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Development of Assays for Assessing Induced Systemic Resistance by Plant Growth-Promoting Rhizobacteria against Blue Mold of Tobacco

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A microtiter plate and a detached leaf assay were developed to test elicitation of induced systemic resistance (ISR) by plant growth-promoting rhizobacteria (PGPR) against tobacco blue mold disease caused by *Peronospora tabacina*, and results from the bioassays were confirmed in pot trials in the greenhouse. Five PGPR strains, *Serratia marcescens* 90-166, *Bacillus pumilus* SE34, *Pseudomonas fluorescens* 89B-61, *B. pumilus* T4, and *B. pasteurii* C-9, which had been previously demonstrated to elicit ISR against several diseases on different crops, were evaluated for potential elicitation of ISR against blue mold. In the microtiter plate system, three PGPR strains significantly reduced number of leaves with lesions when *P. tabacina* was applied onto leaf surfaces. Sporulation of *P. tabacina* was reduced by two of these three PGPR strains when the pathogen was applied as foliar sprays. No PGPR were detected within upper leaves, indicating that the pathogen and inducing agents were spatially separated. Four PGPR strains elicited significant disease reduction in an assay conducted with detached tobacco leaves placed in petri dishes, and in the same assay, sporulation of *P. tabacina* was reduced by all five tested PGPR strains. Results from greenhouse assays confirmed that treatment with PGPR resulted in significant reduction in blue mold disease severity. Elicitation of ISR varied among PGPR strains on different tobacco cultivars; five strains elicited significant protection on tobacco cv. Ky14, four strains on cv. Xanthi-nc, and three on cv. TN 90. Significant reduction in sporulation of *P. tabacina* was achieved with all five strains in the pot trials. These results indicate that the microtiter plate and detached leaf assays can be used for further studies on mechanisms of ISR elicited by rhizobacteria. © 2002 Elsevier Science

Key Words: **induced systemic resistance (ISR); rhizobacteria; biological control; *Peronospora tabacina*.**

INTRODUCTION

Blue mold, caused by *Peronospora tabacina* Adam, is one of the most important diseases of tobacco in Australia, Europe, Central America, and North America (Johnson, 1989). It is a compound-interest disease that occurs in local and macroscale epidemics (Main, 1997). It develops and spreads rapidly during periods of cool, wet weather because of the polycyclic multiplication of the fungal pathogen. During a blue mold epidemic in 1979, more than $250 million in losses were reported in the United States and Canada (Schiltz, 1981). Blue mold disease of tobacco has been controlled mainly by applying the systemic fungicide metalaxyl (Schiltz, 1981). However, strains of *P. tabacina* resistant to metalaxyl have developed in several countries (Schiltz, 1981; Wiglesworth *et al.*, 1988). It is important, therefore, to develop alternative means for controlling this disease.

Induced resistance of tobacco to blue mold disease was first reported by Mandryk (1960) and Cruickshank and Mandryk (1960) in greenhouse and field experiments. They found that stem infection with *P. tabacina* markedly reduced the development of *P. tabacina* subsequently inoculated on the foliage, and this protection lasted through flowering and seed set. However, the stem-infected plants were severely stunted. Sporangia of *P. tabacina* applied to the soil surface around stems caused stem necrosis and systemically induced resistance, although plant growth was also stunted (Cohen and Kuæ, 1981). Stem inoculation with sporangia was subsequently used to induce resistance against blue mold and increase growth of tobacco plants (Tuzun and Kuæ, 1985), but stem injection is impractical in commercial tobacco production.

Since the early 1990s, induction of systemic resistance by plant growth-promoting rhizobacteria (PGPR) has been investigated as a possible practical way to use induced resistance in agriculture. PGPR have been tested in the greenhouse and field for induced systemic resistance (ISR) to fungal (Chen *et al.*, 1995; Liu *et al.*, 1995c; Pieterse *et al.*, 1996; van Peer *et al.*, 1991; Wei *et al.*, 1991, 1996), bacterial (Alström, 1991; Liu *et al.*, 1995b; Park and Kloepper, 2000), and viral (Maurhofer *et al.*, 1994; Raupach *et al.*, 1996) pathogens in various crops such as bean, carnation, cucumber, radish, tobacco, tomato, and *Arabidopsis*.

