Competition Between Ice Nucleation-Active Wild Type and Ice Nucleation-Deficient Deletion Mutant Strains of *Pseudomonas syringae* and *P. fluorescens* Biovar I and Biological Control of Frost Injury on Strawberry Blossoms

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ABSTRACT

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Ice nucleation-deficient (INA$^-$) mutants of *Pseudomonas* strains derived by site-directed mutagenesis were tested for their efficacy as biological control agents of frost injury on blossoms of greenhouse-grown strawberry plants. Inhibition of one bacterial strain by its near-isogenic counterpart was dose dependent rather than strain dependent. The INA$^-$ deletion mutants of *P. syringae* and *P. fluorescens* biovar I, inoculated at 10^7 cfu per blossom, inhibited growth of their ice nucleation-active (INA$^+$) parental strains inoculated at 10^2 cfu per blossom. The INA$^+$ parental strains inhibited INA$^-$ derivatives when inoculum doses were reversed. Inhibition was incomplete unless doses differed by about 10^4-fold. No inhibition occurred when two strains were inoculated simultaneously at equal doses (either 10^2 or 10^7 cfu per blossom). The INA$^-$ *P. syringae* strain protected blossoms against freezing by other *P. syringae* strains but did not inhibit or protect against INA$^+$ *P. fluorescens*. The INA$^-$ *P. fluorescens* strain was much more effective as an inhibitor of *P. syringae* strains than of other *P. fluorescens* strains.

Procedures for screening the efficacy of potential biological control agents frequently involve assays for inhibition in vitro. However, in some cases, protection from infection by phytopathogenic bacteria has been achieved with microbes that demonstrated no inhibitory properties in vitro (2,10,16,19). Lindow isolated mutants of antagonistic bacteria after chemical mutagenesis that were no longer able to inhibit *Erwinia amylovora* and *Pseudomonas syringae* van Hall in plate assays (10). In most cases, these mutant organisms were still effective biological control agents in greenhouse assays.

Since detectable antibiosis is apparently not necessary for efficacy in vivo, bacterial pests containing deletion mutations (which attenuate their harmful property) may be effective biological control agents. Lindow and co-workers have reported that chemical mutants deficient in ice nucleation activity (INA$^-$) gave protection from frost injury under field conditions (9,15). Lindow also found that genetically engineered INA$^-$ deletion mutants were effective in greenhouse tests (11). It has been proposed that genetically engineered pathogenicity-minus mutants of phytopathogenic bacteria may be effective disease control agents (6,17).

The most rigorous method of evaluating the effects of single genes or single phenotypic characteristics on competition and biological control is construction of isogenic lines of an organism, each with a specific mutation in a uniform genetic background. In the present study, near-isogenic lines of *Pseudomonas* strains (differing only in ice nucleation activity and antibiotic resistance) were used to study the phenomena of reciprocal competition, competitive exclusion, and the effect of the INA phenotype on epiphytic colonization ability. To our knowledge, this is the first study showing that, under appropriate circumstances, a plant pest (in this case, an INA$^+$ bacterial strain) can inhibit epiphytic multiplication of a biological control agent (the INA$^-$ bacterial strain). This phenomenon, therefore, places limits on biological control strategies that rely on competition rather than antibiosis and also has important implications for the fate of recombinant bacteria that may be released into the environment.

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MATERIALS AND METHODS

Bacterial cultures. Ice nucleation-active (INA$^+$) strains S203 and MS1650 were originally isolated from healthy strawberry plants in commercial fruit production fields in California. Based on the combined results from 19 diagnostic tests, S203 was identified as *P. syringae* and MS1650 was identified as *P. fluorescens* biovar I (Trevisan) Migula (5,18). Strain S203 closely resembled the group Ib pathovars of *P. syringae*, based on the LOPAT tests (5), but could not be assigned to a specific pathovar since it demonstrated no pathogenic potential on the 26 plant species tested (7; *unpublished data*).

