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Altered Epiphytic Colonization of Mannityl Opine-Producing Transgenic Tobacco Plants by a Mannityl Opine-Catabolizing Strain of *Pseudomonas syringae*

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The plasmid pYDH208, which confers the ability to catabolize the mannityl opines mannopine and agropine, was mobilized into the nonpathogenic *Pseudomonas syringae* strain Cit7. The growth of the mannityl opine-catabolizing strain Cit7(pYDH208) was compared with that of the near-isogenic non-opine-catabolizing strain Cit7xylE on leaves of wild-type tobacco (*Nicotiana tabacum* cv. Xanthi) and transgenic mannityl opine-producing tobacco plants (*N. tabacum* cv. Xanthi, line 2-26). The population size of Cit7(pYDH208) was significantly greater on the lower leaves of transgenic plants than on middle or upper leaves of those plants. The population size of Cit7(pYDH208) on lower leaves of transgenic plants was also significantly greater than the population size of Cit7xylE on similar leaves of wild-type plants. High-voltage paper electrophoresis demonstrated higher levels of mannityl opines in washings from lower- and mid-level leaves than in washings from upper-level leaves. The ability of Cit7(pYDH208) to catabolize mannityl opines in the carbon-limited phyllosphere increased the carrying capacity of the lower leaves of transgenic plants for Cit7(pYDH208). In coinoculations, the increase in the ratio of population sizes of Cit7(pYDH208) to Cit7xylE on transgenic plants was apparently due to a subtle difference in the growth rates of the two strains and to the difference in final population sizes. An ability to utilize additional carbon sources on the transgenic plants also enabled Cit7(pYDH208) to achieve a higher degree of coexistence with Cit7xylE on transgenic plants than on wild-type plants. This supports the hypothesis that the level of coexistence between epiphytic bacterial populations can be altered through nutritional resource partitioning.

The role of nutrients in bacterial colonization of plants has been investigated most frequently with pathogenic and symbiotic root-associated microorganisms such as *Agrobacterium* and *Rhizobium* species. Transformation of plants by the pathogens *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* results in production of specific amino acid derivatives, called opines, which are catabolized by the inducing *Agrobacterium* strains in the rhizosphere (10). The opine concept proposes that these opines act as nutritional substrates which provide a selective advantage for the virulent opine-catabolizing *Agrobacterium* strains in rhizosphere colonization (10). The opine concept was extended to include symbiotic rhizobia when opine-like compounds were also detected in legume root nodules induced by *Rhizobium meliloti* (31, 32, 34). These rhizopines, synthesized by the bacteroids in the nodules, have been hypothesized to provide a selective advantage for free-living rhizobia in the rhizosphere of the nodulated legumes (39). In a similar manner, the compound mimosine, found in the roots of *Leucaena* spp., may provide a selective advantage to those *Leucaena*-nodulating *Rhizobium* strains which are able to utilize the compound as a sole carbon and nitrogen source (43).

The occurrence of selective substrates in plant-bacterium interactions may not be restricted to pathogenic and symbiotic associations. For example, the ability to catabolize calystegins, which occur in the rhizosphere of plants of the genus *Calystegium*, is found only in a few strains of *R. meliloti* (44, 45). These *R. meliloti* strains occur in the rhizosphere of *Calystegium* plants, but they do not initiate a symbiotic association with this genus (4, 44, 45). The ability to catabolize host-specific substrates may be a phenomenon of more-widespread occurrence than is currently recognized, contributing to host specificity in pathogenic, symbiotic, and saprophytic plant-associated bacteria in both the rhizosphere and phyllosphere environments.

Despite the detection of opines, rhizopines, and other host-specific substrates which are catabolized only by particular bacterial populations, the hypothesized ecological basis of the opine concept has received only preliminary experimental support to date. In a gnotobiotic system consisting of transformed opine-producing *Lotus corniculatus* plants, the growth of root-associated opine-catabolizing *Agrobacterium* strains was favored over that of non-opine-catabolizing strains (16, 17). In another study, a naturally occurring mannopine-utilizing (Mut^+) *Pseudomonas putida* strain was more competitive than a Tn*5* nonutilizing mutant derivative (Mut^-) in the rhizosphere of transformed mannopine-producing (Mop^+) tobacco (22, 23). Further support for the selective advantage proposed by the opine concept has come from the work of Savka and Farrand (12, 13, 40–42). In coinoculations of two *Pseudomonas fluorescens* strains, the ratio of the agropine-utilizing strain *P. fluorescens* AGR to the near-isogenic nonutilizing strain *P. fluorescens* Km was larger in the rhizosphere of agropine-producing transgenic tobacco plants than in the rhizosphere of wild-type plants (42). Similar studies are under way to test the selective advantage conferred by the ability to catabolize rhizopines (33, 37, 38), but as yet no such studies have been reported with other substrates such as calystegins or mimosine.

