resistance against blue mold of tobacco, caused by *Peronospora tabacina*. A microtitre plate assay was developed and has been used for studies of mechanisms of PGPR-mediated ISR. Disease severity was consistently reduced significantly by strains 90-166 SE34, and T4, while results with 89B-61 were variable, and no disease reduction was obtained by C-9. Similar results were obtained in greenhouse assays, except that all five strains significantly reduced blue mold symptoms. In the microtitre assays, PGPR-mediated ISR was associated with significantly reduced numbers of infected leaves, and reduced *P. tabacina* spore production. In greenhouse tests, ISR was associated with a reduction of leaf area covered with blue mold lesions and reduced *P. tabacina* sporulation. Trypan blue staining of *P. tabacina* –infected tobacco leaves from microtitre plates demonstrated fewer hyphae in PGPR-treated tobacco leaves than in nontreated leaves. Similar significant reductions in numbers of infected leaves were also obtained by application of four PGPR to NahG tobacco plants indicating that salicylic acid (SA) is not required for ISR to blue mold in tobacco by these PGPR. Northern analysis indicated that there was no difference in expression of PR-1a, basic β-1,3-glucanase, 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, or proteinase inhibitor between PGPR-induced and nontreated tobacco plants. These results suggest that ISR mediated by these tested PGPR strains is SA-independent and may be associated with activation of novel defense pathways.

INTRODUCTION

Induced resistance is a promising tool for plant protection, which is based on the activation of plant resistance mechanisms. It is considered natural and safe, and provides resistance against a broad spectrum of pathogens (Sticher et al., 1997). Both chemical and pathogen-induced systemic acquired resistance (SAR) or PGPR-mediated induced systemic resistance (ISR) can confer season-long resistance for some crops (Liu et al.,

1995). ISR has been shown to be effective in commercial greenhouse and under field conditions (Alström, 1991; Wei et al., 1996). Research on mechanisms of pathogen activated SAR has been extensive, but proportionally much less in known about mechanisms of PGPR-mediated ISR. An understanding of the underlying mechanisms involved in PGPR-mediated ISR should help exploitation of the potential of ISR in plant disease control.

Blue mold, caused by *Peronospora tabacina*, is an economically important disease in tobacco throughout the world. The disease has been mainly controlled by sprays of the fungicide metalaxyl. However, resistant strains of *P. tabacina* have developed in several countries (Wiglesworth et al., 1988). It is important, therefore, to develop alternatives to control this disease. In preliminary studies, we found that blue mold severity was significantly reduced by application of some PGPR in the greenhouse and in microtitre plates (Zhang et al., 1999).

The objectives of this research were to select PGPR strains for ISR activity in the tobacco-blue mold system and to investigate plant defense responses in the microtitre plate system, particularly those in comparison to pathogen-induced SAR.

MATERIALS AND METHODS

Bacterial PGPR cultures: PGPR strains *Serratia marcescens* 90-166, *Bacillus pumilus* SE34, *Pseudomonas fluorescens* 89B-61, *Bacillus pumilus* T4, and *Bacillus Pasteurii* C-9 were tested for ISR in these assays. For long-term storage, bacterial strains were maintained at -80EC in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI) amended with 20% glycerol.

Bacterial spore preparation: Bacteria were taken from – 80 °C, streaked on tryptic soy agar (TSA), and incubated at 28 °C for 24 h. Bacterial cells were then scraped off plates, resuspended in sterile distilled water, and the bacterial density was adjusted to 10^9 cfu/ml.

Preparation of *P. tabacina* inoculum: Sporangiospores of *P. tabacina* were washed off with distilled water using a paintbrush from sporulating tobacco leaves with blue mold symptoms stored at –80 °C. The suspension was filtered through a 0.45 µm nylon filter, and spores were resuspended in water. The spore density was adjusted to 10^5 spores/ml.

