Evaluation of new bactericides for control of fire blight of pears caused by *Erwinia amylovora*

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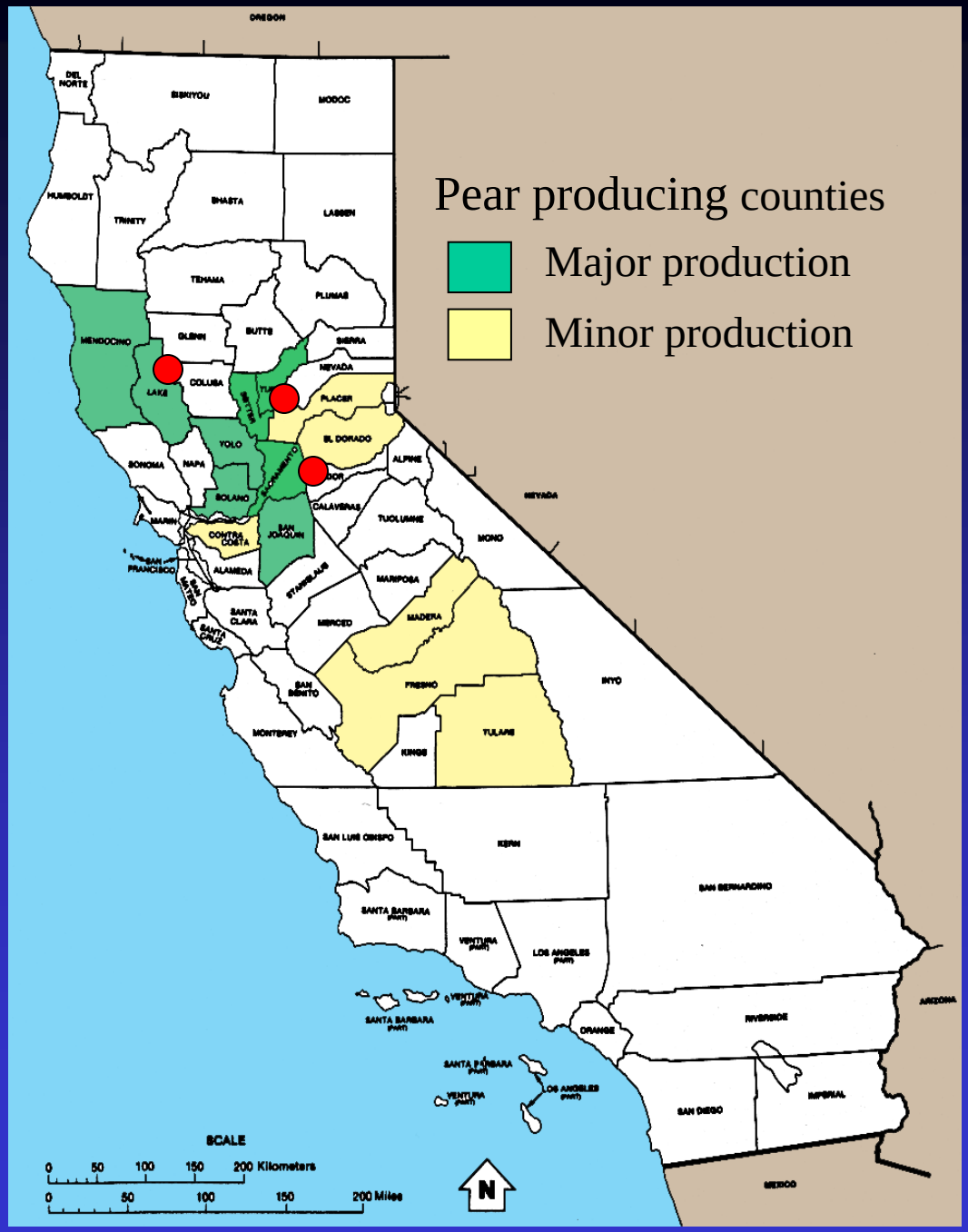
Fire blight - Chemical and biological control
- Currently registered and new materials -

Copper compounds	Antibiotics	Biologicals
Used only during dormant and bloom periods because of phytotoxic effects on developing fruit (russetting)	Streptomycin (1950s): ***Resistance widespread***	Bloomtime Biological *Pantoea agglomerans* E325 Efficacy variable
Effcacy intermediate	Terramycin (oxytetracycline) (1970s): less effective, resistance limited	Blightban *Pseudomonas syringae* A506 Efficacy variable
New copper formulations less phytotoxic? (e.g., Kocide 3000; Badge X2)	Kasugamycin - high efficacy in multiple years' trials, registration expected 2011	Other materials have been evaluated: Cerebrocide, Actinovate

Few materials are available for fire blight control – Goal is to evaluate new conventional and biologicals

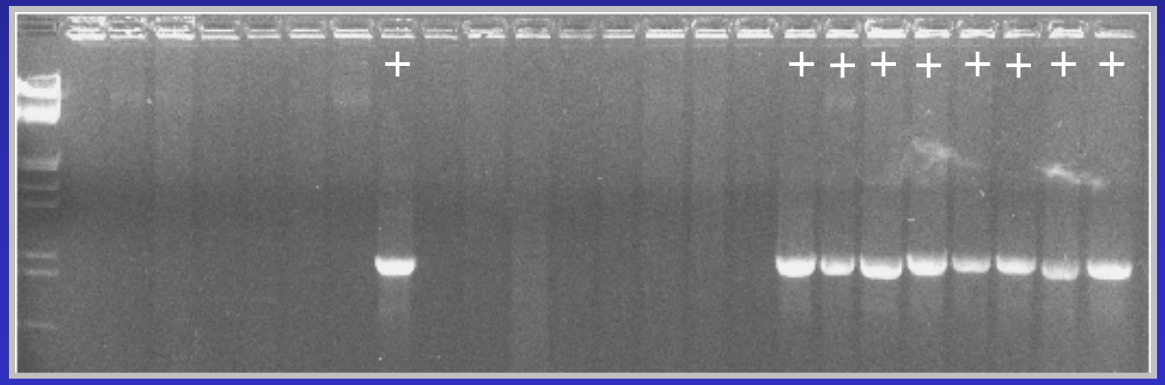


Surveys on antibiotic resistance in populations of *Erwinia amylovora*

- Collect isolates from orchards in major pear growing regions in CA (2006 – 20010)
  - Sacramento valley – Sacramento, Sutter/ Yuba Co.
  - Lake Co.
- Evaluate sensitivity against streptomycin, oxytetracycline, kasugamycin