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One of the characteristics of PGPR-mediated ISR is that a broad spectrum of pathogens could be suppressed by the same PGPR strain(s). In cucumber, for example, PGPR strains *Serratia marcescens* Bizio 90-166 and *Pseudomonas putida* (Trevisan) Migula 89B-27 were first reported to induce systemic protection against cucumber anthracnose caused by *Colletotrichum orbiculare* (Berk. & Mont.) Arx. in the greenhouse (Wei *et al.,* 1991) and then in field trials (Wei *et al.,* 1996). These two strains also induced systemic disease protection against Fusarium wilt caused by *Fusarium oxysporum* (Schlecht.) Snyd. & Hans. f.sp. *cucumerinum* Owen (Liu *et al.,* 1995c), bacterial angular leaf spot caused by *P. syringae* van Hall pv. *lachrymans* (Smith & Bryan) Young, Dye, & Wilkie (Liu *et al.,* 1995b), cucumber mosaic *cucumovirus* (CMV) (Raupach *et al.,* 1996), and naturally occurring cucurbit wilt disease caused by *Erwinia tracheiphila* (Smith) Bergey *et al.* (Kloepper *et al.,* 1993). Some PGPR have elicited ISR in tobacco against wild fire, caused by *P. syringae* (van Hall) Ash *et al.* pv. *tabaci* (Park and Kloepper, 2000; Press *et al.,* 1997), but protection against blue mold has not been investigated with PGPR.

The objective of this research was to determine whether PGPR strains that have previously elicited ISR on other crops could induce resistance in tobacco to blue mold. To achieve this objective and to facilitate future work on mechanisms of bacterial signal transduction in tobacco, miniaturized *in vitro* assays were developed. The responses of the PGPR/tobacco blue mold system in these assays were compared to responses in pot trials in the greenhouse.

MATERIALS AND METHODS

PGPR Strains and Inoculum Preparation

Five PGPR strains, *S. marcescens* 90-166, *Bacillus pumilus* Meyer and Gottheil SE34, *Pseudomonas fluorescens* (Trevisan) Migula 89B-61, *B. pumilus* T4, and *B. pasteurii* (Miquel) Chester C-9, were used in these studies. All of the selected PGPR strains led to significant reduction in foliar disease following application as seed treatments on cucumber, and some have been reported to induce disease resistance in tomato against foliar diseases. The PGPR strains used and the references in which they have been reported are T4 (Raupach *et al.,* 1997), SE34 (Jetiyanon, 1997; Benhamou *et al.,* 1996; Yao *et al.,* 1997; Ji *et al.,* 1997), 90-166 (Liu *et al.,* 1995a,b,c; Raupach *et al.,* 1996; Kloepper *et al.,* 1996; Wei *et al.,* 1996; Zehnder *et al.,* 1997b; Ji *et al.,* 1997), and C-9 (Ji *et al.,* 1997). For long-term storage, bacterial strains were maintained at −80°C in tryptic soy broth (Difco Laboratories, Detroit, MI) that contained 20% glycerol.

Inoculum for treatment of tobacco was prepared by streaking strains from storage at −80°C onto tryptic soy agar (TSA) plates and incubating the plates at 28°C for 24 h. Bacterial cells were harvested from the TSA plates in sterile distilled water to yield 10^9 colony-forming units (CFU) per milliliter.

Preparation of P. tabacina Inoculum

Isolate No. 79 of *P. tabacina* was obtained from W. C. Nesmith, University of Kentucky. Inoculum was obtained from infected leaves with freshly sporulating blue mold lesions of tobacco (*Nicotiana tabacum* L.) cv. Ky14 on 6- to 8-week-old plants, 7 or 8 days after inoculation. Sporangia were gently brushed into a small quantity of distilled water, washed three times again with distilled water on a 0.45-μm nylon filter, and then resuspended in sterile distilled water to make the sporangial suspension (4×10^5 sporangia/ml).