Greenhouse assays of PGPR for ISR against *P. tabacina*: Tobacco plants (cv. Xanthi-nc) were grown in 10-cm plastic pots containing soilless "Pro-Mix" growing medium (Premier Peat Ltd., Rivière-du-Loup, Québec, Canada) for 4 weeks and then PGPR (10^9 cfu/ml) were applied as a soil drench, 50 ml per plant. One week after the treatment with PGPR, tobacco plants were challenged with *P. tabacina* (10^5 spores/ml) by spraying the inoculum onto tobacco leaves until run-off. Five PGPR strains were tested for ISR. Salicylic acid (1.0 mM) was used as a positive control for SAR. Soil drench with water was used as a nontreated control. The experiment was a randomized complete block design with six replications.

Two to three weeks after pathogen challenge, blue mold disease was rated by assessing the percentage of leaf surface area covered with lesions from three middle leaves on each plant. At the same time, ten leaf discs (1 cm in diameter) were cut from each leaf, placed in 10 ml of water, and shaken for 10 min. The spores were examined using a hemacytometer under a light microscope, and the number of spores per leaf disc was calculated.

Microtitre plate assays: Tobacco (cv. Xanthi-nc and NahG) seeds were surface disinfested with 70% ethanol for 1-2 min., then with 1% sodium hypochlorite for 30 min, rinsed in sterilized distilled water three times. The seeds were placed on MS agar (Murashige and Skoog salt and vitamin mixture, GIBCOBRL; with addition of 0.8% agar and 3% sucrose) in multiwell plates (12-well, Falcon), 1 ml in each well. Three weeks after seeding, 10 µl of PGPR bacterial suspension (10^9 cfu / ml), salicylic acid (1.0 mM) or sterilized distilled water were dropped on the medium at the base of the stem at the appropriate treatments. One week after treatments, tobacco plants were challenged with *P. tabacina* by pipetting 2 µl of inoculum (10^5 spores/ml) on a leaf. Two fully developed top leaves from each plant were inoculated. The experiment was a RCB with five replications, and 12 plants per replication. One to two weeks after pathogen challenge, blue mold disease was rated by counting number of symptomatic leaves per plant.

Test for movement of PGPR bacteria to the upper leaves: This test was conducted in the microtitre plate system with tobacco cv. Xanthi-nc. All 5 PGPR were tested for potential from the stem base to upper leaves. The same method was employed as described above regarding induction with PGPR and other conditions in growth chamber. This experiment was designed as randomized blocks with 6 replications per treatment, with 12 plants per replication.

The top two leaves collected from each plant were tested for the population of the bacteria. Ten leaves were detached from one replication, put in 10 ml of sterile water, and then homogenized in a mortar with a pestle. Suspensions of 200 µl were spread onto a TSA plate. Colonies of bacteria were counted after 36 h incubation at 28°C.

Trypan blue staining of tobacco leaves and northern analysis of PR gene expression: Induced tobacco plants were challenged with *P. tabacina* by spraying pathogen inoculum (10^5 spores/ml) evenly onto leaves. Three PGPR strains were tested in this bioassay. Salicylic acid (1.0 mM) was used as a positive control for SAR, and *E. coli* HB101 as a negative control, and treatment with water was a blank control. The experiment was designed as randomized blocks with six replications per treatment. One week after pathogen challenge, spores of *P. tabacina* from a whole plant were washed off with 10 ml of water. Blue mold disease was assessed by determining the mean number of spores per plant. Spores were washed from entire plants and the total number/plant was determined using a light microscope.

Tobacco leaves were completely cleared in farmer's fluid, incubated in trypan blue solution (0.03%) at 60°C until they were black in color. Then, the leaves were

destained in chloral hydrate (2.5 mg/ml). The samples were mounted in 50% glycerol and examined under a light microscope.

Northern blotting was conducted using 4 tobacco cDNA clones as probes. These encoded the PR-1a, basic β-1,3-glucanase, proteinase inhibitor (PI-II) and ACC oxidase genes.

Data analysis: Data was statistically analyzed with analysis of variance using JMP software (SAS Institute Inc., Cary, NC). Significant F -value was calculated at $P = 0.05$, and treatment means were separated using LSD at $P = 0.05$.