Surveys on antibiotic resistance in populations of *Erwinia amylovora*

- Bacteria are isolated from diseased pear tissue (blossoms, woody tissues, fruit) using standard practices
- Isolates are verified for their identity by PCR

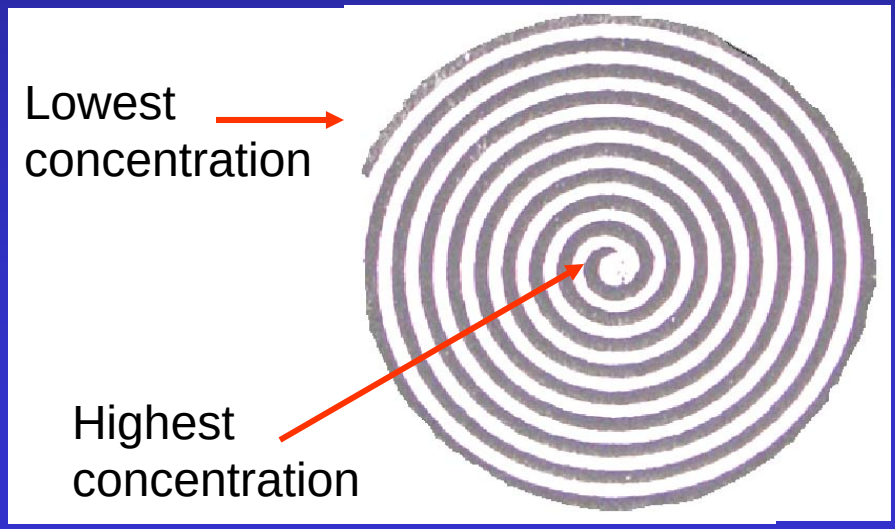


Verification of identify of isolates of *E. amylovora* using species-specific PCR primers (Bereswill et al., Appl. Environ. Microbiol. 58:3522-2536).

Determining inhibitory concentrations using the spiral gradient dilution method

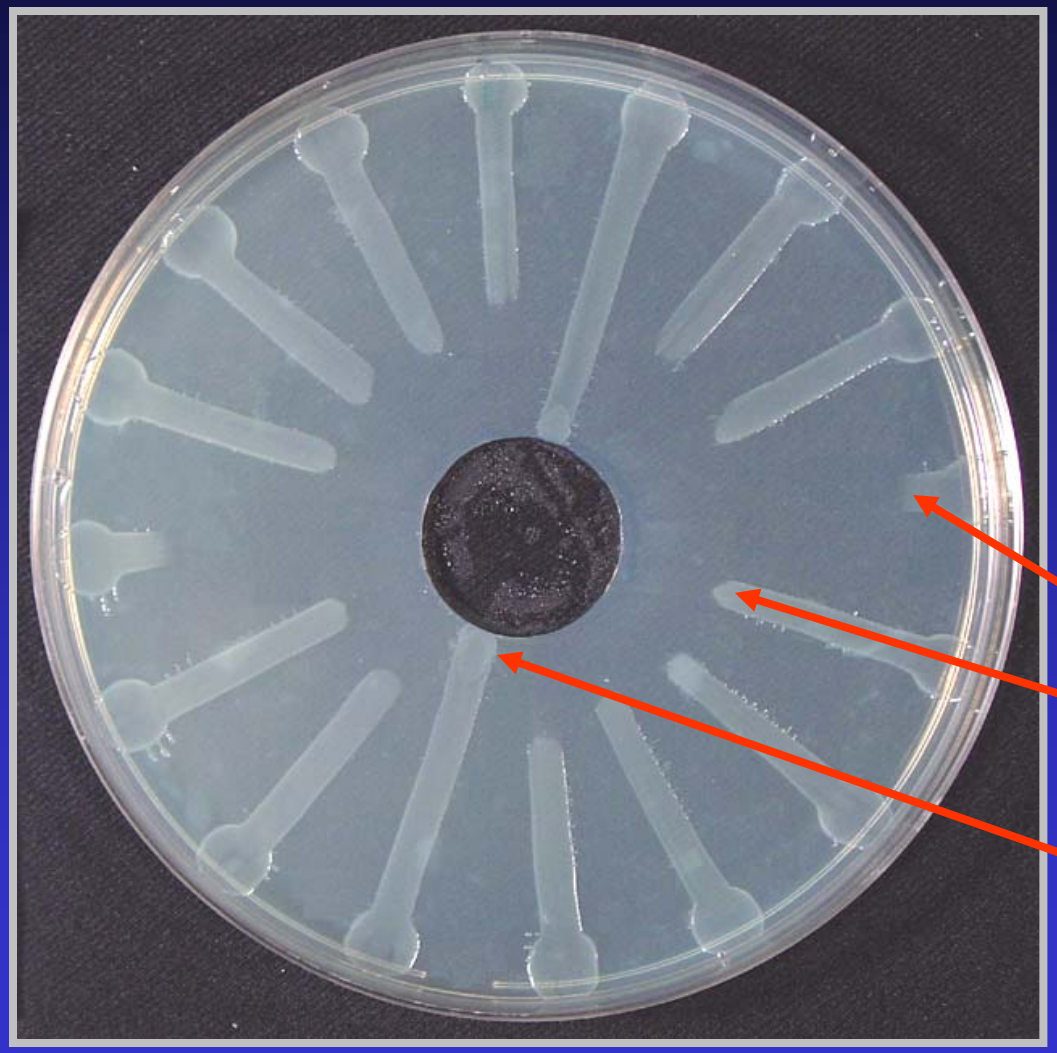
Spiral plater

- A continuous 2.5-log antibiotic gradient is produced on an agar plate using a spiral plater.
- Bacteria are streaked along the gradient and after a 2-day incubation, growth measurements are taken.