The role of nutrients in bacterial colonization of the phyllo-

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sphere has received much less attention than their role in the rhizosphere. Although it is known that a diverse array of nutrients is leaked, leached, or exuded onto the leaf surface and is available to the epiphytic microbial community (14, 15, 30, 47, 48), the occurrence of specific nutritional relationships between pathogens or saprophytes and the host plant have not yet been demonstrated. It has been demonstrated that under nitrogen-sufficient plant growth conditions, the phyllosphere is a carbon-limited environment (50, 51). While epiphytic bacterial populations compete for many of the carbon sources in the phyllosphere, other carbon sources appear to be utilized only by certain genera, permitting these genera to achieve a high population size in the phyllosphere, even in the presence of competing strains (50, 51). The coexistence of epiphytic bacterial populations in the carbon-limited phyllosphere mediated through the utilization of different carbon sources has been termed nutritional resource partitioning (50, 51). These studies suggested that the coexistence of one species with another was achieved through the unique utilization of either a single abundant carbon source or of several less-abundant carbon sources in the phyllosphere. Support for this hypothesis was provided through the use of the near-isogenic salicylate-catabolizing strain *P. putida* R20(pNAH7) and the non-salicylate-catabolizing parental strain *P. putida* R20 (52). It was demonstrated that the differential ability to catabolize a single carbon source, exogenously added salicylate, permitted increased coexistence of the catabolizing strain with the noncatabolizing strain (52). It has not yet been demonstrated, however, that individual endogenous carbon sources are leaked or exuded into the phyllosphere in quantities sufficient either to affect the level of coexistence between epiphytic bacterial populations or to alter the dynamics of colonization and competition.

This study, therefore, used a model system similar to that employed by Wilson and Lindow (52), except that the differential carbon source was produced endogenously by a transgenic plant rather than added exogenously. The model consisted of a near-isogenic bacterial strain pair, the mannityl opine-catabolizing *Pseudomonas syringae* strain Cit7 (pYDH208) and the non-opine-catabolizing *P. syringae* strain Cit7xylE, inoculated onto near-isogenic wild-type and transgenic mannityl opine-producing tobacco plants. The hypothesis that coexistence among epiphytic populations in the phyllosphere can be mediated through nutritional resource partitioning and differential utilization of carbon sources was tested. The model was also used to further investigate the role of carbon nutrition in epiphytic colonization and competition.

MATERIALS AND METHODS

Bacterial strains. The characteristics of *P. syringae* Cit7, which was isolated from a healthy leaf of a navel orange, have been described previously (27). *P. syringae* Cit7 was not pathogenic on any of 45 plant species tested and, hence, has not been assigned to any pathovar grouping (27). *P. syringae* Cit7xylE was derived from Cit7 by insertion of the *xylE* gene into the chromosomal *iceC* gene (6), a location which caused no significant loss of fitness or reduction in competitive ability on potato (50) or bean (51) plants.

Plasmid pYDH208 contains a 21-kb *Hin*dIII fragment from plasmid pTil5955 of *A. tumefaciens* 15955, cloned in the broad-host-range vector pCP13/B (11, 19, 20). This plasmid confers tetracycline resistance and the ability to catabolize the mannityl opines mannopine and agropine but not mannopinic acid and agropinic acid (11). Plasmid pYDH208 was mobilized into *P. syringae* Cit7 from *Escherichia coli* DH1 by triparental mating with pRK2013 (11). A fast-growing transconjugant, designated Cit7(pYDH208), was selected on minimal medium A containing 5 mM mannopine as a sole carbon source.

Growth of wild-type and transgenic tobacco plants. The construction and physiological characterization of transgenic mannityl opine-producing tobacco (*Nicotiana tabacum* cv. Xanthi, line 2-26) has been described previously (41). Seeds of wild-type tobacco (*N. tabacum* cv. Xanthi) and transgenic tobacco (*N. tabacum* cv. Xanthi, line 2-26) were germinated in pots of a peat-sand potting mix. Seedlings were transplanted at about 4 weeks into individual 10-cm pots. Tobacco plants were irrigated through drip lines, with no overhead irrigation, to minimize growth of epiphytic bacterial populations prior to experimental inoculation. Plants were fertilized once a day with half-strength Hoagland's solution. Plants were inoculated when approximately 30 cm tall.

Preparation of inocula and plant inoculation. *P. syringae* Cit7xylE and Cit7(pYDH208) were cultured on King's medium B (KB) for 18 h at 28°C. Bacterial cells were removed from the plate and suspended in phosphate buffer (0.01 M, pH 7.0). The cell suspensions were adjusted turbidimetrically to the appropriate concentration by use of a standard curve relating optical density (λ = 600 nm) with culturable cells per milliliter. For coinoculations, cell suspensions were combined in equal proportions immediately prior to plant inoculation.

The replacement series (18) has been used previously to investigate competitive interactions in the phyllosphere between phytopathogenic fungi (1) and epiphytic bacteria (50–52). For replacement series experiments, cell suspensions were combined in six different proportions [Cit7xylE to Cit7(pYDH208), 0:1, 0.2:0.8, 0.4:0.6, 0.6:0.4, 0.8:0.2, and 1:0], at a constant total concentration of 10^6 CFU/ml. Wild-type tobacco plants (*N. tabacum* cv. Xanthi) and transgenic plants (*N. tabacum* cv. Xanthi, line 2-26) (41) were spray inoculated with suspensions of *P. syringae* Cit7xylE and Cit7(pYDH208), alone and in combination. Each treatment was replicated on five plants. The tobacco plants were covered with plastic bags to maintain a high relative humidity and moisture on the leaves. The plants were randomized within a growth chamber and incubated at 26°C under constant illumination for a period of 96 h. All experiments were repeated at least twice to ensure reproducibility.