RESULTS

Greenhouse assays

In greenhouse bioassays, among five PGPR strains tested for ISR against blue mold disease in tobacco cv. Xanthi-nc, four PGPR strains 90-166, SE34, T4 and C-9 significantly reduced percentage of leaf area with lesions compared to water control at $P = 0.05$ in two repeated trials, while 89B-61 had variable effects on blue mold disease (Table 1). Induction of tobacco plants by PGPR not only significantly decreases lesion area on leaves, but also reduced the sporulation of *P. tabacina*. All of 5 PGPR had significant effects on the reduction of sporulation of *P. tabacina* on tobacco cv. Xanthi-nc (Table 2).

Table 1. Induced disease protection by PGPR against blue mold of tobacco (cv. Xanthi-nc) in the greenhouse

	Percentage of lesion area (%) [2]	
Treatment[1]	Trial 1	Trial 2
90-166	25.3 d [3]	43.3 f
SE-34	23.3 d	48.6 de
89B-61	79.9 a	79.4 b
T4	36.7 c	50.3 d
C-9	55.6 b	72.8 c
SA	28.6 d	43.9 ef
Water control	83.9 a	85.3 a
LSD (P=0.05)	8.0	5.3

[1]PGPR were applied as a root drench by adding 50 ml of bacterial suspension into the soilless potting Pro-Mix in which tobacco plants were grown. Before application, PGPR were cultivated on TSA for 24 h, and spores were scrapped with sterile distilled water.

[2]Tobacco plants were challenged by spraying *P. tabacina* inoculum on to leaves till run-off. Disease parameter represents mean value of percentage of leaf area covered with disease lesions of three middle leaves of each tobacco plant, from 6 replications per treatment.

[3]Means with different letters are significantly different at $P = 0.05$ according to LSD test.

Table 2. Effect of PGPR on sporulation of *P. tabacina* of tobacco (cv. Xanthi-nc) in the greenhouse.

	Mean number of spores ($\times 10^5$)/ leaf disc (1 cm in diameter)[2]	
Treatment[1]	Trial 1	Trial 2
90-166	0.83 ef [3]	0.32 f
SE-34	1.23 e	0.93 e
89B-61	5.03 c	4.02 c
T4	3.02 d	2.98 d
C-9	7.00 b	6.27 b
SA	0.63 f	0.40 f
Water control	7.88 a	8.07 a
LSD (P=0.05)	0.45	0.29

[1]PGPR were applied as a root drench by pouring 50 ml of bacterial suspension into the soilless potting Pro-Mix in which tobacco plants were grown. Before application, PGPR were cultivated on TSA for 24 h, and spores were scrapped with sterile distilled water.

[2]Tobacco plants were challenged by spraying *P. tabacina* inoculum onto leaves till run-off. Disease parameter represents mean number of spores per leaf discs with 1-cm in diameter cut from three middle leaves of each tobacco plant, 6 replications per treatment.

[3]Means with different letters are significantly different at P = 0.05 according to LSD test procedure using JMP software.

Microtitre plate assays

In the microtitre assays with tobacco cv. Xanthi-nc, where *P. tabacina* was applied by dropping spore suspensions onto the leaf surface, 90-166, SE34 and T4 consistently showed significant disease protection in number of symptomatic leaves per plant compared to the water control in two repeated bioassays (Table 3). Treatment with C-9 did not show disease protection in any of these bioassays. The effect of 89B-61 on blue mold in these two assays was variable.

In a separate experiment, *P. tabacina* was applied as foliar spray, the effect of 90-166 and SE34 on reduction of sporulation was significant compared to the water control (Table 4). SA consistently gave significant disease protection against blue mold disease *E. coli* HB101 had no effect on *P. tabacina* sporulation in the microtitre plate system. PGPR strain, 89B-61 did not significantly affect sporulation of *P. tabacina* in this system.

Table 3. Effect of PGPR on blue mold disease of tobacco (cv. Xanthi-nc) in microtitre plates.

