Measuring applied antagonistic bacterial populations for management of fire blight within the state of New York.

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Abstract
The blossom blight phase of fire blight, caused by the bacterium *Erwinia amylovora,* is typically managed by applying the antibiotic, streptomycin sulfate, to trees during bloom. Biological control of fire blight can be achieved by applying non-pathogenic bacteria to open flowers that then colonize flower part surfaces and compete with the pathogen for space and nutrients. The objective of this study was to measure the growth of three applied biological control organisms at three orchard locations in New York. We hope to identify safe and effective alternatives to streptomycin because the pathogen is likely to become resistant to streptomycin if this product is used too often. This has already occurred in some western and mid-western states. We detected population levels of the biological control organisms that were slightly lower than those reported in the Pacific Northwest. We also determined that the spread of the biological control organisms increased over time at each location. Application of the bacteria with a handgun sprayer appeared to result in higher populations than when applied with a handheld sprayer. A mixture of antagonists was detected at more than one location, indicating either tank contamination or natural dispersal of the organisms via pollinators. Our results confirmed the ability of these three biological control organisms to colonize and multiply on flowers in New York state. Further studies with larger sample sizes would allow us to make stronger correlations between population levels and incidence of the biological control agents, and their ability to prevent disease.

Background and Justification
In NY 45,000 acres of apples were in production with receipts worth $160 million in 2004 (14). Fire blight, caused by the bacterium *Erwinia amylovora* (Ea), is an economically important disease of apple and pear that affects nearly every part of the plant and can kill established trees. Streptomycin sulfate is the standard tool for management of this disease. Many growers in NY rely on the forecasting model, MARYBLYT^{TM}, to determine the best times to apply the antibiotic. Overuse of streptomycin has led to the development of resistant pathogen populations in CA, WA, OR, MO, and MI in addition to New Zealand, Israel and Lebanon (9,12). In those areas, such as NY, where resistant strains have not yet emerged, alternatives to streptomycin will be useful in themselves and in delaying the development of resistant strains of Ea.

Biological control of fire blight can be achieved by applying non-pathogenic, antagonistic bacteria to open flowers that then colonize the stigmatic surfaces (17). Through preemptive competitive exclusion the antagonist reaches population levels sufficient to prevent floral

infection by Ea, ultimately decreasing yield loss. One such antagonistic bacterium, *Pseudomonas fluorescens* strain A506 (PfA506), is currently labeled as a microbial pesticide commercially known as BlightBan A506 (NuFarm Americas Inc., Houston, TX). A second antagonistic bacterium, *Pantoea agglomerans* strain C9-1 (PaC9-1), was isolated from apple in MI is in advanced stages of federal registration by NuFarm (5). A third organism, *P. agglomerans* strain E325 (PaE325), was isolated from apple in WA (11) and is registered for use in the US and Canada as Bloomtime Biological (Northwest Agricultural Products, Pasco, WA).

Studies in OR and WA have demonstrated that PfA506 and PaC9-1 reduced blossom blight infection by 40% to 80% (6). Several efficacy studies conducted in NY demonstrated that PaC9-1 provided 33%-48% suppression of blossom blight and was more effective than PfA506, which provided between 8%-40% control (1,3,10,15,16). Field studies performed in NY over the last 3 years reported that PaE325 provided 9%-58% control (2,15,16). Suppression of the disease is not always consistent, as was the case in a 2005 field trial in NY, when none of the antagonistic bacteria tested was effective in suppressing fire blight.

Researchers in OR and WA have reported that PfA506 and PaC9-1 reached population levels of 10^4-10^6 CFU/blossom and colonized 40%-70% of blossom clusters after application (6). In a field trial in NY, PfA506, PaC9-1 and PaE325 were detected on 24%-85% of the blossoms imprinted onto selective media. Although populations of the antagonists were detected on clusters one or two days after application, they were not efficacious against fire blight. Lower temperatures during their application may have suppressed population levels, allowing the pathogen to colonize the stigmatic surfaces and cause disease.

Objectives

Our objective was to better understand the growth response of biological control organisms to weather conditions in NY by monitoring the population levels and incidence of the applied bacteria PaC9-1, PaE325, and PfA506 on apple blossoms in three orchard locations. By determining the population dynamics of the antagonistic bacteria, we aim to improve application timing recommendations in conjunction with weather before and after forecasted infection periods. Studies of these population levels have been performed on the West Coast where the efficacy of these antagonists in managing fire blight has been comparatively higher than in NY. Data from NY should enable their more efficacious use in this region.