Enumeration of bacterial populations. Twenty leaves, four per replicate plant, were collected at each sample time. Individual leaves were placed in 200 ml of sterile phosphate buffer (0.01 M potassium phosphate buffer [pH 7.0]) in 500-ml Erlenmeyer flasks. The flasks were sonicated in an ultrasonic cleaning bath for 7 min to dislodge the epiphytic populations and then agitated briefly (ca. 30 s) to suspend the bacteria before removal of aliquots of the suspension. Serial dilutions of leaf washings were plated on KB amended with 100 μg of cycloheximide per ml, 50 μg of benomyl (Benlate; DuPont) per ml, and 100 μg of rifampin per ml (KBR). Plates were incubated for 72 h at 28°C. Bacterial population sizes were expressed in CFU per leaf or per gram (fresh weight) of leaf tissue. To distinguish Cit7 and Cit7xylE, bacterial colonies were lifted from the plates by applying uniform pressure to a 10-cm-diameter filter paper (Whatman no. 1) placed onto the agar surface. The filter papers were oversprayed with catechol (0.1 M), incubated at room temperature for 5 min, and then dried in an oven at 60°C (6). The total number of colonies, the number of yellow colonies (Cit7xylE), and the number of colonies that remained white after spraying with catechol [Cit7(pYDH208)] were determined for each filter paper. Stability of the plasmid pYDH208 was assessed by replica plating colonies onto KBR amended with 30 μg of tetracycline per ml.

Data analysis. Bacterial population sizes were $\log_{10}$-transformed to achieve normality of distribution prior to calculation of mean population size for each treatment. Population sizes were compared statistically through analysis of variance and Fisher's protected least significant difference test with Proc GLM of SAS (release 6.08; SAS Institute Inc., Cary, N.C.).

For replacement series data, the arithmetic back-transformed values of the mean $\log_{10}$-transformed population size of each strain and the arithmetic back-transformed values of the mean $\log_{10}$-transformed total population size were plotted against the inoculum proportion. An increased level of coexistence of Cit7(pYDH208) with respect to Cit7xylE on transgenic plants would be indicated by (i) a significant positive deviation from linearity in the relationship between population size and inoculum proportion and (ii) a total population size which is not constant and is greater in the coinoculations than when a single strain is inoculated alone. The linearity of the relationship between population size and inoculum proportion was tested by the model described previously by Wilson and Lindow (50). The model is described by the equation $\log_{10}(\text{population size}_{ij}) - \log_{10}(\text{inoculum proportion}_i) = \text{mean}_i + \text{normal error}_{ij}$, where i is the inoculum proportion and j is the leaf replicate. In this model, the population size is log-normally distributed and the relationship is linear only if all of the means are equal. Equality of the means was determined by an F test as determined by Proc GLM in SAS.

Quantification of mannityl opines on leaves. The presence of mannityl opines in washings of leaves from transgenic tobacco plants was confirmed by high-voltage paper electrophoresis by methods described previously (41). Leaves were sampled from the upper-level (leaf 15), mid-level (leaf 10), and lower-level (leaf 5) leaf positions (where the first true leaf was designated leaf 1) of three different transgenic tobacco plants. Each leaf was washed over an area of approximately 9 cm^2 with 200 μl of sterile distilled water. All electrophoretograms included a 20 mM standard of each mannityl opine; however, one of the electrophoretograms also included an agropine dilution series to permit estimation of the quantities of agropine present. By comparing the staining intensities of leaf washings in the electrophoretograms with those of the dilution series, estimates of the quantities of agropine could be obtained and expressed in micrograms per square centimeter of leaf surface.

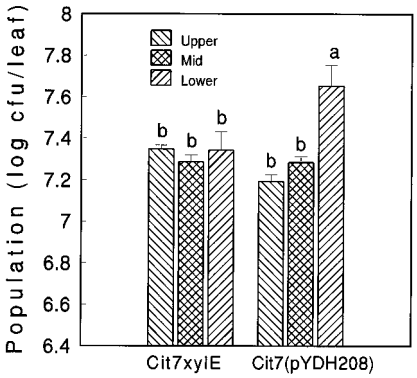

FIG. 1. Population size of near-isogenic bacterial strains from transgenic tobacco plants producing mannityl opines. Tobacco plants (*N. tabacum* cv. Xanthi, line 2-26) were inoculated with *P. syringae* Cit7xylE or Cit7(pYDH208). Plants were incubated for 96 h at high relative humidity prior to enumeration of bacterial populations on leaves at different positions, designated upper, mid, and lower levels. Population sizes represent the means of 20 leaves, and error bars represent standard errors of the means. Bars labelled with the same letter are not significantly different ($P = 0.05$).

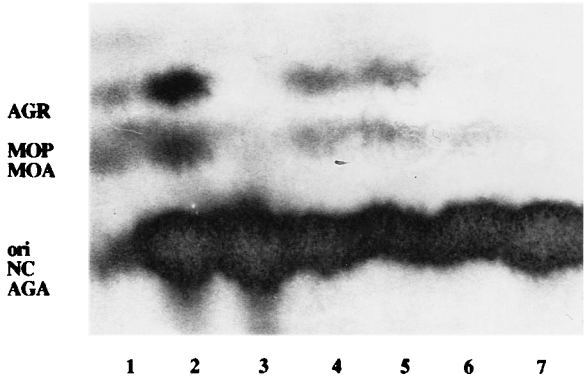

FIG. 2. High-voltage paper electrophoretogram of washings of leaves of transgenic mannityl opine-producing tobacco plants (*N. tabacum* cv. Xanthi), line 2-26. Lanes 1, standards of the four mannityl opines at 20 mM each; 2, tissue extracts from homogenized leaves of line 2-26; 3, tissue extracts from homogenized leaves of wild-type plants; 4 to 6, samples of the leaf washes from lower, middle, and upper leaves of the agropine line 2-26, respectively; 7, wash sample from the lower leaves of the wild-type plant. Abbreviations AGR, agropine; MOP, mannopine, MOA, mannopinic acid; AGA, agropinic acid; ori, origin; NC, neutral compounds.