Treatment[1]	Mean number of symptomatic leaves per plant[2]	
	Trial 1	Trial 2
90-166	0.12 c [3]	0.17 d
SE-34	0.68 b	0.53 bc
89B-61	1.64 a	0.26 cd
T4	0.80 b	0.67 b
C-9	1.68 a	1.45 a
SA	0.82 b	0.34 bcd
Water control	1.87 a	1.20 a
LSD (P=0.05)	0.42	0.34

[1]PGPR were applied as a root drench by dropping 10 µl of bacterial suspension on MS agar at the stem base of tobacco plants. Before application, PGPR were cultivated on TSA for 24 h, and spores were scrapped with sterile distilled water.
[2]Two µl of *P. tabacina* inoculum was dropped on a top leaf. Two leaves were challenged per tobacco plant. Disease parameter represents the mean number of leaves with blue mold symptom of each tobacco plant, from 6 replications per treatment, 12 plants per replication.
[3]Means with different letters are significantly different at P = 0.05 according to LSD test.

Table 4. Effect of PGPR on sporulation of *P. tabacina* of tobacco (cv. Xanthi-nc) in microtitre plates.

Treatment [1]	Mean number of spores per plant ($\times 10^4$) [2]	
	Trial 1	Trial 2
90-166	8.90 b[3]	8.96 cd
SE-34	4.46 c	4.42 e
89B-61	10.40 ab	9.33 bc
E. coli HB101	11.95 ab	13.00 a
SA	6.38 bc	5.79 de
Water control	14.66 a	12.38 ab
LSD (P=0.05)	4.34	3.40

[1]PGPR were applied as a root drench by dropping 10 µl of bacterial suspension on MS agar at the stem base of tobacco plants. Before application, PGPR were cultivated on TSA for 24 h, and spores were scrapped with sterile distilled water.
[2]Tobacco plants were challenged with *P. tabacina* by spraying the pathogen inoculum onto plant leaves. Tobacco plants were placed in water, shaken for 10 min. The spores were counted under a light microscope. Disease parameter represents the mean number of spores of each tobacco plant, from 6 replications per treatment, 12 plants per replication.
[3]Means with different letters are significantly different at P = 0.05 according to LSD test procedure using JMP software.

Movement of PGPR to tobacco leaves in the microtitre plate system

In the microtitre plate system, no bacteria were detected from the top two leaves of tobacco plants within two weeks after PGPR treatment (Table 5), but one week later, bacteria were detected. In general, the population of PGPR on the first leaf from the top (top leaf 1) was much lower than that on the second leaf (top leaf 2). In addition, the capacity of PGPR to move varied with strains. Bacterial populations on the top two leaves were highest with 89B-61 and T4.

Table 5. Colonization of PGPR on tobacco (cv. Xanthi-nc) leaves in microtitre plates.

Treatment [1]	Population of bacteria detected from tobacco leaf (cfu/leaf)					
	Top leaf 1			Top leaf 2		
	7 DAT [2]	14 DAT	21 DAT	7 DAT	14 DAT	21 DAT
90-166	- [3]	-	-	-	-	20
SE-34	-	-	10	-	-	40
89B-61	-	-	30	-	-	1.1×10^3
T4	-	-	-	-	-	6.0×10^3
C-9	-	-	5	-	-	25
Water control	-	-	-	-	-	-

[1]PGPR were applied as a root drench by dropping 10 µl of bacterial suspension on MS agar at the stem base of tobacco plants. Before application, PGPR were cultivated on TSA for 24 h, and spores were scrapped with sterile distilled water.

[2]DAT represents days after PGPR treatment. Samples were collected at 7, 14 and 21 days after PGPR treatment.

[3]indicates that no bacteria were detected. The limit of the detection was 5 cfu/leaf. The data was the mean value of 6 replications.

Effect of PGPR on blue mold in NahG tobacco

All four PGPR strains, which showed significant disease protection against blue mold on Xanthi-nc tobacco in the microtitre plate system, significantly reduced blue mold on transgenic NahG tobacco plants compared to water control (Table 6), indicating that induced systemic resistance by PGPR is SA independent. SA, which was degraded into catechol by salicylate hydrolase encoded by the transgenic *nahG* gene in tobacco plants transferred from *Pseudomonas putida*, didn't protect tobacco plants from blue mold disease.

Table 6. Effect of PGPR on blue mold disease in transgenic NahG tobacco in micro-titer plates[1].