Ice nucleation-deficient (INA$^-$) deletion mutants of these two strains, derived by site-directed mutagenesis as described elsewhere (21), are designated RGP36 (*P. syringae*) and GJP17B (*P. fluorescens*). The deletion mutant strains had no detectable ice nucleation activity (less than one ice nucleus per 10^9 cells) at −10 C when assayed by a droplet freezing assay (13,20). Wild type and deletion mutant strains were subjected to a battery of 19 standard diagnostic tests to verify their identity as described previously (7). These strains were also screened for their ability to utilize 31 compounds as sole carbon sources in a minimal medium (1), their sensitivity to 19 antibiotics, and their growth rates in vitro and in vivo. The INA$^-$ deletion mutant strains were identical to their INA$^+$ wild type parental strains in all tests except ice nucleation. Variants expressing spontaneous resistance to rifampicin and nalidixate were selected by plating about 10^8 cells from log phase cultures onto plates containing the appropriate antibiotic at 50 μg/ml and isolating individual colonies after incubation. The antibiotic-resistant mutants that were chosen for this study were those that demonstrated a colonization competence on strawberry blossoms equivalent to that of the parental strain. Parental and derivative strains used in greenhouse studies are referred to as near isogenic, rather than isogenic, because they differed in both antibiotic resistance and ice nucleation activity. Additional strains of *P. syringae* and *P. fluorescens* were isolated from strawberry plants. Two strains (from different locations) of each species were included in competition studies.

Bacterial stock cultures were maintained in a freezer at −80 C in 1:10 (v/v) DMSO:Luria broth. Fresh inoculum for colonization studies was always prepared from the frozen stock cultures. Bacteria were streaked onto King's medium B (KB) (4)

supplemented with cycloheximide at 100 μg/ml (KB + C) and an appropriate antibiotic, either rifampicin or nalidixic acid at 50 μg/ml. Plates were incubated at 28 C for 24–36 hr and the bacteria were suspended in 0.01 M $KHPO_4$ buffer, pH 7.0. Concentrations were adjusted turbidimetrically to about 10^8 cfu/ml (OD_{600nm} = 0.1) and diluted in the same buffer. Bacterial concentrations were verified by dilution plating. Bacterial suspensions were applied to plant surfaces by aerosol spray in a Plexiglas inoculation chamber until plants were wet to runoff.

Plants. Dormant, leafless, bareroot strawberry plants, *Fragaria* × *ananassa* (Duchesne) 'Douglas' were removed from cold storage, trimmed and planted in steamed potting mix, and grown in a greenhouse. Plants were fertilized with Hoagland's solution. Strawberry plants from cold storage began to blossom within 5–6 wk after planting and, at that time, possessed three to four new leaves. All experiments were performed with young plants in the first flush of blossoming. Individual blossoms retained their petals for 4–5 days under greenhouse conditions. Thus, most tests were a maximum of 4 days' duration. After spray inoculation, plants were randomized and incubated under intermittent mist in a temperature-controlled greenhouse maintained at 21 ± 3 C. In some experiments, plants were misted only during the evening (1800–0600 hr) or were incubated without mist. The frequency of misting was regulated by a leaf wetness simulator.

Treatments. The growth of each strain individually on strawberry blossoms was compared with the growth of strains challenged by their near-isogenic relative (parental INA^+ strain or INA^- derivative) or by a nonparental INA^+ strain. Except where noted, co-colonization studies were conducted by mixing suspensions of differentially marked strains in buffer immediately before spray inoculation. For most experiments, the near-isogenic lines used were *P. syringae* strains S203 R1 (INA^+) and RGP36 Nal 1 (INA^-) and *P. fluorescens* strains MS1650 Nal 3 (INA^+) and GJP17B R2 (INA^-).