RESULTS

The population sizes of the near-isogenic *P. syringae* strains Cit7xylE and Cit7(pYDH208) were compared on leaves at different positions on transgenic tobacco plants to determine whether there was any enhancement of the population size of the mannityl opine-catabolizing strain on the mannityl opine-producing plants and whether the amount of enhancement was the same on leaves of different physiological ages. The population sizes of *P. syringae* Cit7xylE at the upper, middle, and lower leaf positions and the population sizes of *P. syringae* Cit7(pYDH208) on the upper and middle leaf positions were not significantly different ($P = 0.05$) (Fig. 1). However, the population size of *P. syringae* Cit7(pYDH208) at the lower leaf position was significantly ($P = 0.05$) greater than the population size of Cit7(pYDH208) at the middle and upper leaf positions and the population size of Cit7xylE at any leaf position (Fig. 1). These data suggest that *P. syringae* Cit7 (pYDH208) was able to achieve a higher final population size than Cit7xylE by catabolism of mannityl opines on the lower leaves of transgenic plants.

To correlate the enhancement of the population size of *P. syringae* Cit7(pYDH208) with the presence of mannityl opines in the phyllosphere of transgenic tobacco plants, the presence of mannityl opines in leaf washings was determined by high-voltage paper electrophoresis. No mannityl opines were detected in washings of leaves from wild-type plants (Fig. 2). In all three transgenic plants tested, mannityl opines were present in leaf washings, and quantities in washings from lower leaves were consistently greater than those in washings from upper leaves. For one electrophoretogram, the quantities of agropine in washings of leaves from different positions were estimated by comparison of staining intensities of the test lanes with an agropine dilution series. The quantities of agropine estimated per unit leaf area were approximately 1.9 $\mu g/cm^2$ for the lower leaves, approximately 1.3 $\mu g/cm^2$ for the mid-level leaves, and <0.1 $\mu g/cm^2$ for the upper leaves. The enhancement of the population size of *P. syringae* Cit7(pYDH208) on transgenic plants was correlated with the presence of mannityl opines in washings of leaves from these plants, and the greater population enhancement on lower leaves may have been related to the greater availability of mannityl opines on the lower leaves or to other factors relating to the nature of resource limitation on mid-level and upper leaves.

P. syringae Cit7xylE and Cit7(pYDH208) were both inoculated individually onto lower leaves of wild-type and transgenic plants, and their population sizes were determined over a period of 6 days. On wild-type plants, the final population sizes attained by *P. syringae* Cit7xylE and Cit7(pYDH208) were not significantly different (Fig. 3A and C); however, on transgenic plants, the final population size attained by Cit7(pYDH208) was significantly higher than the final population size attained by Cit7xylE (Fig. 3B and D). These data further suggest that *P. syringae* Cit7(pYDH208) was able to achieve a higher final population size than Cit7xylE by catabolism of mannityl opines on the leaves of transgenic plants. Although the initial population size of *P. syringae* Cit7(pYDH208) was less on the transgenic plants than on the wild-type plants, the final population sizes were not significantly different (Fig. 4A and C). Although the initial population size of *P. syringae* Cit7(pYDH208) was less on the transgenic plants than on the wild-type plants, the final population size was significantly greater on transgenic plants than on wild-type plants (Fig. 4B and D). Insufficient data were available to accurately determine growth rates of the strains on wild-type and transgenic plants; however, there were no obvious differences between the growth rates of Cit7 (pYDH208) and Cit7xylE when inoculated alone on either wild-type or transgenic plants.

P. syringae Cit7xylE and Cit7(pYDH208) were coinoculated onto the lower leaves of wild-type and transgenic tobacco plants to determine whether the ability to catabolize mannityl opines on transgenic plants provided Cit7(pYDH208) with a competitive advantage over Cit7xylE (Fig. 5). On wild-type plants, the growth rates were apparently similar, final population sizes of strains Cit7xylE and Cit7(pYDH208) were not significantly different (Fig. 5A), and the ratio of the population sizes remained almost constant (Fig. 5C). On transgenic plants, while the growth rates of Cit7xylE and Cit7(pYDH208) were apparently similar, the final population size attained by Cit7(pYDH208) was significantly higher than that attained by Cit7xylE (Fig. 5B), and the ratio of the population size of Cit7(pYDH208) to Cit7xylE increased from 0.66 to 4.08 (Fig. 5C). In these coinoculation studies, strain identification was