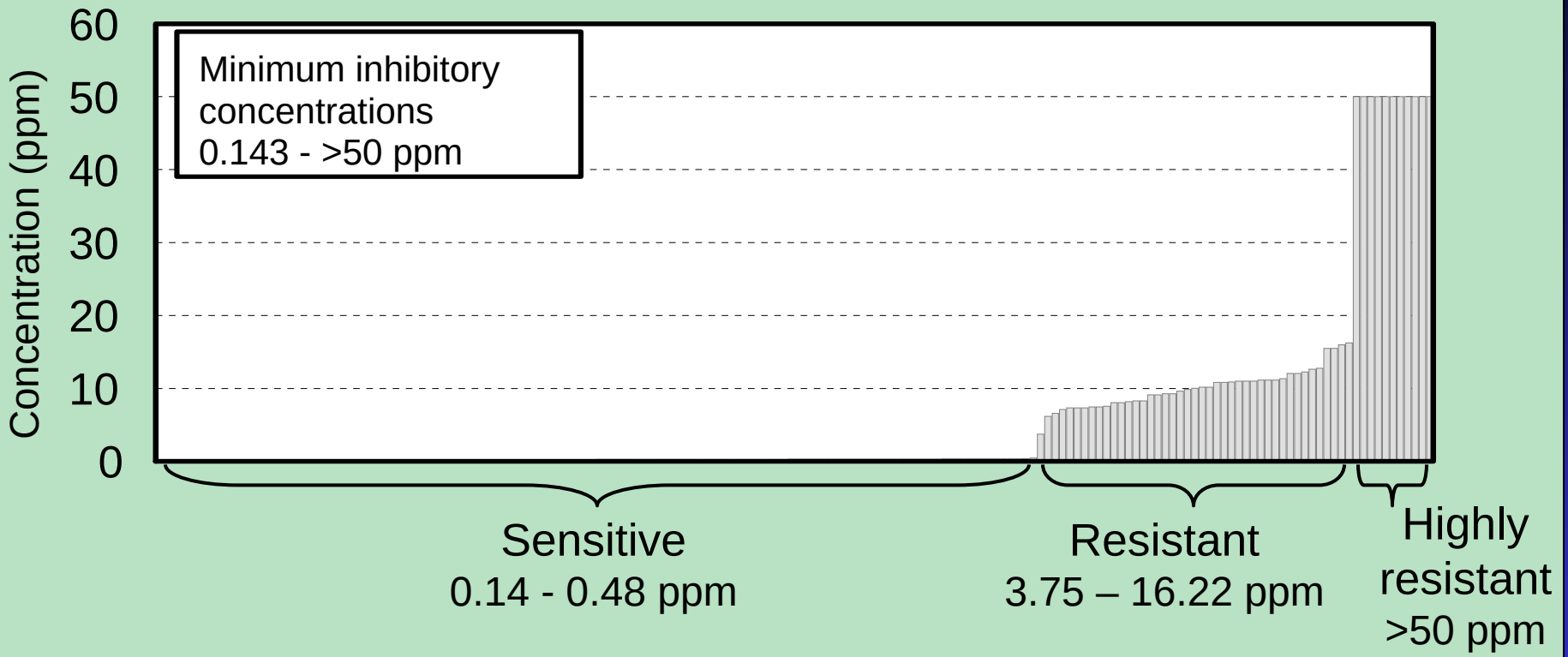
Results:

In vitro sensitivity of *E. amylovora* isolates to antibiotics



Spiral gradient dilution plate showing isolates with different sensitivity against streptomycin