Treatment	Mean no. of symptomatic leaves per plant[2]	
	Trial I	**Trial II**
90-166	0.02 b	0.02 b
SE-34	0 b	0.02 b
89B-61	0 b	0.29 b
T4	0.08 b	0.32 b
SA[3]	1.12 a	2.71 a
Water	1.50 a	3.38 a
LSD ($P = 0.05$)	0.44	0.88

[1]Tobacco seeds were placed on MS agar (Murashige and Skoog salt and vitamin mixture, GIBCOBRL) amended with 3% sucrose in multiwell (12-well, Falcon) plates after being disinfested with 70% ethanol and 1% sodium hypochlorite. Three weeks after seeding, 10 µl of PGPR bacterial suspension (10^8 cfu / ml), salicylic acid (1.0 mM) or water (as a control) were dropped on the medium at the base of the stem. One week after PGPR induction, plants were challenged with *P. tabacina* by pipetting 2 µl of inoculum (10^5 sporangiospores/ml) on a leaf. Two fully developed top leaves from each plant were inoculated. This experiment was a RCB with 5 replications, and 12 plants per replication.
[2]One to Two weeks after pathogen challenge, blue mold disease was rated by counting no. of symptomatic leaves per plant. JMP software (SAS Institute Inc., Cary, NC 27513) was used to analyze the data.
[3]The concentration of SA was 1 mM.

Trypan blue staining of tobacco leaves

Among the samples collected at 2 days after pathogen challenge, very few pathogen hyphae were observed in tobacco leaf tissues regardless of treatment. However, differences were apparent in tobacco leaves observed 7 days after pathogen challenge between the SA or PGPR induced plant and the untreated control plant. In control tobacco leaves, there were many well-developed hyphae of *P. tabacina*. In the leaves treated with salicylic acid, less extensive *P. tabacina* hyphal development was observed compared to the control plant. In addition, some necrosis occurred in SA-treated, pathogen-challenged tissues. Similar observations were made from plants receiving the SE34 treatment. In the leaves from plants treated with strain 90-166, although there was more pathogen hyphae in the tobacco tissues compared to other treatments, e.g. SA or SE34, the frequency of the necrosis seen in the plant tissue was much higher than in other treatments. Furthermore, the staining of the necrotic areas was much more extensive than other treatments. The response to treatment with PGPR strain 89B-61 was quite distinct. Small necrotic spots were observed in the leaves from 89B-61- treated tobacco plants. In addition, there were less hyphae in the leaves of 89B-61- treated plants than in the water treated control**.**

Northern Blotting

No significant difference was found in the expression of genes responsible for PR-1a, basic β–1, 3-glucanase, proteinase inhibitor and ACC oxidase in PGPR-treated and nontreated control tobacco plants before and after challenge with *P. tabacina*.

DISCUSSION

Results presented here demonstrate that PGPR strains, which previously exhibited induced protection in cucumber and tomato against several pathogens, induced significant disease protection against blue mold in tobacco. Studies on the movement of PGPR on tobacco plants in the microtitre plate system indicate that disease protection due to the PGPR treatments was mainly a systemic phenomenon because no bacteria were detected from the two top leaves two weeks after PGPR inoculation in the microtitre plates. The ISR by PGPR not only resulted in reduction of blue mold lesion areas and number of symptomatic leaves, but also resulted in a decrease of *P. tabacina* sporulation. *P. tabacina* is an obligate biotrophic pathogen dependent upon living tissue for life cycle completion. The results of this study are interesting because not only did PGPR treatment reduce visual symptoms and pathogen development *in planta*, but also reduced sporulation This suggests that PGPR-mediated ISR may affect the efficiency of sporulation, an important component of an obligate biotroph's life cycle.

Three PGPR substantially reduced blue mold disease both in Xanthi-nc and NahG tobacco, indicating that PGPR-mediated ISR is independent of SA. Northern analysis using four probes derived from cDNA clones of plant defense-related genes, including the SA-inducible PR-1a gene, suggest that ISR mediated by the tested PGPR strains may be associated with novel defense pathways. No qualitative differences in transcript accumulation were observed due to PGPR treatment prior to or after pathogen challenge.

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