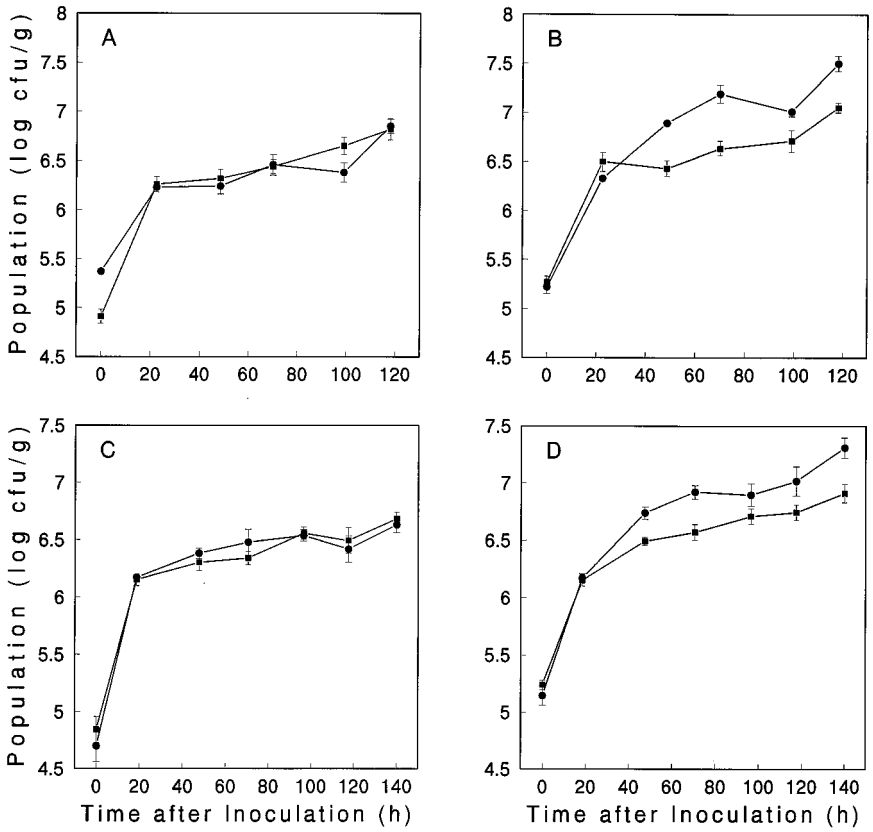

FIG. 3. Population sizes of *P. syringae* Cit7xylE (squares) and Cit7(pYDH208) (circles) on wild-type (A and C) and transgenic mannityl opine-producing (B and D) tobacco plants, as a function of time after inoculation, in two separate experiments. The vertical bars represent standard errors of the means.

based on the XylE phenotype; hence, strain identification and enumeration could not be affected by plasmid loss from Cit7 (pYDH208) or transfer to Cit7xylE. Plasmid retention by the transconjugant *P. syringae* strain Cit7(pYDH208) was determined to be >90% over the duration of these experiments. Furthermore, there was no evidence of plasmid transfer from Cit7(pYDH208) to Cit7xylE, since no $Tet^r/XylE^+$ colonies were detected.

The relative competitiveness and the level of coexistence of *P. syringae* Cit7 with respect to that of Cit7xylE on transgenic plants were investigated by replacement series experiments (Fig. 6). The near-isogenic strains developed similar population sizes when inoculated alone, and although Cit7xylE appeared slightly less competitive than the parental strain Cit7, the two strains replaced each other, indicating competition for limiting resources (Fig. 6). Subsequently, the relative competitiveness and the level of coexistence of *P. syringae* Cit7 (pYDH208) with respect to that of Cit7xylE were investigated on wild-type and transgenic plants (Fig. 7). The results of the replacement series experiments between Cit7xylE and Cit7 (pYDH208) on transgenic tobacco plants were consistent in four separate experiments. On wild-type plants, the population size of Cit7(pYDH208) when inoculated alone was consistently slightly less than the population size of Cit7xylE; however, the strains replaced each other, indicating competition for limiting resources (Fig. 7A). In contrast, on transgenic plants, the population size of Cit7(pYDH208) was significantly higher than the population size of Cit7xylE, and the strains no longer replaced each other (Fig. 7B). The relationship between population size and inoculum proportion deviated significantly from linearity, suggesting that Cit7(pYDH208) was not limited by the same resources as Cit7xylE (Fig. 7B). Similar replacement series graphs were obtained in four replications of this experiment.

DISCUSSION

Colonization of the phyllosphere by the mannityl opine-catabolizing strain *P. syringae* Cit7(pYDH208) was significantly enhanced compared with that of the near-isogenic strain Cit7xylE by its ability to catabolize the mannityl opines produced by transgenic tobacco plants. The final population size of Cit7(pYDH208) was on average, over several experiments, 3.8-fold higher than that of the noncatabolizing strain Cit7xylE on the lower leaves of mannityl opine-producing transgenic plants. In other words, the lower leaves of transgenic plants exhibited a significantly higher carrying capacity for Cit7 (pYDH208) than for Cit7xylE.

Mannityl opines were detected in the washings of leaves from transgenic plants but not in washings of leaves from wild-type plants. The concentrations of the mannityl opines mannopine and agropine were consistently higher in washings of leaves from the lower part of the plant than in washings of leaves from the upper part of the plant. The levels of mannopine and agropine may have been higher in washings from the lower leaves because of either greater synthesis in these leaves or greater exudation or leakage in the older leaves. Leaf samples were not homogenized for estimation of tissue opine concentrations; hence, the basis of the gradient of opine concentration in washings cannot be determined. However, levels of