Development of in Vitro Bioassays for Testing PGPR for ISR Activity against P. tabacina

Detached leaf assay. Two fully expanded top leaves were detached from a 7-week-old tobacco cv. Ky14 seedling grown in the greenhouse. Suspensions of PGPR (200 μl) were injected into leaf petioles. The injected leaves were placed in a plastic petri dish (150 mm) containing 5% solution of Murashige and Skoog (MS) medium with salt and vitamin mixture (GIBCO-BRL, Grand Island, NY). The petri dish was tilted so that the cut end of the petiole was in MS solution and was placed in a growth chamber with a 14-h-light and 10-h-darkness cycle at 22°C. Five PGPR strains (90-166, SE34, 89B-61, T4, and C-9) were tested in this assay; 1.0 mM salicylic acid (SA) was used as a chemical control and injection with water was a nontreated control. Treatments were arranged in a randomized complete block with six replications. Seven days after PGPR injection into leaf petioles, tobacco leaves were challenged by spraying evenly with the sporangial inoculum of *P. tabacina.*

One week after pathogen challenge, blue mold disease was visually rated by assessing the percentage of leaf surface covered by blue mold lesions. Ten leaf disks (1 cm in diameter) were cut from each leaf, placed in 10 ml of sterile distilled water, and shaken for 10 min. The sporangia were enumerated using a hemacytometer under a light microscope, and the number of sporangia per leaf disk was calculated.

Microtiter plate assay. Tobacco cv. Xanthi-nc seeds provided by J. Ryals (Novartis Agricultural Biotechnology Research Unit, Research Triangle Park, NC) were used in this assay. Seeds were surface-disinfected with 70% ethanol for 1–2 min and then with 1% sodium hypochlorite for 30 min and rinsed in sterile distilled water three times. One seed was placed on 1 ml of 0.8% MS agar supplemented with 3% sucrose in each well of a 12-well microtiter plate. Three weeks after seeding, treatments were applied. Ten microliters of PGPR bacterial suspension (10^9 CFU/ml), SA, or sterile distilled

water were applied on the medium at the base of the stem. The same five PGPR strains were used in this assay. One week after PGPR treatments, tobacco plants were challenged with *P. tabacina* by pipetting 2 μl of the sporangial inoculum onto a leaf. Two fully developed top leaves from each plant were inoculated. Treatments were arranged in a randomized complete block with five replications and 12 plants per treatment per replication. One to 2 weeks after pathogen challenge, blue mold disease was rated by counting the number of symptomatic leaves per plant.

In a separate bioassay conducted in the microtiter plate system, induced tobacco plants were challenged with *P. tabacina* by spraying pathogen inoculum (10^5 sporangia/ml) evenly onto leaves. Three PGPR strains, 90-166, SE34, and 89B-61, were tested in this bioassay. SA was used as a positive control; *Escherichia coli* (Migula) Castellani and Chalmers strain HB101, provided by Brent L. Nielson, Auburn University, was a negative control, and treatment with water was the nontreated control. Treatments were arranged in a randomized complete block with six replications and 12 plants per replication. One week after pathogen challenge, sporangia of *P. tabacina* were washed from whole plants with 10 ml of water. Blue mold disease was assessed by counting the mean number of sporangia per plant under a light microscope.

Test for Movement of PGPR to the Upper Leaves

This test was conducted in the microtiter plate system with tobacco cv. Xanthi-nc. All five PGPR were tested for potential movement from the stem base to upper leaves. The same methods were employed as described above with regard to induction with PGPR. Treatments were arranged in a randomized complete block with six replications and 12 plants per replication.

The top two leaves from each plant were collected and tested for bacterial presence. Ten leaves were detached from plants in 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 and were compared to reference strains of the inoculated PGPR for identification by typical colony morphologies, although bacteria not representative of the inoculated PGPR were not recovered.

Confirmation of ISR by PGPR in Greenhouse Pot Trials

Tobacco plants (cvs. Xanthi-nc and Ky14) were grown in 10-cm plastic pots containing soilless "Pro-Mix" growing medium (Premier Peat Ltd., Rivière-du-Loup, Québec, Canada) for 4 weeks when PGPR (10^9 CFU/ml) were applied as a soil drench, 50 ml per plant. One week after treatment with PGPR, the tobacco plants were challenged with sporangial inoculum of *P. tabacina* by spraying onto the leaves until runoff. All five PGPR strains were tested; SA was used as a positive control, and a water drench was used as the nontreated control. Treatments were arranged in a randomized complete block with six replications of each treatment and one plant per replication.