Procedures

A company funded efficacy trial evaluating the three bacterial antagonists was conducted in an apple orchard located at the New York State Agricultural Experiment Station in Geneva, NY. Individual plots of two adjacent trees within a row (one inoculated and one non-inoculated), were arranged in a randomized complete block design with five replications. Two blocks included alternately planted 'Idared' (inoculated) and 'Empire' (non-inoculated) trees; three blocks included only 'Idared' trees, all on M.7 rootstock. Trees were inoculated at full bloom (10 May) with *Erwinia amylovora* strain Ea 273 at 10^7 cfu/ml using a Solo backpack sprayer equipped with a hollow cone nozzle (Figure 1). Treatments of PaC9-1 (1 x10^{11} cfu/ml), PaE325 (1 x 10^{10} cfu/ml) and PfA506 (1 x 10^{10} cfu/ml) were applied to runoff at 20-30% (5 May) and 70-80% (8 May) bloom, using a handgun sprayer (Table 1). Weather conditions were monitored by the weather station located less than 1 mile from the orchard block. Incidence of blossom

blight was evaluated by counting the number of infected clusters out of a maximum of 200 clusters per tree 4 wk after inoculation.

With the assistance of Debbie Breth, Area Extension Educator, two grower orchards in Wayne County were chosen. The first was located in Sodus, NY and was planted to Idared trees on M.26 rootstock with a recent history of shoot blight (Figure 2). The second was located in Newark, NY and was planted to Gala trees (Figure 3). The orchard located in Sodus is closer to Lake Ontario, where bloom tends to occur later than in the Geneva location. This allowed us to compare the bacterial populations under varying weather conditions within the state during one season. Weather conditions at the Newark location were recorded using a Hobo environmental sensor (Spectrum Technologies, Plainfield, IL) and Rain Collector II dump bucket (Davis Instruments, Hayward, CA) (Figure 2). Weather conditions for the Sodus site were collected from the closest Sodus weather station operated by the NYS IPM's Network for Environment and Weather Awareness.

Antagonistic bacterial treatments at the grower orchards were applied using a one gallon handheld pump sprayer equipped with a hollow cone nozzle, twice during bloom (Table 2). Five adjacent trees within a row were chosen per treatment leaving five non-treated buffer trees between each treatment. Natural blossom blight infection was not observed in either grower orchard this year. MARYBLYT did not forecast any natural infection events for these orchards.

Three flowers per tree were collected (15 flowers per treatment) with alcohol treated, gloved hands, one to six days after each application. Each flower was placed in a small zip-lock bag and placed in an ice chest until further processing in the lab. Under sterile conditions, the pistil and nectary of each blossom were removed and placed in 1 ml sterile 10 mM potassium phosphate buffer, pH 7.0 in eppendorf tubes. The tubes were placed in racks, sonicated for 2 min, and vortexed to dislodge the bacteria from the plant surfaces (Figure 4). The sample buffer from each blossom was serially diluted three times by 10- or 100-fold intervals, and three 10ul drops of each dilution were plated onto Pseudomonas agar F (Sigma, St. Louis, MO) amended with rifampicin (100µg/ml) selective media (Figure 5). The morphology and number of colonies were observed 2-3 days after plating and final concentrations per blossom were calculated. Colonies with a fluorescent phenotype were identified as PfA506; colonies with a yellow phenotype were identified as PaC9-1; and colonies with a white, mucoid phenotype were identified as PaE325. We assume that naturally present bacteria were excluded from our cultures by the addition of rifampicin in the culture media. In addition, 24 flowers per tree (120 flowers per treatment) were randomly chosen and imprinted onto Pseudomonas agar F amended with rifampicin (100µg/ml) *in situ* (Figures 6a and 6b). Bacterial growth and morphology were observed two days after imprinting. Data were statistically analyzed with the glm procedure and the Student-Newman-Keuls multiple comparison test using SAS software (SAS Institute, Cary, NC).

Results and Discussion

Natural infection did not occur at either grower orchard. Non-treated, artificially inoculated trees at the Geneva location resulted in 77% blossom cluster blight (Table 1). Among inoculated trees, PaE325 followed by Agrimycin (66% control) was statistically similar in blossom blight control efficacy to that of trees treated with Agrimycin one time after inoculation (45% control). PaE325

(20% control) and PaC9-1 (23% control) did not differ significantly from each other, Agrimycin, or the non-treated control. PfA506 was no different from the non-treated control.