Range of streptomycin sensitivity in populations collected in 2009



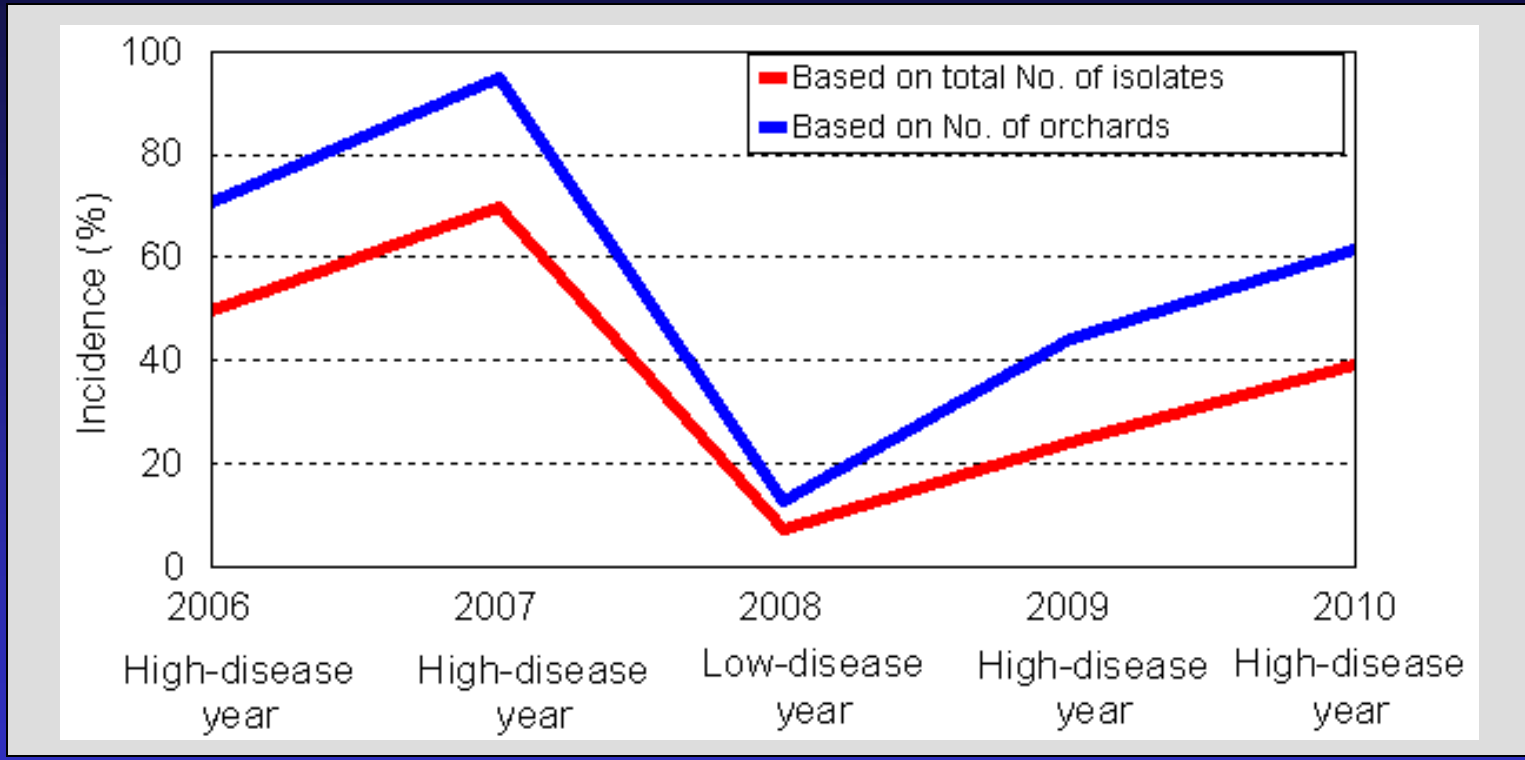
- 36 locations, 177 isolates.
- 24.3% of the isolates (44.4% of the orchards) were resistant (6.2% were highly resistant; 18.1% moderately resistant)

Distribution of isolates of *Erwinia amylovora* sensitive or less sensitive to streptomycin or oxytetracycline in a survey of 13 CA pear orchards in 2010

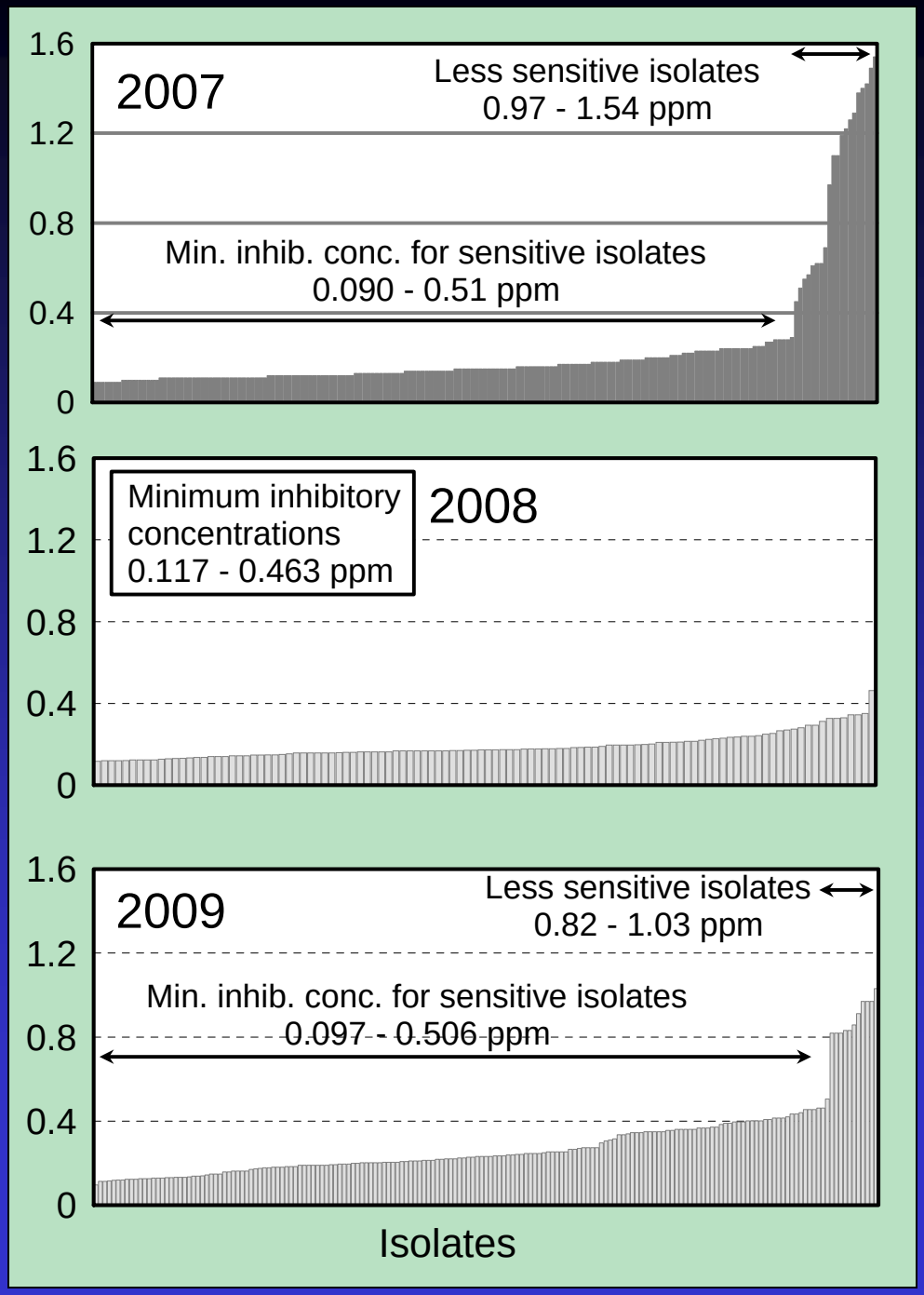
No.	County	No. isolates	Incidence Steptomycin Resistance (%)*	Incidence Oxyteracycline Resistance (%)*	Incidence Kasugamycin Resistance (%)*
1	Placer	5	0	0	0
2	Placer	2	0	0	0
3	Placer	3	0	0	0
4	Sacramento	6	100	0	0
5	Sacramento	7	85.7	0	0
6	Sacramento	5	100	0	0
7	Sacramento	5	20	0	0
8	Sacramento	6	33.3	0	0
9	Sacramento	6	16.6	0	0
10	Sacramento	9	44.4	0	0
11	Sacramento	4	0	0	0
12	Sacramento	3	33.3	0	0
13	Sacramento	5	0	0	0
Total		66			