Sampling procedure. Individual strawberry blossoms were placed in 125-ml Erlenmeyer flasks containing 25 ml of washing buffer, pH 7.0 (12). The flasks were shaken for 2 hr at room temperature (21–24 C) on a rotary shaker at 250 rpm. The undiluted wash and serial 10-fold dilutions of the wash in sterile 0.01 M $KHPO_4$ buffer, pH 7.0, were plated (0.1 ml per plate) onto KB + C + appropriate antibiotics. Plates were incubated 3–4 days at room temperature before colonies were counted. Bacterial populations determined for each sample were transformed to $\log_{10}$ cfu per blossom before mean populations were calculated. Means and standard errors are based on five replicate samples of individual blossoms per treatment. The limit of detection in these studies was approximately 10^2 cfu per blossom.

Efficacy assessment. The INA^- strains were tested for their ability to reduce the freezing temperatures of detached strawberry blossoms using a tube nucleation assay (3). The INA^- strains were sprayed onto strawberry blossoms at 10^8 cfu/ml (~10^7 cfu per blossom) and the INA^+ strains were applied at 10^3 cfu/ml (~10^2 cfu per blossom.) After 72 hr of incubation under intermittent mist, 10 blossoms per treatment were removed and submerged in 0.01 M $KHPO_4$, pH 7.0 (1 blossom per test tube) and subjected to freezing temperatures between −1.5 C and −8 C at half-degree increments in a refrigerated, constant-temperature bath. Tubes were held at each temperature for 20 min and the numbers of frozen blossoms were recorded at each temperature.

RESULTS

On strawberry blossoms, competitive inhibition of one bacterial strain by its near-isogenic relative was reciprocal, rather than strain specific in nature. For example, application of the INA^- strain of *P. syringae*, RGP36, at 10^8 cfu/ml inhibited growth of the INA^+ parental strain, S203 R1, applied at 10^3 cfu/ml (Fig. 1A). S203 R1 inhibited growth of RGP36 Nal 1 when the inoculum dosages were reversed (Fig. 1B). Competition between the near-isogenic lines of *P. fluorescens* was also reciprocal. All strains had generation times of about 2 hr during logarithmic growth when grown axenically on strawberry blossoms (*data not shown*).

During growth on strawberry blossoms, mixtures of the near-isogenic strains behaved as a single population, with the two strains exhibiting equivalent growth rates until the maximum carrying capacity of the blossoms was reached. For example, in Figure 1A, both strains increased 10-fold in 24 hr and then ceased multiplying. Thus, the apparent competition between the strains is probably not the result of any direct interaction (either competitive or inhibitory) between the strains but rather of growth limitations imposed upon both strains by the plant and the environment. For convenience, however, we will continue to refer to the observed phenomenon as growth inhibition.