During the first collection time, all antagonistic bacteria treatments were of statistically similar population levels, yet overall, bacterial levels at the Geneva location were statistically higher than at the other locations. By the second collection time, PaC9-1 had significantly higher population values than the other two bacteria. Population levels on flowers collected at Geneva were significantly higher than those collected at Newark, which in turn, had significantly higher populations than at Sodus. When data were pooled over both collection times, PaC9-1 had significantly higher highest population levels than PfA506 or PaE325. Also, higher levels of bacteria were detected at Geneva than at Newark or Sodus.

A summary of the average population levels and incidence of all treatments of this experiment can be found in Table 2. Overall, we have found PfA506 to be a poor colonizer of stigmas in New York. Under field conditions, this organism ranged from 1.10×10^2 cfu/flower to 4.0×10^3 cfu/flower. This is very low compared to results reported from the Pacific Northwest, where this organism has been shown to reach 10^5 to 10^6 cfu/flower (13). We found this organism to colonize 41% to 48% of the imprinted flowers, which is comparable to the 40% to 60% reported by Stockwell, et al. PaC9-1 tended to be a slightly better colonizer ranging from 1.77×10^1 to 2.17×10^4 cfu/flower. Again, this is lower than results found in the Pacific Northwest, where levels can reach 10^4 to 10^6 cfu/flower. PaC9-1 colonized between 15% to 77% of the flowers imprinted, which is also comparable to the results from the Pacific Northwest. Populations of PaE325 ranged from 2.37×10^3 to 1.58×10^4 cfu/flower. This range does not include the results of the second collection time at the Sodus location when no bacteria were isolated from the 15 flowers collected from trees treated with PaE325. However, PaE325 was detected on 9% to 59% of the stigmas imprinted at this time. There are no known data from other locations to compare these results to.

There seems to be a correlation between population level and efficacy. PfA506 had the least effectiveness for controlling disease in Geneva and had the lowest population levels at the second collection time. PaC9-1 and PaE325 provided 23% and 20% control of blossom blight, respectively. Population levels of these two bacteria were detected at 2.17×10^4 and 1.58×10^4 cfu/flower, respectively. While, PfA506 provided 3% control of blossom blight and was found to reach a population level of 1.65×10^3 cfu/flower. These results are not statistically stringent, as they are based on only 15 flowers per treatment. Further studies with larger sample sizes are needed to strengthen these observations.

The population of antagonistic bacteria detected at the Geneva location was statistically higher than at the other two locations. This may be explained by the large volume of spray applied with a handgun sprayer, compared to the volume applied with a handheld pump sprayer. In a commercial setting, growers would most likely use an air-blast sprayer, which can be calibrated to emit varying volumes of output. Further studies could be conducted to determine the best volume of water required for the best coverage of these organisms.

Reducing the number of streptomycin sprays also reduces the environmental impact on natural bacterial populations. Maintaining the natural balance of microbes present in an orchard may aid

in overall productivity, although studies are lacking. The risk of water contamination is also reduced by applying less streptomycin sprays per season. In addition, decreasing the use of antibiotics reduces the user exposure to these chemicals.

Bloomtime Biological has just gained federal registration and is currently undergoing the New York registration process. BlightBan A506, however, is not yet available in NY. According to the 2003 USDA, National Agricultural Statistics Service, PfA506 has been used on apples and pears in CA, OR and WA. Out of the total acres of apples in WA, 4% were treated with PfA506. Also, out of the total acres of pears in OR, 24% were treated with PfA506. All states in which apple or pear are grown, including NY, would benefit from the experiences of western growers who currently use biological control agents as a part of their fire blight management plan.

Table 1. Efficacy of bacterial antagonist and control treatments when applied to open blossoms before or after artificial inoculation with *Erwinia amylovora* at Geneva, NY in 2006.

Material(s), rate/100 gal	Spray timings[z]	% blossom clusters blighted[y] I[x]	N[w]
Agrimycin 17WP 8.0 oz	3	42.5 ab	0.9 a
Pantoea agglomerans E325 (1 x 10^{10} cfu/g) 7.2 oz	1,2	61.4 bcd	2.7 a
Pantoea agglomerans C9-1 (1 x 10^{11} cfu/g) 9.4 oz	1,2	59.3 bcd	1.3 a
Pseudomonas fluorescens A506 (5x10^{10} cfu/g) 9.4 oz	1,2	74.6 d	3.9 a
Non-treated	--	76.9 d	9.2 a

[z] Spray timing designations: 1=20-30% bloom (5 May), 2=70-80% bloom (8 May), 3=inoculation+24 hr (11 May).