Two to 3 weeks after pathogen challenge, blue mold disease was visually rated by assessing the percentage of leaf surface area covered with lesions from three middle leaves on each plant. At the same time, 10 leaf disks (1 cm in diameter) were cut from each leaf, placed in 10 ml of water, and shaken for 10 min. The sporangia were enumerated using a hemacytometer under a light microscope, and the number of sporangia per leaf disk was calculated.

Separate greenhouse bioassays were conducted with tobacco cv. TN90 in Speedling trays (Speedling Inc., Sun City, Florida). PGPR were applied as seed treatments by pipetting 1 ml of bacterial suspension onto a seed at the time of sowing and as two soil drenches (50 ml of bacterial suspension at each drench) at 2 and 3 weeks after planting. Tobacco plants were challenged with *P. tabacina* (10^5 sporangia/ml) at 6 weeks after the final drench in one trial and at 8 weeks in a second trial. Five PGPR were tested in this assay, and treatment with water was the control. Treatments were arranged in a RCB with six replications, one plant per replication.

Two weeks after pathogen challenge, blue mold disease was rated by assessing the percentage of leaf surface covered with lesions.

Statistical Analyses

Data from microtiter, detached leaf, and greenhouse assays were analyzed by analysis of variance using JMP software (SAS Institute Inc., Cary, NC). The significance of effect of PGPR treatments was determined by the magnitude of the F value ($P = 0.05$). When a significant F test was obtained for treatments, separation of means was accomplished by Fisher's protected Least Significant Difference (LSD).

RESULTS

In Vitro Assays

Detached leaf assay. In the assays using detached leaves from tobacco plants (cv. Ky14), all PGPR treatments except C-9 reduced blue mold disease compared to the water control (Table 1). The level of protection resulting from treatment with PGPR strains 90-166, SE34, and T4 was statistically equivalent to the protection resulting from treatment with SA. Sporulation of *P. tabacina* in all treatments was reduced compared to that of the water control (Table 2). The reduction of

TABLE 1

Systemic Resistance Induced by PGPR against Blue Mold in Tobacco cv. Ky14 in Detached Leaf Bioassays

Treatment[a]	Percentage of leaf area with lesions[b]	
	Bioassay 1	Bioassay 2
Serratia marcescens 90-166	21.7 b[c]	14.2 bc[c]
Bacillus pumilus SE34	23.3 b	16.7 bc
Pseudomonas fluorescens 89B-61	30.0 b	28.3 b
B. pumilus T4	28.3 b	25.0 bc
B. pasteurii C-9	77.6 a	76.7 a
Salicylic acid (1.0 mM)	13.3 b	11.7 c
Water control	85.3 a	83.2 a

[a] PGPR was injected into the petiole of detached leaves (200 μl/leaf) of tobacco cv. Ky14. Salicylic acid was applied at 200 μl/leaf and water was used as a control at the same rate.

[b] Seven days after PGPR, SA, and water treatments, detached leaves were challenged with *P. tabacina* by spraying until runoff. Seven days later, blue mold was assessed as percentage of leaf area covered with lesions to evaluate the treatment effects.

[c] Data are the means of six replications, 12 detached leaves per replication. Means in columns followed by different letters are significantly different at $P = 0.05$ according to the LSD test.

sporulation, which occurred with strains 90-166 and SE34, was statistically equivalent to that from the SA treatment. Treatment with strains 89B-61 and T4 also reduced sporulation of *P. tabacina* compared to the nontreated control (water), but not as substantially as caused by strains 90-166 and SE34.

Microtiter plate assay. In the microtiter assays with tobacco cv. Xanthi-nc, where PGPR were drenched onto the medium at the stem base and *P. tabacina* inoculum was placed onto the leaf lamina surface, 90-166, SE34, and T4 reduced the 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. At the time of disease rating, tobacco plants in this system typically had six to eight leaves and were 1–2 cm in height.