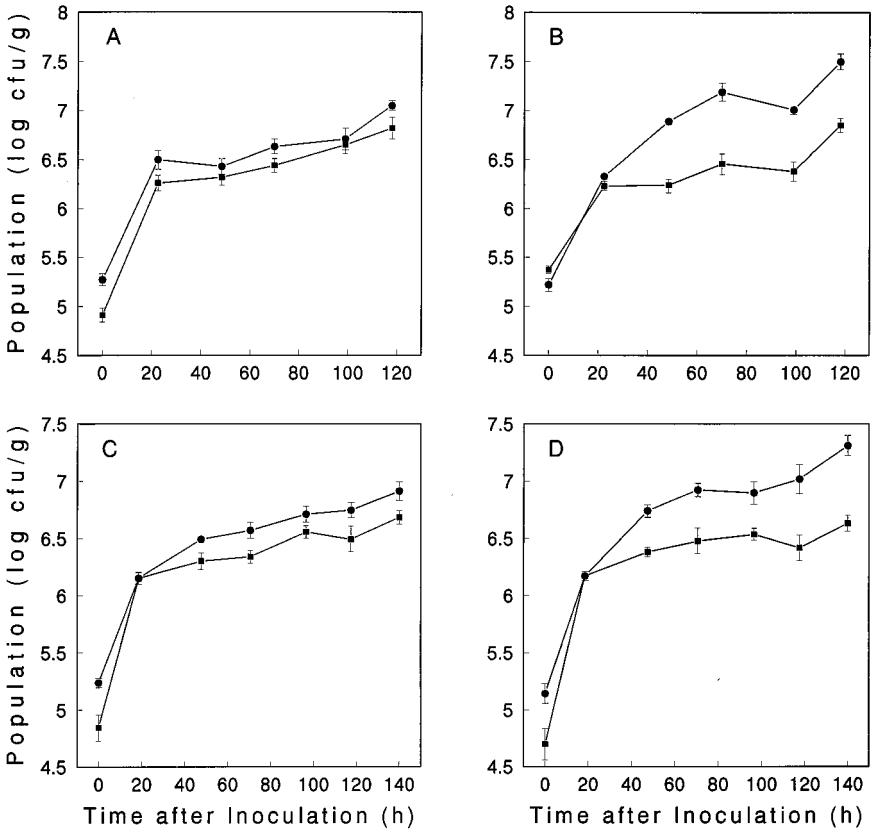

FIG. 4. Population sizes of *P. syringae* Cit7xylE on wild-type (squares) and transgenic mannityl opine-producing (circles) tobacco plants (A and C) and of *P. syringae* Cit7(pYDH208) on wild-type (squares) and transgenic mannityl opine-producing (circles) tobacco plants (B and D), as a function of time after inoculation, in two separate experiments. The vertical bars represent standard errors of the means.

synthesis may have been greater in lower leaves, since the mannopine synthase (*mas*) 1′ and 2′ dual promoters are regulated by the relative levels of auxin and cytokinin and greater promoter activity has been detected in the lower stem and roots of transgenic tobacco plants (24).

The enhancement in the carrying capacity of lower leaves of transgenic plants for Cit7(pYDH208) was apparently related to the presence of high levels of agropine and mannopine in exudates or leachates from these leaves. Agropine was estimated to be present at 1 to 2 $\mu g/cm^2$ in the phyllosphere of lower leaves of transgenic plants. The agropine concentration estimated for exudates of lower leaves was slightly higher than the concentrations estimated for carbohydrates on the leaves of *Zea mays* (approximately 0.1 $\mu g/cm^2$) (9, 14) and *Antirrhi-*

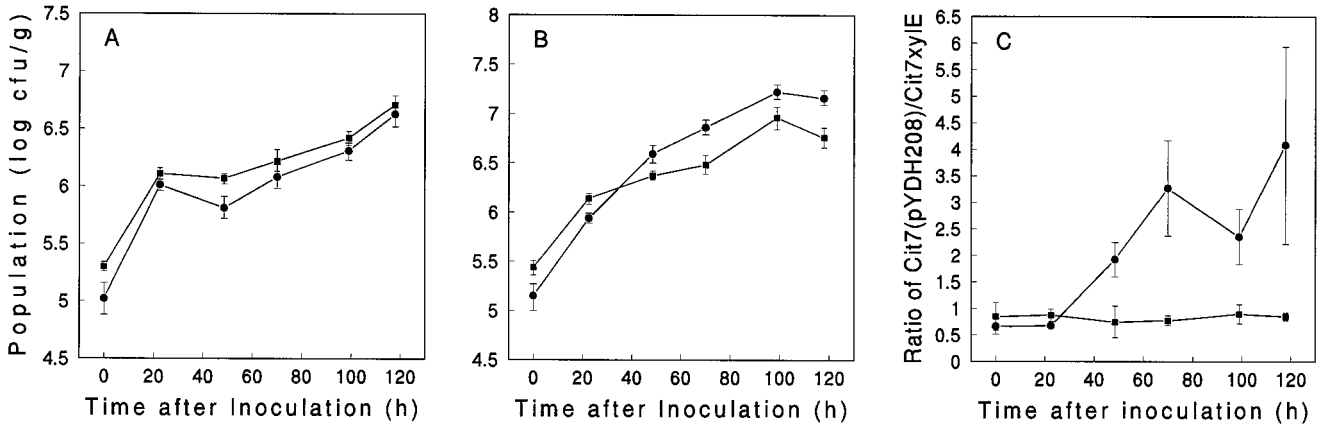

FIG. 5. Population sizes of *P. syringae* Cit7xylE (squares) and Cit7(pYDH208) (circles) following coinoculation onto wild-type (A) and transgenic mannityl opine-producing (B) tobacco plants. The mean ratio of strain Cit7(pYDH208) to Cit7xylE on wild-type plants (squares) and transgenic mannityl opine-producing tobacco plants (circles), determined from populations from each of 20 individual leaves for each treatment, is also shown (C). The vertical bars represent standard errors of the means.