[y] Results are per tree and represent mean values from five replicates per treatment. Means within a column followed by a common letter are not significantly different (P>0.05) as determined by Waller-Duncan *k*-ratio *t* test.

[x] Blossoms for all treatments were inoculated 10 May with *E. amylovora* strain Ea 273 at 1 x 10^7 cfu/ml.

[w] Non-inoculated tree was within the row and adjacent to inoculated tree of the same treatment.

Table 2. Average population levels and incidence of three antagonistic bacteria measured after their applications to blossoms of apple trees at three locations in New York.

Location	Organism Applied	Timing	Average Population (cfu/flower) n=15 flowers	% Incidence (dilutions plates) n=15 flowers	% Incidence[g] (stigma imprints) n=120 flowers
Geneva	A506	1[a]	1.65×10^2	86	--
	C9-1	1	5.59×10^3	93	--
	E325	1	2.39×10^2	92	--
	A506	2[b]	1.65×10^3	60	53
	C9-1	2	2.17×10^4	93	55
	E325	2	1.58×10^4	50	35[h]
Newark	A506	1[c]	4.03×10^3	20	7
	C9-1	1	2.96×10^1	40	29
	E325	1	3.50×10^3	33	5
	A506	2[d]	2.80×10^3	53	49
	C9-1	2	1.06×10^3	73	78
	E325	2	2.48×10^2	67	62
Sodus	A506	1[e]	1.22×10^3	60	4
	C9-1	1	8.34×10^3	67	15
	E325	1	2.37×10^2	20	8
	A506	2[f]	1.10×10^2	33	35
	C9-1	2	1.77×10^3	67	50
	E325	2	0	0	28

[a] Application date was May 5 (20-30% bloom) and collection date was May 7 (70-80% bloom).
[b] Application date was May 8 (70-80% bloom) and collection date was May 9 (90-100% bloom).
[c] Application date was May 8 (1-5% bloom) and collection date was May 10 (10-20% bloom).
[d] Application date was May 10 (20-30% bloom) and collection date was May 16 (70-80% bloom).
[e] Application date was May 10 (10% bloom) and collection date was May 16 (20-30% bloom).
[f] Application date was May 10 (30-40% bloom) and collection date was May 16 (90-100% bloom).
[g] May represent a mixed population of antagonistic bacteria.
[h] n=96 flowers

Literature Cited

1. Aldwinckle, H.S., Gustafson, H.L., Heidenreich, G., Penev, R., and LoGiudice, N. 2002. Field evaluation of materials for control of blossom blight of apple, 2001. FN Tests 57:PF02.
2. Aldwinckle, H.S. and R.P. Penev. 2004. Field evaluation of materials for control of fire blight infection of apple blossoms, 2003. FN Tests 59:PF016.
3. Bhaskara Reddy, M.V., Norelli, J.L., and Aldwinckle, H.S. 2000. Control of fire blight infection of apple blossoms, 1999. FN Tests 55:22.
4. Bhaskara Reddy, M.V., Norelli, J.L., and Aldwinckle, H.S. Biologicals, SAR inducers, copper compounds, and other chemicals for blossom blight control on apples, 2000. FN Tests PF34.
5. Ishimaru, C.A., et al. 1998. Multiple antibiotic production by *Erwinia herbicola*. Phytopathology 78:746-750.
6. Johnson and Stockwell. 1998. Management of fire blight: A case study in microbial ecology. Annu. Rev. Phytopathology 36:227-48.
8. Johnson and Stockwell. 2004. Adaptation of fire blight forecasting to optimize the use of biological controls. Plant Dis. 88:41-48.
9. Jones, A.L. and E. Schnabel. 2001. The development of Streptomycin-resistant strains of *Erwinia amylovora*. CAB Intl. Fire Blight: The disease and its causal agent, *Erwinia amylovora* (ed. J.L. Vanneste).
10. Momol, M.T., Aldwinckle, H.S., and Norelli, J.L. 1998. Evaluation of several products for control of fire blight on apple blossom, 1997. FN Tests 53:22.
11. Pusey, P.L. 1997. Crab apple blossoms as a model for research on biological control of fire blight. Phytopathology 87:1096-1102.
12. Saad, A.T., Hanna L., and Choueiri, E. 2000. Evaluation of streptomycin and oxytetracycline resistance of *Erwinia amylovora* populations in Lebanon. Phytopathology 90:S68 (Abstr.).
13. Stockwell, VO, Loper JE, Johson KB. 1992. Establishment of bacterial antagonists on blossom so fpear. Phytopathology 82:1128 (Abstr.).
14. United States Department of Agriculture, New York Agricultural Statistics Service. 2004. Fruit Annual Summary (Publication No. 975-1-05). On-line at: http://www.nass.usda.gov/ny/01jan/frt0105.htm/.
15. Werner, N.A., Heidenreich, G., Aldwinckle, H.S. Field evaluation of materials for control of fire blight infection of apple, 2004. FN Tests 60:PF030.
16. Werner, N.A., Heidenreich, G., Aldwinckle, H.S. Field evaluation of materials for control of fire blight infection of apple, 2005. FN Tests 61:PF020 .
17. Wilson, M. and Lindow, S.E. 1993. Interactions between the biological control agent *Pseudomonas fluorescens* strain A506 and *Erwinia amylovora* in pear blossoms. Phytopathology 83:117-123.