TABLE 2

Effect of PGPR on Sporulation of *P. tabacina* of Tobacco cv. Ky14 in Detached Leaf Bioassays

Treatment[a]	Mean number of sporangia ($\times 10^5$)/leaf disk[b]	
	Bioassay 1	Bioassay 2
Serratia marcescens 90-166	1.50 e[c]	1.65 e[c]
Bacillus pumilus SE34	1.23 e	1.60 e
Pseudomonas fluorescens 89B-61	5.03 c	4.02 c
B. pumilus T4	3.02 d	2.98 d
B. pasteurii C-9	7.00 b	6.23 b
Salicylic acid (1.0 mM)	1.63 e	1.62 e
Water control	7.88 a	8.07 a

[a] PGPR was injected into the petiole of detached leaves (200 μl/leaf) of tobacco cv. Ky14. Salicylic acid was applied at 200 μl/leaf and water was used as a control at the same rate.

[b] Seven days after PGPR, SA, and water treatments, detached leaves were challenged with *P. tabacina* by spraying until runoff. Seven days later, sporulation of *P. tabacina* per leaf disk with 1 cm in diameter was assessed to evaluate the treatment effects.

[c] Data are the means of six replications, 12 detached leaves per replication. Means in columns followed by different letters are significantly different at $P = 0.05$ according to the LSD test.

TABLE 3

Systemic Resistance Induced by PGPR against Blue Mold of Tobacco cv. Xanthi-nc in Microtiter Plate Bioassays

Treatment[a]	Mean number of symptomatic leaves per seedling[b]	
	Bioassay 1	Bioassay 2
Serratia marcescens 90-166	0.12 c[c]	0.17 d[c]
Bacillus pumilus SE34	0.68 b	0.53 bc
Pseudomonas fluorescens 89B-61	1.64 a	0.26 cd
B. pumilus T4	0.80 b	0.67 b
B. pasteurii C-9	1.68 a	1.45 a
Salicylic acid (1.0 mM)	0.82 b	0.34 bcd
Water control	1.87 a	1.20 a

[a] PGPR were applied as a root drench (10 μl of bacterial suspension/seedling) of tobacco cv. Xanthi-nc seedlings grown on MS agar in microtiter plates. Salicylic acid was applied at 200 μl/seedling and water was used as a control at the same rate.

[b] Two microliters of *P. tabacina* inoculum was placed on two top leaves of each tobacco seedling at 7 days after PGPR application. Seven days later, blue mold was assessed as number of leaves with blue mold symptoms of each tobacco seedling.

[c] Data are the means of six replications, 12 seedlings per replication. Means in columns followed by different letters are significantly different at $P = 0.05$ according to the LSD test.

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

Movement of PGPR to Tobacco Leaves in the Microtiter Plate Assay

In the microtiter plate system, no bacteria were detected from the top two leaves of tobacco plants within 2 weeks after PGPR treatment (Table 5), but at 3 weeks three or four of the bacterial strains 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 in the top two leaves were highest with 89B-61 and T4. The only bacteria recovered matched the colony type of the introduced PGPR.

TABLE 4

Effect of PGPR on Sporulation of *P. tabacina* of Tobacco cv. Xanthi-nc in Microtiter Plate Bioassays

Treatment[a]	Mean number of sporangia per seedling ($\times 10^4$)[b]	
	Bioassay 1	Bioassay 2
Serratia marcescens 90-166	8.90 b[c]	8.96 cd[c]
Bacillus pumilus SE34	4.46 c	4.42 e
Pseudomonas fluorescens 89B-61	10.40 ab	9.33 bc
E. coli HB101	11.95 ab	13.00 a
Salicylic acid (1.0 mM)	6.38 bc	5.79 de
Water control	14.66 a	12.38 ab

[a] PGPR were applied as a root drench (10 μl of bacterial suspension/seedling) of tobacco cv. Xanthi-nc seedlings grown on MS agar in microtiter plates. Salicylic acid was applied at 200 μl/seedling and water was used as a control at the same rate.

[b] Tobacco seedlings were challenged with *P. tabacina* by spraying onto leaves until runoff at 7 days after PGPR treatment. After 7 days of pathogen challenge, number of sporangia was estimated per seedling to evaluate the PGPR effect.