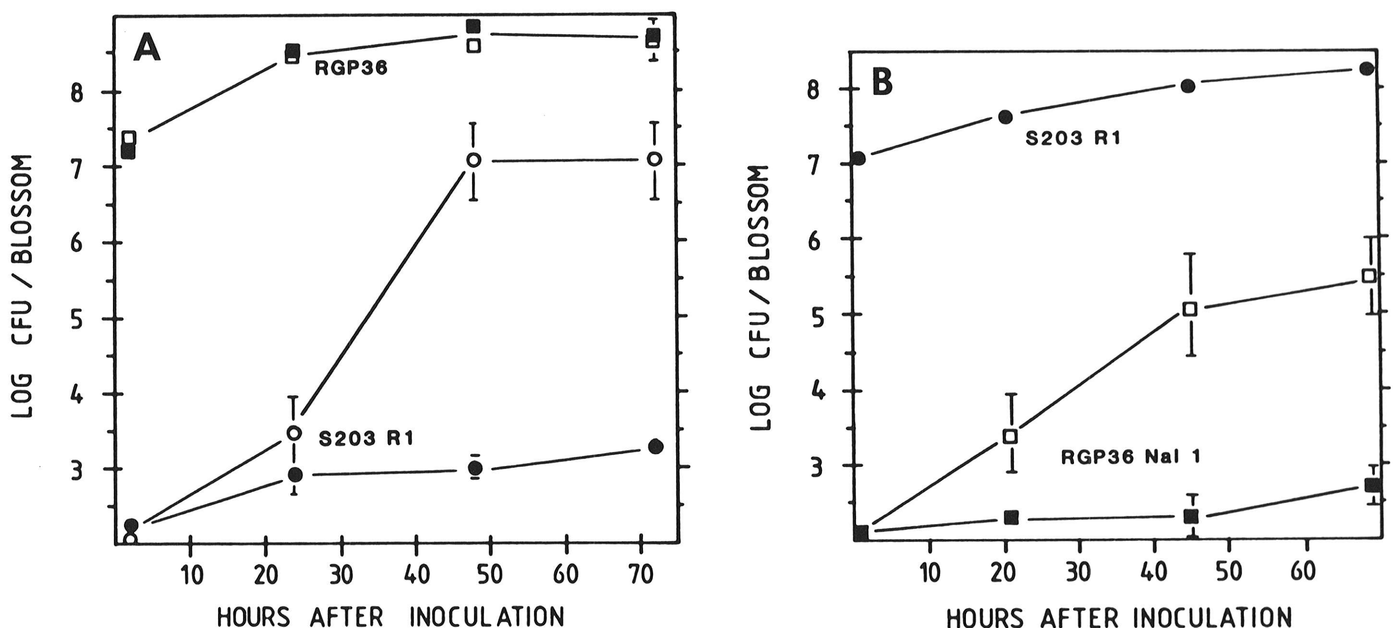

Fig. 1. Comparison of co-colonization and axenic colonization of strawberry blossoms by INA^+ S203 R1 and INA^- deletion mutant RGP36 strains of *Pseudomonas syringae*. Bacterial suspensions were applied by spray inoculation to open blossoms: S203 R1 alone (○), S203 R1 coinoculated with RGP36 or RGP36 Nal 1 (●), RGP36 or RGP36 Nal 1 alone (□), and RGP36 or RGP36 Nal 1 coinoculated with S203 R1 (■). Plants were incubated under intermittent mist. **A,** Inhibition of INA^+ by INA^- strain; **B,** inhibition of INA^- by INA^+ strain.

Within this general framework, the magnitude of the growth inhibition was dose dependent and roughly proportional to the difference in inoculum doses between the two strains. Dosage differences of 10^3-fold resulted in a reduced growth rate of the challenge strain, rather than a complete inhibition of its growth (Fig. 2). Inhibition was not detected when the two bacterial strains were applied simultaneously at equal dosages, either 10^2 (Fig. 3) or 10^7 cfu per blossom (Fig. 4).

Inhibition of less closely related strains was also primarily dose dependent, although some strain specificity was apparent. Generally, RGP36 Nal 1 (INA⁻ *P. syringae*) was inhibitory to other strains of *P. syringae* but did not inhibit the growth of *P. fluorescens*. Strains of *P. fluorescens* could inhibit the *P. syringae* strains as long as the inoculum doses were biased in favor of the *P. fluorescens*, as discussed above (Fig. 5). The inhibition by GJP17B R2 of other strains of *P. fluorescens* varied with the challenge strain (*data not shown*).

Blossoms coinoculated with an INA⁻ and an INA⁺ strain were less likely to freeze at warm temperatures than blossoms sprayed only with an INA⁺ strain as long as inhibition of the INA⁺ strain was successful; therefore, reductions in blossom-freezing temperatures paralleled reductions in INA⁺ populations. For example, the same population of blossoms was sampled to generate the bacterial population data shown in Figure 1A and the blossom-freezing spectra shown in Figure 6. The strain RGP36 Nal 1 reduced the mean freezing temperature of strawberry blossoms challenged with INA⁺ *P. syringae* by about 2 C but was ineffective against strains of *P. fluorescens* (Figs. 6 and 7). Strain GJP17B R2 gave about 2 C protection against strains of *P. syringae* but only 0.5–1.0 C protection against strains of *P. fluorescens* (Fig. 8). Thus, even a 1,000-fold reduction in populations of the *P. fluorescens*