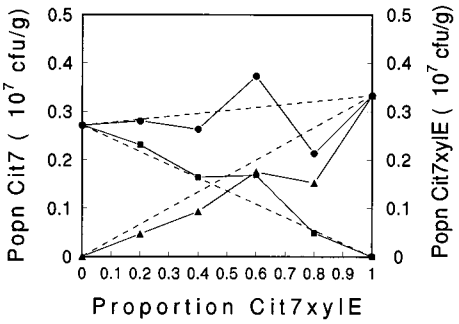

FIG. 6. Competition between *P. syringae* Cit7xylE and the parental strain Cit7 on transgenic mannityl opine-producing tobacco plants in replacement series experiments. Leaves were harvested 96 h after inoculation. The population size of Cit7 (squares), Cit7xylE (triangles), and the total population (circles) are plotted against the inoculum proportion. The dashed lines represent expected population sizes based upon inoculum proportion, assuming equal competition between the strains.

num nanum (approximately 0.7 μg/cm^2) plants (7). The mannityl opines apparently represented additional carbon sources for the growth of Cit7(pYDH208) in the phyllosphere, which usually is carbon limited under these growth conditions (50, 51). This suggests that under carbon-limited conditions in the phyllosphere, the differential ability to catabolize a substrate can increase the population size of the catabolizing strain relative to those of other competing strains. The absence of a significant enhancement of the carrying capacity of mid-level leaves for Cit7(pYDH208), despite the apparent presence of moderate quantities of mannityl opines, may be attributable either to differences in the plants grown for ecological studies at one institution and physiological studies at a different institution or to differences in the nature of resource limitation of the epiphytic populations on lower, mid-level, and upper leaves.

In contrast to the increase in carrying capacity observed in the phyllosphere, the population size of the agropine-catabolizing strain *P. fluorescens* AGR was not significantly different in the rhizosphere of wild-type and transgenic tobacco plants (12, 13, 40, 42). This may indicate that unlike the phyllosphere, the rhizosphere was not carbon limited (12, 13). Alternatively, the difference may relate to the source of the carbon diverted to opine synthesis. In the phyllosphere, the population size of the non-opine-catabolizing strain Cit7xylE was not significantly different on transgenic and wild-type plants, suggesting that carbon for synthesis of mannityl opines was not diverted from the synthesis of carbon sources ordinarily utilized by Cit7. By contrast, in the rhizosphere, agropine may have been synthesized at the expense of carbon sources in the roots which would otherwise have been catabolized by *P. fluorescens* AGR. Hence, no additional carbon was made available to *P. fluorescens* AGR in the rhizosphere of transgenic plants.

Following coinoculation of the near-isogenic strains onto transgenic plants, the ratio of the population size of Cit7 (pYDH208) to Cit7xylE increased by over fivefold. This change in the ratio of population sizes could indicate merely that the population of Cit7xylE ceased to increase before that of Cit7(pYDH208). This appears not to be the entire reason, since the ratio started to change at 24 to 48 h, well before the growth rate of Cit7xylE decreased as the carrying capacity was approached. Hence, the initial change in the ratio of Cit7 (pYDH208) to Cit7xylE may reflect a subtle difference in the growth rates of the two populations. The increase in the ratio of the population sizes of *P. fluorescens* AGR to *P. fluorescens* Km in the rhizosphere of transgenic mannityl opine-producing tobacco, which occurred in the absence of any difference in final population sizes (12, 13, 40, 42), may also be attributable to different growth rates.

Replacement series experiments were used by Wilson and Lindow (52) to demonstrate that exogenous provision of the carbon source salicylate, which was catabolized uniquely by one member of a near-isogenic strain pair, permitted an increased level of coexistence of that strain with the competing strain. An increased level of coexistence was defined as an increase in the population size of the salicylate-catabolizing strain, in the presence of the non-salicylate-catabolizing strain, without any depression in the population size of the latter strain. The concentration of salicylate applied (approximately 10 μg/cm^2), however, was 10- to 100-fold higher than carbohydrate concentrations estimated from leaf washings (7, 9, 14). Hence, it was necessary to determine whether the quantities of individual carbon sources leaked or exuded into the phyllosphere are sufficient to permit coexistence of strains differing in the utilization of only one or two compounds. The replacement series graphs in this study demonstrated an increase in coex-

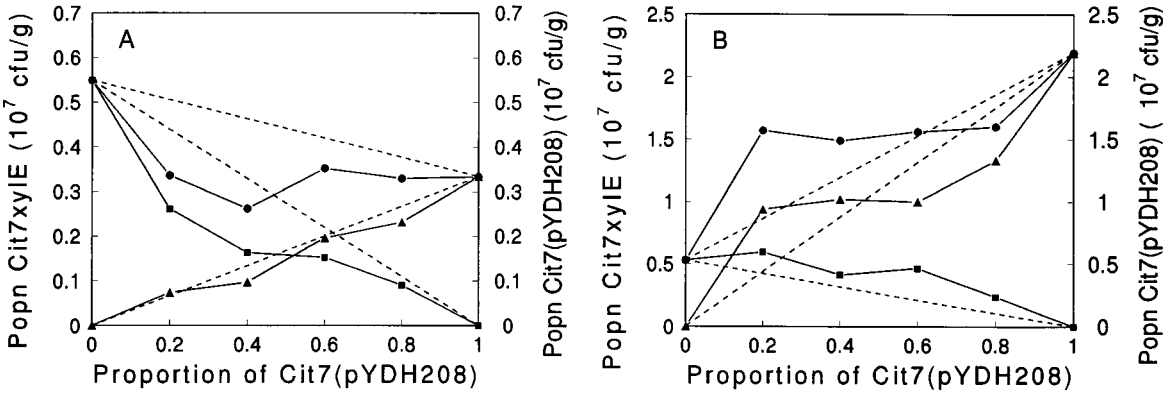

FIG. 7. Competition between *P. syringae* Cit7xylE and Cit7(pYDH208) in replacement experiments. Leaves were harvested 96 h after inoculation. Population sizes of *P. syringae* Cit7xylE (squares) and Cit7(pYDH208) (triangles) and total population size (circles) are plotted against inoculum proportion. The dashed lines represent expected population sizes based upon inoculum proportion, assuming equal competition between the strains. (A) Strains were inoculated onto wild-type tobacco plants. Both populations exhibited significant deviations from linearity [for Cit7xylE, $F = 6.58$ and $P = 0.0001$; for Cit7(pYDH208), $F = 3.07$ and $P = 0.0199$]. (B) Strains were inoculated onto transgenic mannityl opine-producing tobacco plants. Both populations exhibited significant deviations from linearity [for Cit7xylE, $F = 10.75$ and $P = 0.0001$; for Cit7(pYDH208), $F = 11.57$ and $P = 0.0001$].