[c] Data are the means of six replications, 12 seedlings per replication. Means in columns followed by different letters are significantly different at $P = 0.05$ according to the LSD test.

TABLE 6

Induced Systemic Resistance by PGPR against Blue Mold in Tobacco cv. Xanthi-nc in Greenhouse Bioassays

Treatment[a]	Percentage of leaf area with lesions[b]	
	Bioassay 1	Bioassay 2
Serratia marcescens 90-166	25.3 d[c]	43.3 f[c]
Bacillus pumilus SE34	23.3 d	48.6 de
Pseudomonas fluorescens 89B-61	79.9 a	79.4 b
B. pumilus T4	36.7 c	0.3 d
B. pasteurii C-9	55.6 b	72.8 c
Salicylic acid (1.0 mM)	28.6 d	43.9 ef
Water control	83.9 a	85.3 a

[a] PGPR were applied as a root drench by pouring 50 ml of bacterial suspension into a soilless potting medium (Pro-Mix) in which tobacco plants were grown. Before application, PGPR were cultivated on TSA for 24 h, and bacterial cells were removed from TSA plates with sterile distilled water.

[b] Tobacco plants were challenged by spraying *P. tabacina* inoculum onto leaves until runoff. Disease parameter represents mean value of the percentage of leaf area covered with lesions of three middle leaves of each tobacco plant, from six replications per treatment.

[c] Means in columns followed by different letters are significantly different at $P = 0.05$ according to the LSD test.

Confirmation of ISR by PGPR in Greenhouse Pot Trials

In greenhouse bioassays, five PGPR strains were tested for ISR against blue mold disease in three tobacco cultivars. With tobacco cv. Xanthi-nc (Table 6), PGPR strains 90-166, SE34, T4, and C-9 reduced the percentage of leaf area with lesions compared to the water control in two repeated trials. PGPR strain 89B-61 had variable effects on blue mold disease in these assays. On tobacco cv. Ky14, all treatments resulted in significant disease reductions, as measured by the percentage of lesions on leaves, compared to the water control in two repeated trials (Table 7). In a bioassay with tobacco cv. TN90, treatment with 90-166, SE34, and T4 resulted in a significant disease reduction compared to the water control in the repeated trials (Table 8), while 89B-61 and C-9 showed inconsistent effects. Treatment of tobacco plants by PGPR not only significantly decreased lesion area on leaves, but also reduced sporulation of *P. tabacina*. All

TABLE 5

Movement of PGPR from Tobacco Seedling Rhizosphere to Upper Parts of Leaves in Tobacco cv. Xanthi-nc in Microtiter Plate Bioassays

Treatment[a]	PGPR densities (CFU/leaf)					
	Upper leaf 1[b]			Upper leaf 2[b]		
	7 DAT	14 DAT	21 DAT	7 DAT	14 DAT	21 DAT
Serratia marcescens 90-166	—	—	—	—	—	—
Bacillus pumilus SE34	—	—	10	—	—	40
Pseudomonas fluorescens 89B-61	—	—	30	—	—	1100
B. pumilus T4	—	—	—	—	—	6000
B. pasteurii C-9	—	—	5	—	—	25
Water control	—	—	—	—	—	—

Note. —, Not detected. The limit of the detection was 5 CFU/leaf.

[a] PGPR were applied as a root drench (10 μl of bacterial suspension/seedling) of tobacco cv. Xanthi-nc seedlings grown on MS agar in microtiter plates. Salicylic acid was applied at 200 μl/seedling and water was used as a control at the same rate.

[b] Bacterial densities were estimated at 7, 14, and 21 days after PGPR treatment (DAT) from upper leaves of seedlings. Data are the means of six replications.