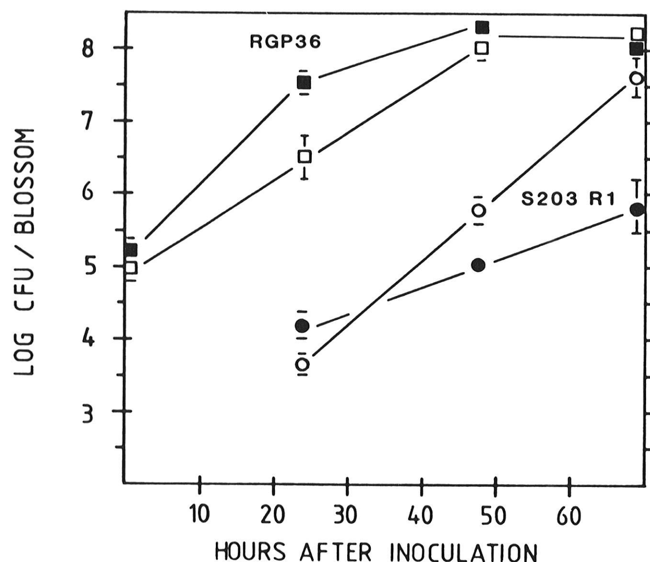

Fig. 2. Comparison of co-colonization and axenic colonization of strawberry blossoms by INA⁺ S203 R1 and INA⁻ deletion mutant RGP36 strains of *Pseudomonas syringae*. Bacterial suspensions were applied by spray inoculation to open blossoms: S203 R1 alone (o), S203 R1 coinoculated with RGP36 (•), RGP36 alone (□), and RGP36 coinoculated with S203 R1 (■). Bacterial suspensions of S203 R1 (10^5 cfu/ml) were applied 23 hr after inoculation of blossoms with RGP36 (10^6 cfu/ml). Plants were incubated under intermittent mist. Incomplete inhibition was dose dependent (compare with Fig. 1A).

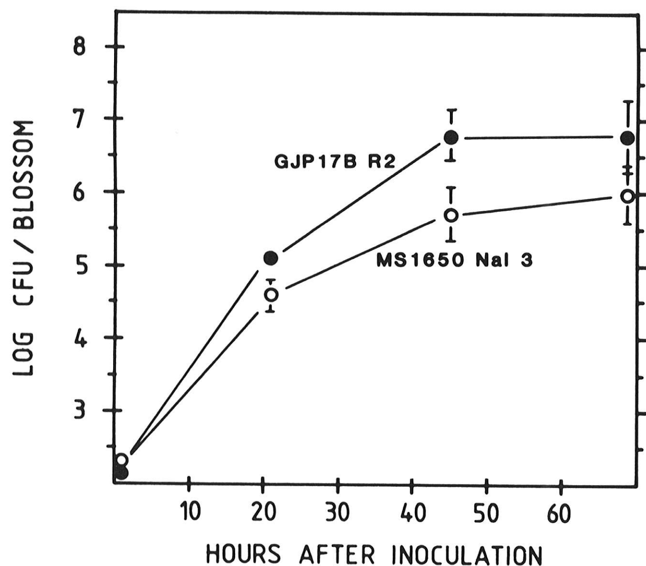

Fig. 3. Co-colonization of strawberry blossoms by INA⁺ MS1650 Nal 3 (o) and INA⁻ deletion mutant GJP17B R2 (•) strains of *Pseudomonas fluorescens*. Suspensions of two bacterial strains (about 8×10^2 cfu/ml) were mixed and applied to open strawberry blossoms by spray inoculation. Plants were incubated under intermittent mist. Populations of two strains are not significantly different at 0, 45, and 69 hr after inoculation by analysis of variance (F test, $P > 0.05$).