Figures.

Figure 1. Artificial inoculation of apple trees with *Erwinia amylovora* strain 273 using a backpack sprayer.

Figure 2. Weather station used to collect temperature and rainfall data in Newark, NY. Orchard is planted to Gala apple trees.

Figure 3. Orchard planted to Idared trees on M.26 rootstock, located in Sodus, NY.

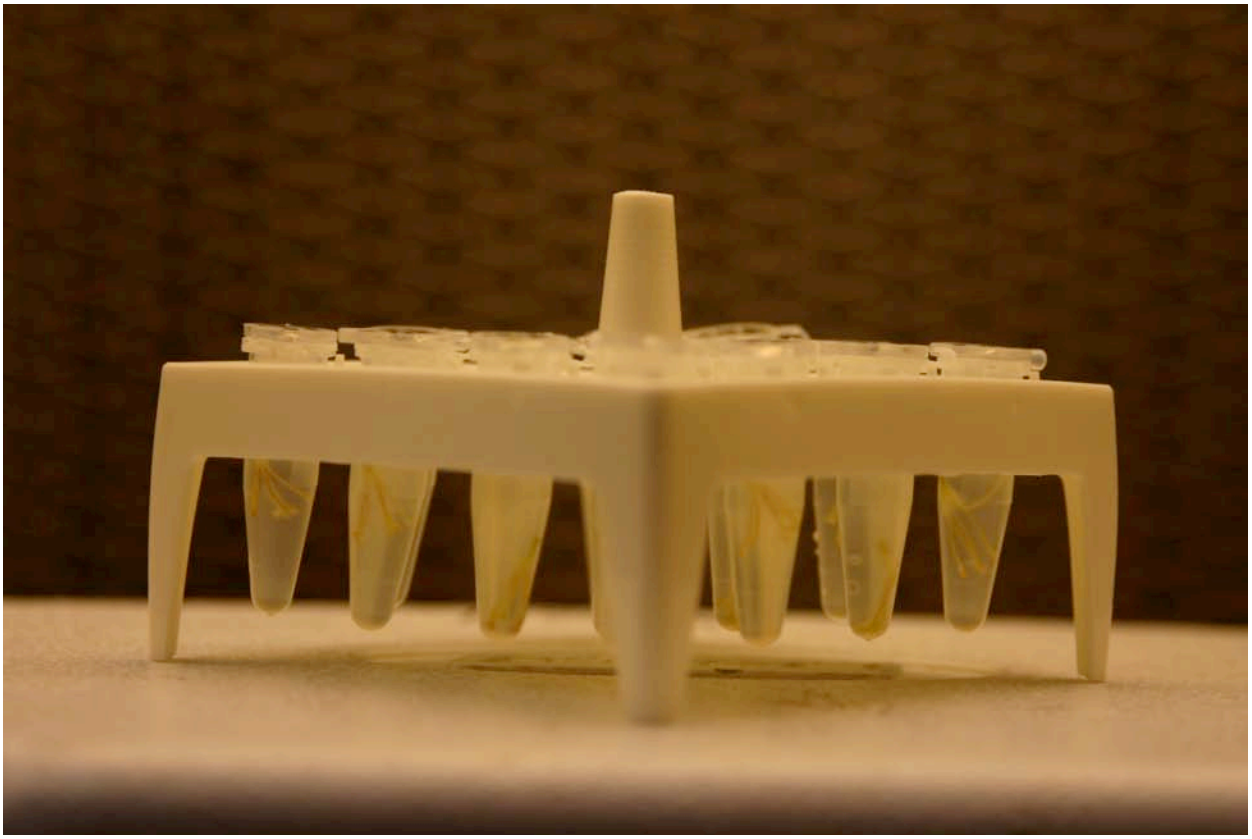

Figure 4. Rack of eppendorf tubes containing phosphate buffer and stigma removed