* - Inhibitory concentrations were determined on nutrient agar using the SGD method. Minimum inhibitory concentrations (LIC) of isolates sensitive to streptomycin were 0.157-0.481 ppm; whereas LIC of isolates resistant to streptomycin were 7.45-15.72 ppm.
** - Minimum inhibitory concentrations (LIC) of isolates for oxytetracycline were 0.097-0.168 ppm; whereas those for kasugamycin were 4.29-9.15 ppm.

Annual fluctuations in streptomycin resistance in isolates of *Erwinia amylovora* 2006-2010 correlate with selection pressure



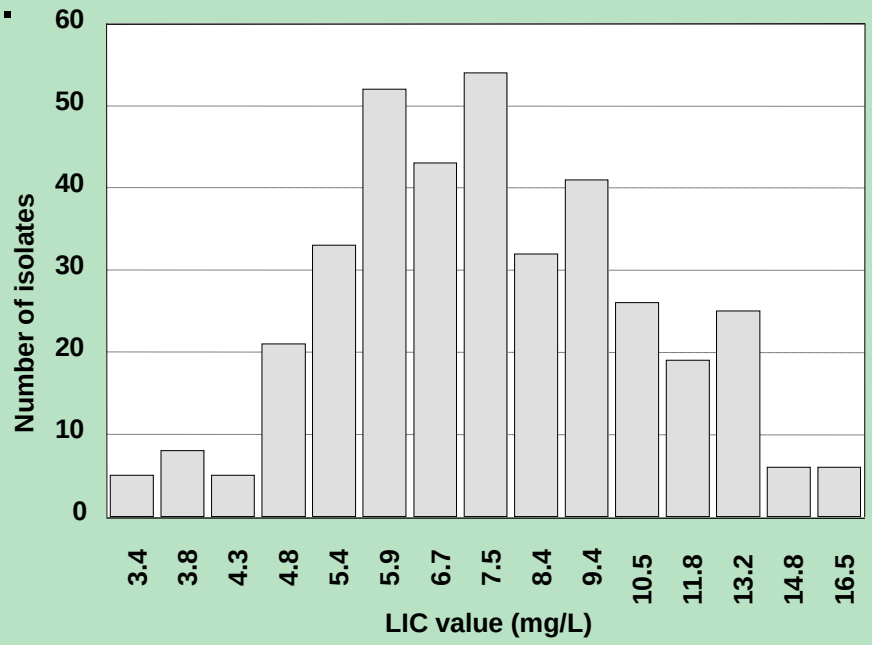
- Isolates of *E. amylovora* with intermediate levels of resistance (MIC <20 ppm) to streptomycin appear to be less fit.
- In contrast, isolates of with high levels of resistance to streptomycin (MIC >50 ppm) are persistent (stable occurrence in several locations).



Range of oxytetracycline sensitivity in populations collected in 2007-2009

- Insensitivity to oxytetracycline first detected in 2007.
- Insensitivity is limited to few locations.
- At some of these locations, however the less sensitive populations are persistent and treatments with Mycoshield lacked efficacy – field resistance.
- **Persistent isolates are highly resistant to streptomycin**
- No resistance detected in 2010

A.



The lowest inhibitory concentration (LIC; the lowest concentration where inhibition of bacterial growth was observed, i.e., where the bacterial streak became less dense visually) determined using the spiral gradient dilution method. Bar height indicates the number of isolates within each bin and bin width was based on Scott's method using $\log_{10}$ transformed values.

In vitro sensitivity to kasugamycin of 376 isolates of *Erwinia amylovora* collected throughout California pear growing regions from 2005-10

- Insensitivity to kasugamycin has not been detected to date (no multiple resistance to the other antibiotics).
- Inhibitory concentrations to kasugamycin are relatively high, but may be dependent on culture media.

In vitro inhibitory effects for kasugamycin, streptomycin, and oxytetracycline against *E. amylovora* using selected agar media

	Kasugamycin		Streptomycin		Oxytetracycline	
Agar medium[a]	LIC (mg/L)[b]	LSD[c]	LIC (mg/L)	LSD	LIC (mg/L)	LSD
NA	6.93	e	0.301	cd	0.106	e
Czapek	12.25	d	0.225	d	0.722	bc
KMB	20.63	c	0.288	cd	0.668	c
523	28.54	b	0.357	c	0.822	b
LBA	43.09	a	0.791	b	0.359	d
YSA	45.58	a	1.260	a	2.156	a

[a] - NA = nutrient agar, KMB = King's medium B, LBA = Luria-Bertani agar, 523 = Kado medium 523, YSA = Yeast-salts agar.

[b] - Inhibitory concentrations were determined using the spiral gradient dilution method. Values are the average of 8 isolates from 2 repeated experiments.