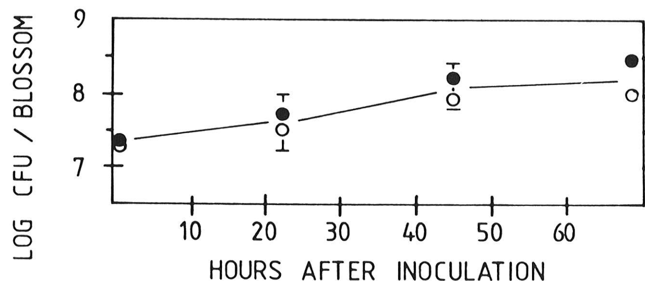

Fig. 4. Coexistence of INA⁺ MS1650 Nal 3 (o) and INA⁻ deletion mutant GJP17B R2 (•) strains of *Pseudomonas fluorescens*. Suspensions of two bacterial strains (10^8 cfu/ml) were mixed and applied to open strawberry blossoms by spray inoculation. Plants were incubated under intermittent mist.

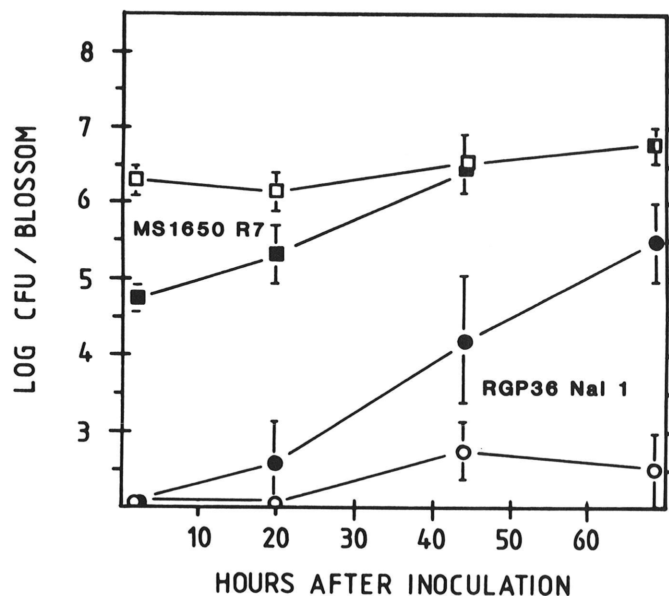

Fig. 5. Dose dependence of inhibition of INA⁻ deletion mutant strain RGP36 Nal 1 of *Pseudomonas syringae* by INA⁺ strain MS1650 R7 of *P. fluorescens* during co-colonization. RGP36 Nal 1 was applied at 3×10^3 cfu/ml and MS1650 R7 was applied at 10^8 cfu/ml (□) and 4×10^6 cfu/ml (■). Suspensions of two strains were mixed and applied by spray inoculation to open strawberry blossoms. Plants were incubated under intermittent mist between 1800 and 0600 hr. RGP36 Nal 1 (o) was inhibited by high dose of MS1650 R7 (10^8 cfu/ml; □), but RGP36 Nal 1 (•) was not inhibited by a lower dose of MS1650 R7 (4×10^6 cfu/ml; ■).

strains was not sufficient to prevent the formation of ice nuclei active at −3.0 C under these test conditions.

The duration of misting profoundly affected the competition between any two bacterial strains. Inhibition of the challenge strain was more complete under incubation conditions of 12 hr of misting or no misting than under 24 hr of intermittent misting (*data not shown*). In the absence of mist, blossoms were also less likely to contain ice nuclei active above −3 C (Fig. 9). Thus, efficacy tests performed under a 24-hr misting program can be considered the most difficult challenge for a potential biological frost protection agent.