istence between the mannityl opine-catabolizing strain Cit7 (pYDH208) and the non-opine-catabolizing strain Cit7xylE on transgenic mannityl opine-producing plants compared with that on wild-type plants. This increased level of coexistence was indicated by the significant positive deviation from linearity in the relationship between the population size of Cit7 (pYDH208) and the inoculum proportion, the absence of a depression of the population size of Cit7xylE, and the increase in the total *P. syringae* population on transgenic plants. This demonstrates that sufficient quantities of individual carbon sources are leaked or exuded into the phyllosphere to permit coexistence between bacterial strains exhibiting ecological differentiation in nutritional resource utilization as proposed by Wilson and Lindow (51, 52). Therefore, the selective advantage proposed in the opine concept appears to be attributable to the production of a novel catabolic niche which can be occupied only by the opine-catabolizing strains. The presence of opines permits the opine-catabolizing populations to coexist with non-opine-catabolizing populations with which they would otherwise compete.

In addition to the increased level of coexistence of Cit7 (pYDH208) with respect to Cit7xylE, the level of coexistence of Cit7xylE with respect to Cit7(pYDH208) was also increased in four of five replacement series experiments. This suggests that Cit7(pYDH208) utilized mannopine and agropine preferentially over other phyllosphere carbon sources and that the intensity of competition for the other phyllosphere carbon sources was reduced. Hence, Cit7xylE was able to achieve a higher population size than that predicted upon the basis of its proportion in the inoculum and the population size of Cit7xylE when inoculated alone. Therefore, the opine-catabolizing strain Cit7(pYDH208) cannot be considered to be competitively superior to the non-opine-catabolizing strain Cit7xylE, because the intensity of competition for non-opine phyllosphere carbon sources was actually reduced. The selective advantage proposed in the opine concept, therefore, does not involve competitive superiority of the opine-catabolizing strains over the non-opine-catabolizing strains in the presence of opines.

Resource competition is probably the predominant interaction involved in the preemptive competitive exclusion of epiphytic pseudomonads (25, 26, 28, 29). Ecologically similar epiphytic *P. syringae* strains competed for carbon sources in the carbon-limited phyllosphere (50), and preemptive exclusion of Ice^+ *P. syringae* strains by an Ice^- *P. syringae* strain was achieved presumably by prior utilization of these carbon sources (50). Many other epiphytic species, however, were ineffective in preemptive exclusion and coexisted with *P. syringae*, apparently by the utilization of alternative carbon sources (51). The results of the replacement series with the near-isogenic *P. syringae* strain pair employed in this model system further substantiate the conclusion of Wilson and Lindow (52) that coexistence between epiphytic bacterial populations can be mediated through nutritional resource partitioning and the differential ability to catabolize certain carbon sources. In addition to substantiating the concept of nutritional resource partitioning, the model also demonstrates that epiphytic bacterial population size in the carbon-limited phyllosphere is determined at least in part by the quality and quantity of available carbon sources.

The concepts developed in this study are significant for the development and application of bacterial biocontrol agents of foliar pathogens and pests. One of the problems limiting biological control in the phyllosphere is the introduction of an antagonistic organism into an established microbial community in which the majority of the nutritional resources already have been sequestered. In such a community, there are no nutritional niches available to the biocontrol agent. Although niche clearing with bactericides may allow the establishment of a bactericide-resistant biocontrol agent, the creation of a novel metabolic niche by the exogenous or endogenous provision of a selective substrate may be a more desirable alternative. While it is possible to apply nutrients exogenously to maintain the population size of an applied biocontrol agent (8, 21), it may be preferable to maintain the population size of an applied biocontrol agent through the endogenous provision of a selective nutritional substrate by a transgenic host plant. Endogenous provision of the selective substrate would obviate the need for repeated exogenous applications. Such a system would be particularly useful for the maintenance of an antibiotic-producing or an insecticidal biocontrol agent in the phyllosphere. Systems employing endogenously supplied selective substrates have been proposed for the introduction of beneficial microbes into the rhizosphere (17, 33, 37, 38); however, this is the first reported attempt to investigate the potential of endogenously provided substrates to selectively support epiphytic bacterial populations.

To selectively support foliar biocontrol agents under field conditions, the chosen substrate should be catabolized only by the engineered biocontrol agent. Not all opines would be sufficiently selective for field use because the ability to catabolize most opines is more widespread than realized originally (2, 3, 5, 35, 36, 46). However, the mannityl opine agropine is to date known to be catabolized only by the inducing agrobacteria and may be useful in this context. To implement such a system, it will be necessary for all leaves, not just those on the lower plant parts, to produce sufficient quantities of the selective substrate. To achieve this, the mannopine synthase gene *mas1* could be placed under the regulatory control of a leaf-specific promoter. This system and a similar system based on rhizopine are currently under investigation by M. Wilson for use in the selective support of foliar biocontrol agents.

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