from flowers that had been treated with antagonistic bacteria in the field.

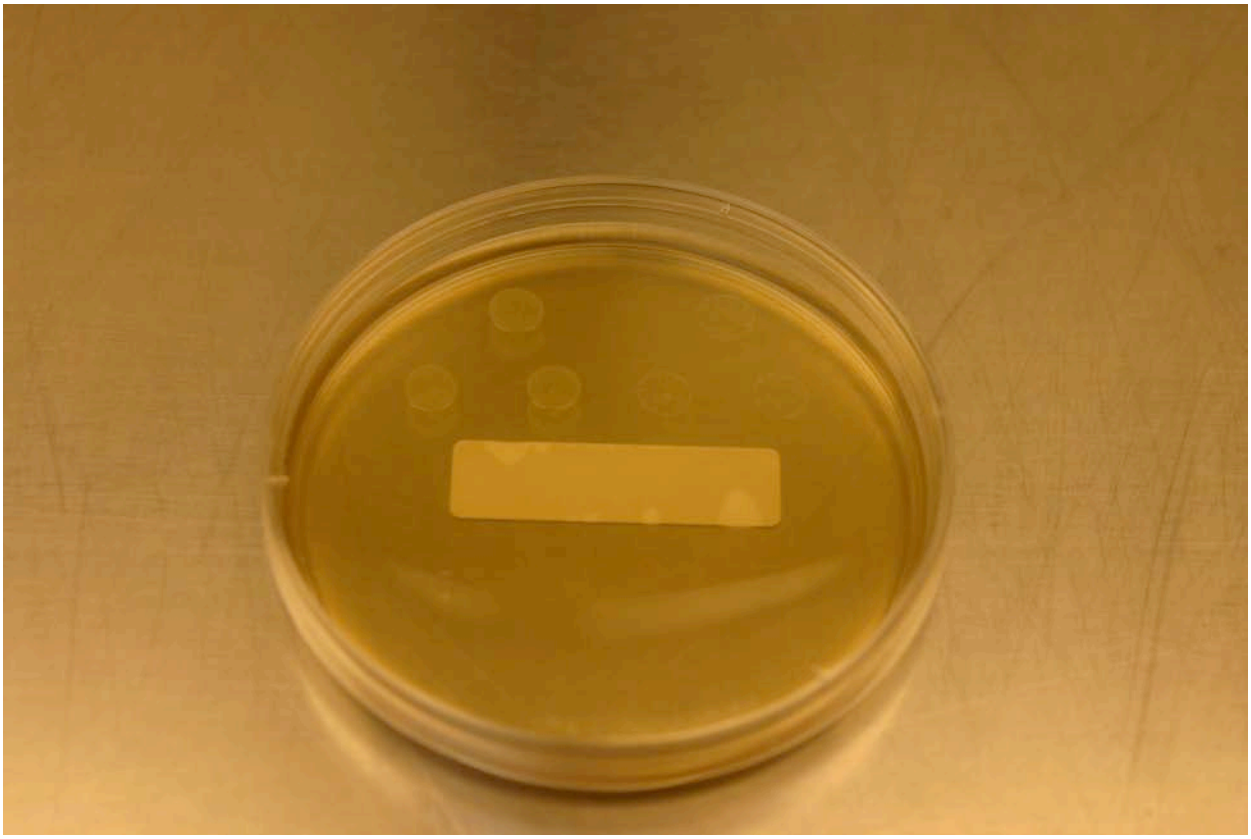

Figure 5. Petri dish of a serial dilution of antagonistic bacteria washed from the stigma of a flower. The number of bacteria growing on the stigma can be determined by counting the bacteria growing on the plate.

Figures 6a and 6b. By imprinting flower stigma onto selective media we can determine the presence of biological control agents.