TABLE 7

Systemic Resistance Induced by PGPR against Blue Mold in Tobacco cv. Ky14 in Greenhouse Bioassays

Treatment[a]	Percentage of leaf area with lesions[b]	
	Bioassay 1	Bioassay 2
Serratia marcescens 90-166	12.5 bcd[c]	7.5 d[c]
Bacillus pumilus SE34	15.6 bcd	20.0 bc
Pseudomonas fluorescens 89B-61	24.4 b	21.3 b
B. pumilus T4	11.3 cd	8.8 cd
B. pasteurii C-9	13.8 bcd	13.1 bcd
Salicylic acid (1.0 mM)	3.8 d	6.3 d
Water control	53.1 a	45.6 a

[a] PGPR (50 ml), SA (50 ml), and water (50 ml) were applied as a root drench to tobacco cv. Ky14 grown in soilless potting medium at 4 weeks after planting.

[b] Seven days after PGPR, SA, and water treatments, tobacco seedlings were challenged by spraying *P. tabacina* until runoff. Seven days after pathogen challenge, blue mold symptoms were assessed as percentage of leaf area covered with lesions from three middle leaves of each tobacco seedling.

[c] Data are the means of six replications, 12 plants per replication. Means in columns followed by different letters are significantly different at $P = 0.05$ according to the LSD test.

TABLE 9

Effect of PGPR on Sporulation of *P. tabacina* of Tobacco cv. Xanthi-nc in Greenhouse Bioassays

Treatment[a]	Mean number of sporangia ($\times 10^5$) per leaf disk (1 cm in diameter)[b]	
	Bioassay 1	Bioassay 2
Serratia marcescens 90-166	0.83 ef[c]	0.32 f[c]
Bacillus pumilus SE34	1.23 e	0.93 e
Pseudomonas fluorescens 89B-61	5.03 c	4.02 c
B. pumilus T4	3.02 d	2.98 d
B. pasteurii C-9	7.00 b	6.27 b
Salicylic acid (1.0 mM)	0.63 f	0.40 f
Water control	7.88 a	8.07 a

[a] PGPR (50 ml), SA (50 ml), and water (50 ml) were applied as a root drench at 4 weeks after planting tobacco cv. Xanthi-nc seedlings grown in soilless potting media.

[b] Seven days after PGPR, SA, and water treatments, tobacco leaves were challenged with *P. tabacina* by spraying until runoff. Seven days later, sporulation was assessed as number of sporangia per leaf disk with 1 cm in diameter to evaluate the treatment effects.

[c] Data are the means of six replications, 12 leaf disks per replication. Means in columns followed by different letters are significantly different at $P = 0.05$ according to the LSD test.

five PGPR strains had significant effects on the sporulation of *P. tabacina* on tobacco cv. Xanthi-nc (Table 9).

DISCUSSION

Results presented here demonstrate that some PGPR strains, previously demonstrated to induce protection in tobacco against *P. syringae* pv. *tabaci* (Jetiyanon, 1997; Park and Kloepper, 2000; Press *et al.*, 1997), in cucumber against bacterial angular leaf spot and Fusarium wilt, caused by *C. orbiculare* and *F. oxysporum* f.sp. *cucumerinum* (Liu *et al.*, 1995a,b,c; Rapauch *et al.*, 1996; Wei *et al.*, 1991, 1996), and in tomato against CMV and *P. syringae* van Hall pv. *tomato* (Ji *et al.*, 1997; Rapauch *et al.*, 1996), induced significant disease protection against blue mold of tobacco. This protection was observed on tobacco cvs. Xanthi-nc, Ky14, and TN90, which differ in their levels of resistance to blue mold. Studies on the movement of PGPR on tobacco plants in the microtiter plate system indicate that disease protection due to the PGPR treatment was mainly a systemic phenomenon because no bacteria were detected from the two top leaves at 2 weeks after PGPR inoculation in the microtiter plates (Table 5).

In the microtiter plate assay, adding SA at the stem base of tobacco plants resulted in significant protection against blue mold. In the detached leaf assays, SA injected into the petiole of tobacco leaves produced 85% reduction of lesion area compared to the control (Table 1) and 80% reduction in sporulation of *P. tabacina* (Table 2). Our results agree with those of Cohen (1994), who showed that foliar spray with SA resulted in 74% reduction in fungal sporulation of *P. tabacina* and that dipping the petiole of tobacco leaves into a solution of SA induced a reduction in sporulation.