In vitro inhibitory effect of kasugamycin on growth of *Erwinia amylovora* at selected pH values.

Kasugamycin	Growth (%)[a]			
(mg/L)	pH 5.1[b]	LSD[c]	pH 7.3	LSD
0	100.0	Aa	100.0	Aa
5	13.8	Bb	78.9	Ba
10	3.9	Cb	64.9	Ca

[a] - Growth in microtiter plates was measured as optical density. For each pH treatment, growth in the presence of kasugamycin was expressed as percent growth compared to the non-amended control. Values are the average of three repeated experiments.

[b] - The pH of nutrient broth was adjusted using a citrate-glycine-potassium phosphate buffer.

Summary: Sensitivity to antibiotics in *Erwinia amylovora* from 36 California pear orchards in a survey in 2010

- Streptomycin resistance was found in 39.4% of the isolates and in 61.5% of the orchards sampled.
- Intermediate-resistance to streptomycin appears to be non-persistent, whereas high-resistance is persistent (stable).
- Populations adapt quickly to changing selection pressure: Resistance management strategies using rotations with new treatments will be very effective in managing the disease.
- Resistance to oxytetracycline seems to be stable.
- All 66 isolates evaluated in 2010 were sensitive to kasugamycin. To date, no resistance has been found in CA populations of *E. amylovora*.
- Media and pH affect toxicity of kasugamycin.

Summary: In vitro sensitivity of *E. amylovora* against antibiotics

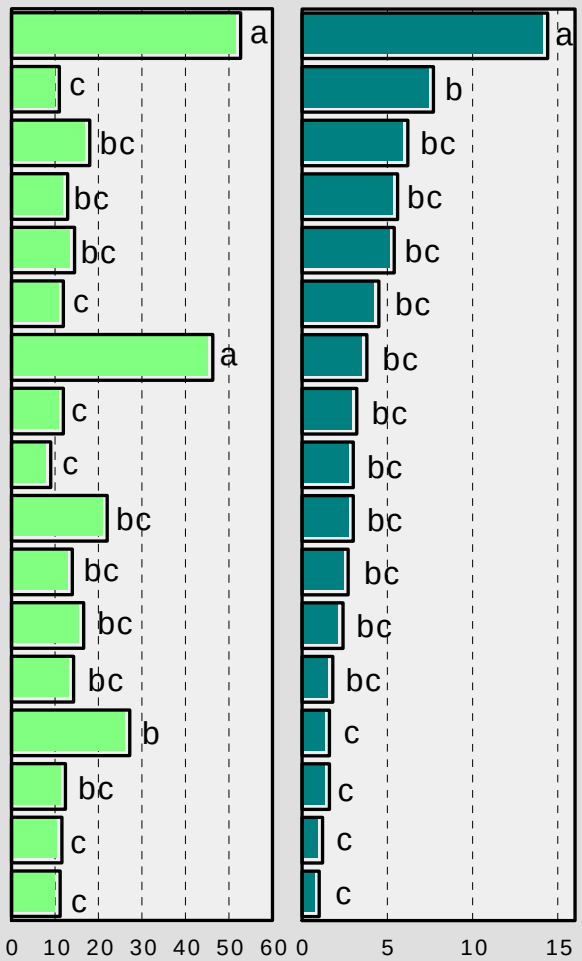
- Under selection pressure, resistance against streptomycin develops quickly and is starting to develop against oxytetracycline.
- This stresses the need for alternative bactericides for management of fire blight so that efficacy of oxytetracycline can be maintained.

Field efficacy studies with new fire blight treatments
Kasugamycin

- Apply kasugamycin (Kasumin 2L or 10L) by itself or in mixtures or rotations with other compounds:
  - Bloomtime Biological (*Pantoea agglomerans*)
  - Blossom Protect (*Aureobasidium* sp.)
  - Actinovate (*Streptomyces lydicus*)
  - Citrox, Proalexin, + others
  - Streptomycin (Agrimycin)
  - Oxytetracycline (Mycoshield)
  - Fixed Coppers (Kocide, Badge)
  - EBDC fungicides
    - Mancozeb (Dithane)
- Small-scale field trials, hand sprayer applications
- Field trials, air blast applications

Evaluation of new bactericides and biologicals for fire blight management on Bartlett pears in a field trial in Live Oak 2010

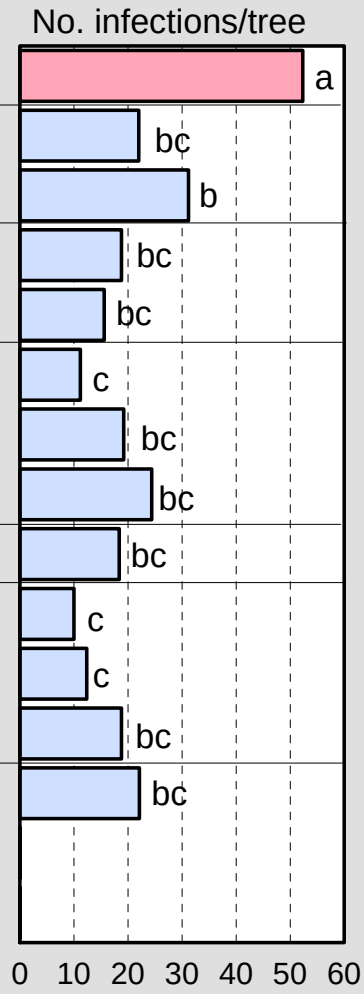
Treatment*	80% bloom (3-23)	Full Bloom (3-31)	FB/PF (4-7)	Petal Fall (4/15)
Control	---	---	---	---
Kasumin + Streptomycin (100+100 ppm)	@	@	@	@
* Kasumin 10L (100 ppm)	@	@	@	@
→ Citrox BC (1%)	@	@	@	@
Kocide 3000 (8 oz)	@	@	@	@
Manzate F45 (150 fl oz)	@	@	@	@
ProAlexin (1%)	@	@	@	@
→ Blossom Protect + Buffer (21.5 oz+9.3 lb)	@	@	@	@
Mycoshield (200 ppm)	@	@	@	@
Badge 2.27SC (14.5 fl oz)	@	@	@	@
Kasumin 10L + Manzate F45 (100 ppm+75 fl oz)	@	@	@	@
→ Actinovate + NuFilm P (12 oz+8 fl oz)	@	@	@	@
Streptomycin (100 ppm)	@	@	@	@
Cerebrocide + Biozyme (67 fl oz+32 fl oz)	@	@	@	@
* Kasumin 2L (100 ppm)	@	@	@	@
* Kasumin 10L, Strep., Myco + Manzate F45**	@	@	@	@
* Kasumin + Mycoshield (100+200 ppm)	@	@	@	@