DISCUSSION

In all of these competition studies, the pattern of interstrain competition was similar whether the INA$^+$ parental strains or the INA$^-$ deletion mutant strains were used. There were no significant differences in growth characteristics in vivo between the parental and deletion mutant strains. The deletion mutant strains did not exhibit any competitive advantage over the INA$^+$ strains. Growth of an INA$^+$ strain could be inhibited by artificially elevating the bacterial population to carrying-capacity level by application of an INA$^-$ strain, but growth cessation was not followed by a significant decline of the population of INA$^+$ bacteria. This finding supports that of Lindow and co-workers (8,10,14) that biological control agents that function as competitors and not as antagonists can be used only as prophylactic treatments and have no therapeutic value (cannot reduce established populations of INA$^+$ bacteria). For a

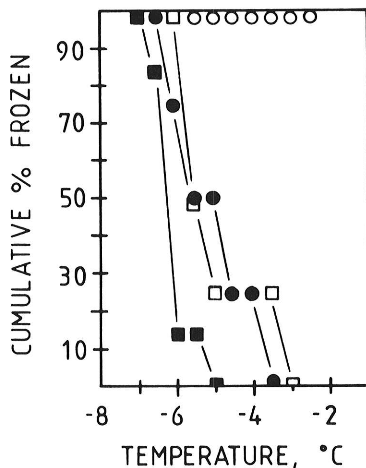

Fig. 6. Freezing temperatures of strawberry blossoms spray inoculated with INA$^+$ strain S203 R1 of *Pseudomonas syringae* at 6×10^2 cfu/ml (○), INA$^-$ strain RGP36 at 2×10^8 cfu/ml (■), water (□), or coinoculated with S203 R1 and RGP36 (●). Plants were incubated under intermittent mist for 73 hr before freezing assay was performed. Bacterial populations are shown in Figure 1A.

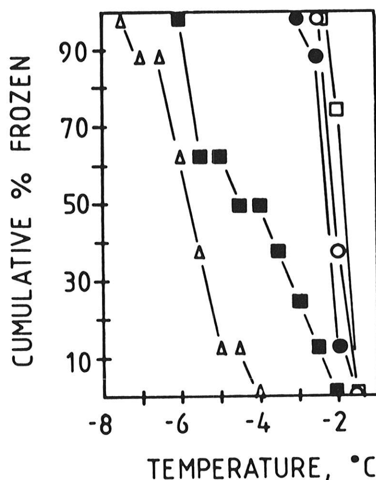

Fig. 7. Freezing temperatures of strawberry blossoms spray inoculated with axenic cultures of INA$^+$ bacteria (*P. syringae* MS287, □; *P. fluorescens* MS1650, ○), INA$^-$ deletion mutant strain RGP36 of *P. syringae* (△), or mixtures of INA$^+$ and INA$^-$ strains (MS287 coinoculated with RGP36, ■; MS1650 coinoculated with RGP36, ●). INA$^+$ strains were applied at about 3×10^2 cfu/ml and RGP36 was applied at 3×10^8 cfu/ml by spray inoculation of open blossoms. Plants were incubated for 67 hr under intermittent mist before freezing assay was performed. RGP36 protected blossoms against warm-temperature freezing by unrelated *P. syringae* strain MS287 (□ vs. ■) but did not protect against *P. fluorescens* strain MS1650 (○ vs. ●). Control (unsprayed) blossoms had freezing spectra similar to those sprayed with RGP36 alone.