It is apparent that levels of induced resistance by PGPR in tobacco against *P. tabacina* depended on the

TABLE 8

Systemic Resistance Induced by PGPR against Blue Mold in Tobacco cv. TN90 in Greenhouse Bioassays

Treatment[a]	Percentage of leaf area with lesions[b]	
	Bioassay 1	Bioassay 2
Serratia marcescens 90-166	10.9 bc[c]	16.5 cd[c]
Bacillus pumilus SE34	12.2 bc	15.8 cd
Pseudomonas fluorescens 89B-61	16.5 abc	13.3 d
B. pumilus T4	9.1 c	19.5 bcd
B. pasteurii C-9	16.8 abc	14.0 d
Salicylic acid (1.0 mM)	9.2 c	13.1 d
Water control	32.0 a	33.8 a

[a] PGPR (1 ml), SA (1 ml), and water were applied to tobacco cv. TN90 seeds immediately after seeding in soilless mix. Also, the same treatments at 50 ml of each plant were applied as soil drenches at 2 and 3 weeks after seeding.

[b] Eight weeks after the final drench in Bioassay 1 and 6 weeks in Bioassay 2, tobacco seedlings were challenged by spraying *P. tabacina* until runoff. Seven days after pathogen challenge, blue mold symptoms were assessed as percentage of leaf area covered with lesions from three middle leaves of each tobacco seedling.

[c] Data are the means of six replications, 12 plants per replication. Means in columns followed by different letters are significantly different at $P = 0.05$ according to the LSD test.

TABLE 10

Overall Summary of Systemic Resistance Induced by PGPR against Blue Mold Disease of Tobacco in Detached Leaf, Microtiter, and Greenhouse Bioassays

	Bioassays		
PGPR strains	Detached leaf	Microtiter plate	Greenhouse
90-166	+	+	+
SE34	+	+	+
89B-61	+	±	±
T4	+	+	+
C-9	—	—	+

Note. +, Significantly different from nontreated water control at $P = 0.05$ according to the LSD test; —, not significant; ±, variable.

PGPR strains. All the strains tested under greenhouse conditions, four strains (90-166, SE34, 89B61, and T4) in the detached leaf assays, and three strains (90-166, SE34, and T4) in microtiter plate assays generally protected tobacco from blue mold disease (Table 10). This may be due to the difference in growing conditions between the greenhouse, detached leaf, and microtiter plate assays. Efficacy of disease reduction in blue mold was best under the greenhouse conditions for the PGPR strains. This is probably due to microbial colonization of soilless growth media used to grow the seedlings in the greenhouse. Overall, strains 90-166, SE34, and T4 were the most effective strains, giving 50–90% protection compared to the water control in all assays. Strain 89B-61 was variable in disease reduction. Although inoculum density of PGPR was the same in all assays, different methods of pathogen challenge were employed in the studies. When tobacco plants were challenged with *P. tabacina* by foliar spray, which resulted in higher disease pressure than that from drops of the inoculum on leaves, protection by strain 89B-61 did not differ from that of the water control (Table 4).

In greenhouse trials, when PGPR were applied as seed treatment and soil drench, effects on disease protection by some PGPR were variable and this may be due to differences in the period of time between the last PGPR treatment and the pathogen challenge since all other factors in these two trials were the same except for the time between the final PGPR treatment and the pathogen challenge (Table 8). When challenged at 8 weeks instead of 6 weeks, PGPR strains 89B-61 and C-9 showed decreased disease protection against blue mold, which indicated that ISR elicited by some PGPR may protect plants for a limited period of time. However, some PGPR strains can prolong the period of protection, as seen with strains 90-166, SE34, and T4.

PGPR not only prevented lesions from spreading on tobacco leaves, but they substantially reduced the sporulation of *P. tabacina*. This is potentially important since tobacco blue mold is a polycyclic disease that could result in macroscale epidemics (Main, 1997). Reduction in sporulation may play a role in decreased disease spread and in disease increase.

The fact that disease protection by some PGPR in the greenhouse was similar to that on seedlings in the microtiter assay indicates that the microtiter gnotobiotic assay can be used for future studies on mechanisms of PGPR. In addition, use of this microtiter system can exclude the role of other microorganisms, such as soil microflora, from the studies.

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