Treatments were applied using an air-blast sprayer at 100 gal/A. Disease was evaluated in 4-10.

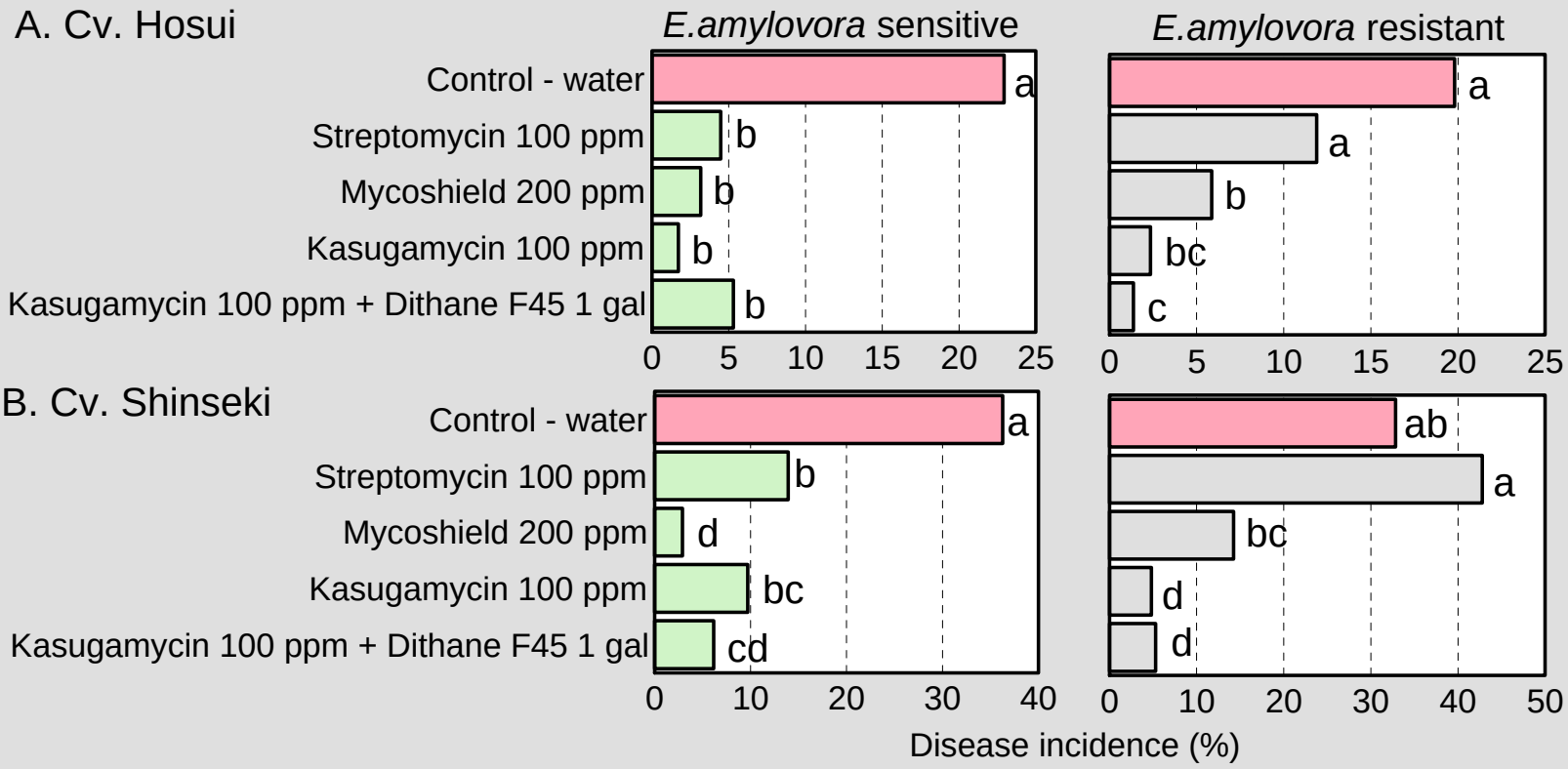
Evaluation of new fire blight treatments
Field trial on Bartlett pear - Live Oak 2009

	Treatment	80% bloom (3-23)	Full Bloom (3-31)	Petal Fall (4-7)
	Control	---	---	---
Natural product and biocontrol	Cerebrocide 0.5%	@	@	@
	Actinovate 12 oz + Breakthru 2 fl oz	@	@	@
Coppers	Kocide 3000 8 lb	@	@	@
	Badge 8 fl oz	@	@	@
Antibiotics	Kasumin 2L 100 ppm	@	@	@
	Streptomycin 100 ppm	@	@	@
	Mycoshield 200 ppm	@	@	@
Fungicide	Dithane 75DF 6 lb	@	@	@
Mixtures	Kasumin 2L 100 ppm + Dithane 6 lb	@	@	@
	Kasumin 2L 100 ppm + Syllit 2 pts	@	@	@
	Kasumin 100 ppm + Streptomycin 100 ppm	@	@	@
Rotation of mixtures	Mycoshield 200 ppm + Syllit 2 pts	@		
	Kasumin 2L 100 ppm + Dithane 6 lb		@	
	Streptomycin 100 ppm + Dithane 6 lb			@



Kasugamycin demonstrated very good efficacy over several years at several locations.