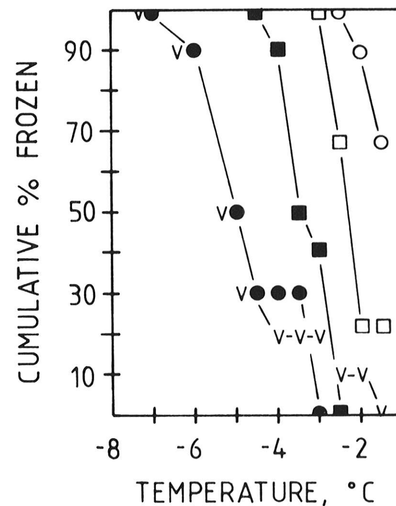

Fig. 8. Freezing temperatures of strawberry blossoms spray inoculated with axenic cultures of INA$^+$ bacteria (*P. syringae* MS287, ○; *P. fluorescens* MS1650, □), INA$^-$ deletion mutant strain GJP17B of *P. fluorescens* (V), or mixtures of INA$^+$ and INA$^-$ strains (MS287 coinoculated with GJP17B, ●; MS1650 coinoculated with GJP17B, ■). INA$^+$ strains were applied at 5×10^2 cfu/ml and GJP17B was applied at 6×10^8 cfu/ml by spray inoculation of open blossoms. Plants were incubated for 67 hr under intermittent mist before freezing assay was performed. GJP17B reduced mean freezing temperature of blossoms challenged with *P. syringae* strain by about 2 C (○ vs. ●) but gave only 1 C protection against strain MS1650 of *P. fluorescens* (□ vs. ■).

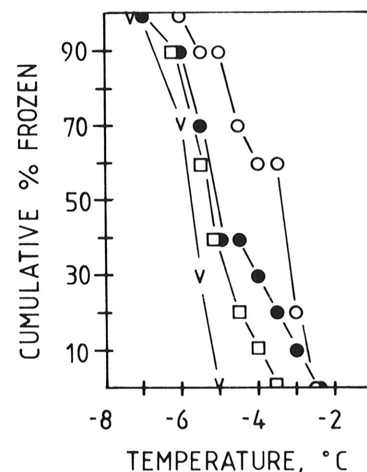

Fig. 9. Freezing temperatures of strawberry blossoms spray inoculated with axenic culture of INA$^+$ strain MS1650 of *P. fluorescens* (○), INA$^-$ strain GJP17B of *P. fluorescens* (V), water (□), or mixture of INA$^+$ and INA$^-$ strains of *P. fluorescens* (●). MS1650 was applied at 7×10^2 cfu/ml and GJP17B at 2×10^8 cfu/ml by spray inoculation of open blossoms. Plants were incubated for 72 hr without misting before freezing assay was performed. GJP17B reduced mean freezing temperature of blossoms sprayed with MS1650 by about 1.5 C in absence of mist (compare with Fig. 8).

nonantagonistic biological control agent to be effective under a wide range of conditions and to maintain its effectiveness during the colonization of expanding plant tissues, it may need to exhibit a faster growth rate than the target pest.

This study supports what has already been shown by others in that biological control may be effective in the absence of apparent antibiosis (2,10,16,19). The apparent growth inhibition could be the result of nutrient depletion and/or the saturation of colonizable sites as the maximum population capacity of the blossoms is reached. The first alternative could be investigated using near-isogenic lines with altered substrate utilization spectra. Since the interspecific competition between *P. syringae* and *P. fluorescens* was not reciprocal, antibiosis cannot be ruled out as a part of the competitive mechanism. This aspect is currently under investigation.

The deletion of the genetic sequences that code for ice nuclei had no demonstrable effect on competence for epiphytic colonization. Thus, there is no evidence to suggest that the release of genetically engineered INA^- deletion mutants would alter the population balance of INA^+ and non-INA bacteria in the natural environment. In a field test, it is hoped that the large dose advantage given to the INA^- bacteria by the applicator would be sufficient to allow successful biological control at the test site. However, once the INA^- strains were dispersed beyond the experimental plot, they would have no dose advantage. In the environment at large, natural bacterial strains would far outnumber the deletion mutants, the result being that the INA^- mutants would themselves be prevented from growing by the presence of established populations of other bacteria. The absence of intrinsic competitive advantage in the INA^- strains is convincing evidence that they can safely be released into the environment but also will probably limit their effectiveness as biological control agents under field conditions.

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