Efficacy against fire blight caused by *E. amylovora* resistant to streptomycin and oxytetracyline
Small-scale field test on Asian pear 2009

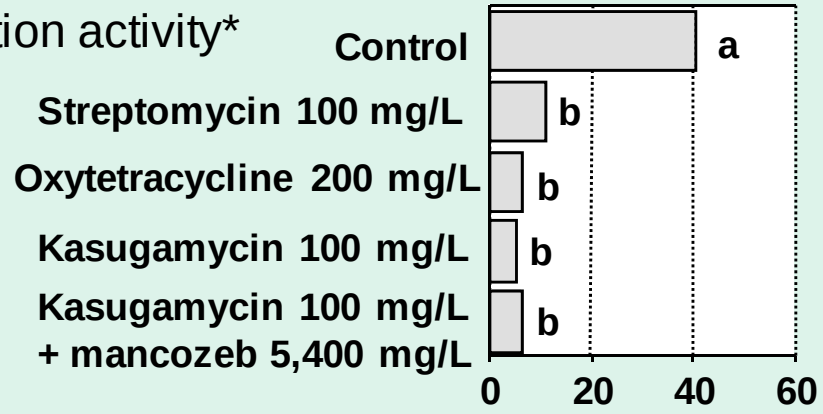


Inoculations 1 h after treatment.

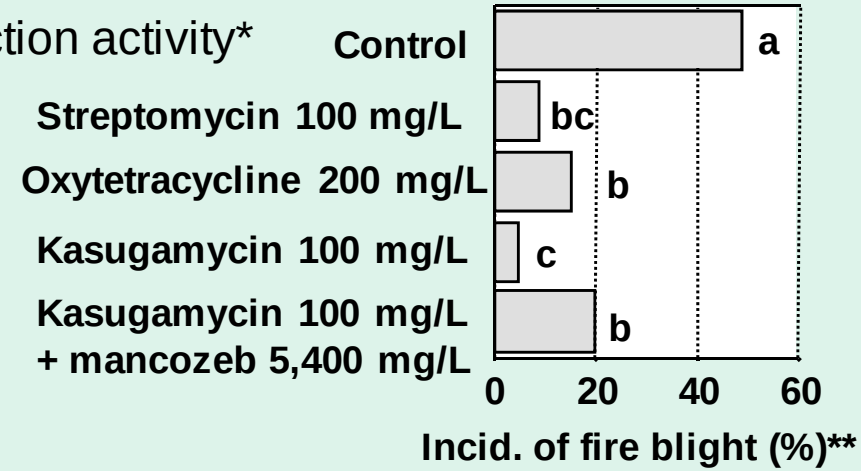
- Kasugamycin was effective against fire blight caused by isolates of *E. amylovora* that were streptomycin/oxytetracycline-sensitive or -resistant.

Pre- and Post-infection activity on Bartlett pear
Small-scale field tests - 2010

A. Pre-Infection activity*



B. Post-infection activity*



* - Treatments were applied to run-off using a hand sprayer either 1 or 2 h before (i.e., pre-infection activity) or 16 h after (i.e., post-infection activity) inoculation with *Erwinia amylovora* (1×10^6 cfu/ml).

** - Disease was evaluated as the incidence of blighted flowers per 20 to 50 flowers for each of the four replications after 10 to14 days. Data were combined for the two cultivars after testing for homogeneity of variance.

The three antibiotics all demonstrated pre- and post-infection activity when treatments were applied 16 h after inoculation.

Residue levels of kasugamycin after field treatments with Kasumin in IR-4 studies 2006

Crop	No. of studies	Kasugamycin residue	Suggested MRL
Pear	12	0.04 - 0.17 ppm	0.3 ppm
Apple	12	0.014 - 0.029 ppm	0.05 ppm

- 6 applications with 100 ppm Kasumin were done between bloom and up to 30 days before harvest.
- Tolerances were calculated by the EPA MRL calculator using EU Method 1.
- Import tolerances have been previously established in many countries around the world.

Summary: Efficacy of kasugamycin

- Kasugamycin efficacy equivalent or better than terramycin or streptomycin.
- Kasugamycin effective against blight caused by strep/oxy-resistant isolates of *E. amylovora*.
- Kasugamycin, oxytetracycline, and streptomycin showed very good post-infection activity (treatments applied 16 h after inoculation).
- Mixture treatments of kasugamycin with EBDC or other fungicides need to be further evaluated. This could be part of a resistance management strategy.
- No phytotoxicity was observed after 3 applications with Kasumin.
- Submission of a pome fruit registration package by IR-4 to EPA was done in 2010 and registration is expected for 2011.

Conclusion: Kasugamycin

- The most promising new bactericide for control of fire blight
- This aminoglycoside antibiotic is not used in medicine
- Antifungal and antibacterial activity
  (Also effective against scab)
- Different mode of action from other antibiotics
- Registered on crops in Asia, Europe, & Central America
- US-EPA approved an import tolerance in 2005
- US-EPA reviewing 2010 submission for 2011 registration

Overall Summary

Etiology: Molecular detection/identification routinely possible

Resistance:
- Ongoing problem with streptomycin (widespread)
- Potentially a problem for terramycin (limited distrib.)
- Not present with kasugamycin (no cross resistance but may still develop with over-usage)

New Bactericides:
- Kasugamycin – First new antibiotic in US agriculture in 40 years
- Some post-infection activity and persistence
- Effective for managing fire blight caused by strep.- or terramycin-resistant strains of the pathogen
- Effective in mixtures with selected fungicides
- New formulations of copper have promise in minimizing injury and being effective
- New biologicals may offer integrated control!