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From: Barbour, Darryl [Darryl.Barbour@biosecurity.gov.au]
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Please find attached a copy of the reference "Hale and Taylor 1999" as discussed during the Senate Estimates hearing on 24 May (specifically Senator Back's comments). We agreed to follow up and provide a copy.

The reference (Acta Horticulturae Volume 489) is on the bottom of page 139 and is as listed in the May 2011 draft report.

Regards
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Hale, C.N. and Taylor, R.K. (1999)

Hale, C.N. and Taylor, R.K. (1999)
Effect of cool storage on survival of Erwinia amylovora in apple calyxes. Acta Horticulturae 489: 139-143.

EFFECT OF COOL STORAGE ON SURVIVAL OF *ERWINIA AMYLOVORA* IN APPLE CALYXES

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Abstract

Coolstorage of mature, export quality apples (cv. Gala) in either the laboratory (0°C ± 0.5°C) or a commercial packhouse (2°C ± 0.5°C) for a period of 25 days reduced the survival of *Erwinia amylovora* in calyxes of both inoculated and naturally infested fruit. Populations of *E. amylovora* did not increase to levels detectable by PCR in fruit which were coolstored and then incubated at room temperature (c. 20°C) for 14 days to simulate likely retail conditions. The results provide evidence that coolstored, mature, export quality fruit are unlikely to be a vector of *E. amylovora*. These same commercial coolstorage conditions required for codling moth disinfestation of apple exports from New Zealand to Japan provide assurance that fruit, either from orchards free of fire blight symptoms or with a low incidence of the disease, will not provide an inoculum level likely to cause new outbreaks in previously blight-free areas.

1. Introduction.

The potential for spread of *Erwinia amylovora* and fire blight via commercial apple fruit has been critically reviewed by Roberts *et al.* (1998) and shown to be extremely low.

Apple fruit exported to Japan from New Zealand is coolstored after harvest as a requirement for codling moth disinfestation. Certified inspections of designated export areas for freedom from fire blight symptoms throughout the growing season and at harvest complete the export requirements for that market.

The research reported here investigates the effects on the survival and multiplication of *E. amylovora* in broth or saline suspensions, and in calyxes of mature apple fruit after either coolstorage or coolstorage and incubation to simulate conditions likely to be encountered during export and retail.

2. Materials and methods

2.1. Culture used

E. amylovora ICMP*1501 isolated from Malus x domestica ex. Auckland, New Zealand.

* International Collection of Micro-organisms from Plants, Manaaki Whenua Landcare Research New Zealand Ltd., Mt Albert Research Centre, Auckland, New Zealand.

2.2. Inoculated apples

2.2.1. Coolstorage and incubation in laboratory conditions

Calyxes of 180 mature, export-quality apples (cv. Gala) were inoculated with 0.1ml suspension of *E. amylovora* containing 10^7, 10^5, 10^4, 10^3, 10^2, 10^1 or 10^0 colony forming

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units (cfu). A further 180 apples were inoculated with 0.1ml bacteriological saline (0.85%$^{w}/_{v}$ NaCl) and used as controls. Fruit were either coolstored (0°C ± 0.5°C) for 25 days or coolstored for 25 days and incubated at room temperature (c.20°C) for a further 14 days in laboratory conditions.

2.2.2. Coolstorage and incubation in commercial conditions

Calyxes of 90 mature, export-quality apples (cv. Gala) were inoculated with 0.1ml suspension of *E. amylovora* containing 10^7, 10^5, 10^3, 10^2, 10^1 cfu. A further 90 apples were inoculated with 0.1ml saline and uses as controls. Fruit were either coolstored (2°C ± 0.5°C) for 25 days or coolstored for 25 days and incubated at room temperature (c. 20°C) for a further 14 days in commercial conditions.

2.3. Orchard-run fruit

Mature apples (c.v Gala) were harvested from an orchard with fire blight symptoms (450 fruit) and from an orchard with no fire blight symptoms (150 fruit). After harvest fruit were either coolstored (2°C ± 0.5°C) for 25 days or coolstored for 25 days and incubated (c. 20°C) for a further 14 days in commercial conditions.

2.4. *Erwinia amylovora* suspensions

Suspensions of *E. amylovora* containing 10^0- 10^8 cfu/ml were prepared in either Luria-Bertani (LB) broth or saline and either coolstored (0°C ± 0.5°C) for 25 days or coolstored and incubated (c. 20°C) for a further 14 days in laboratory conditions.

2.5. Testing for *Erwinia amylovora*

Calyxes of inoculated fruit and orchard-run fruit, and LB broth and saline suspensions were tested for the presence of *E. amylovora* using a modification of the PCR-based technique described by Guilford *et al.* (1996) to enhance the sensitivity of detection. Tissue samples were macerated in 500µl of extraction buffer containing 4g NaCl, 0.2g KCl, 0.25ml Tween 20, 10g PVP, and 2g BSA per 500ml sterile distilled water. Suspensions were vortexed and supernatants transferred to 500µl (Miller and Schroth (1972)) broth and incubated in a shaking waterbath at 27°C for 18 hours. Samples (5µl) of the resultant suspensions were mixed in 0.2ml PCR tubes with 15µl of GeneReleaser™ to bind inhibitors of the PCR, vortexed for 10-20 seconds, heated in a microwave oven at full power for 5 minutes and incubated at 80°C for 5 minutes prior to PCR analysis.

The prepared samples and the *E. amylovora* suspensions were amplified using a Techne Genius thermocycler in 50µl reaction volumes containing 1.5mM $MgCl_2$, 50mM KCl, 10mM Tris HCl pH= 8.3, 200µm of each dNTP, 25pmol of each primer, 400ng/µl BSA, and 1.25 units of Amplitaq polymerase derived from *Thermus aquaticus*. Amplification involved 94°C for 3 minutes (1 cycle), 94°C for 20 seconds, 55°C for 20 seconds, 72°C for 1 minute (35 cycles) and a final extension cycle of 5 minutes at 72°C. Aliquots (20 µl) of the PCR were analysed on 2% $^{w}/_{v}$ agarose gels stained with ethidium bromide (Sambrook , 1989). Positive controls using *E. amylovora* ICMP 1501 and negative controls containing sterile distilled water, extraction buffer and GeneReleaser™ were included in each set of reactions.

Viability checks were made by streaking 0.1ml aliquots of tissue and bacterial suspensions used for PCR on CCT medium (Ishimaru *et al.*, 1984) and incubated for 18 hours at 26°C.

3. Results

3.1. Inoculated apples

3.1.1. Coolstorage and incubation in laboratory conditions (Table 1)

Immediately after inoculation *E. amylovora* was detected in 100% of fruit inoculated with 10^7, 10^5, 10^4, or 10^3 cfu, in 40% of fruit inoculated with 10^2 cfu, in 20% of fruit inoculated with 10^1 cfu, and in 1% of fruit inoculated with 10^0 cfu, but not in fruit inoculated with saline. *E. amylovora* was isolated from 100% of fruit inoculated with 10^7, 10^5, or 10^4 cfu, from 22% of fruit inoculated with 10^3 cfu, but not from any fruit inoculated with 10^2, 10^1 or 10^0 cfu, or saline.

After coolstorage *E. amylovora* was detected in 90% of fruit inoculated with 10^7 cfu, in 20% of fruit inoculated with 10^5 or 10^4 cfu, in <8% of fruit inoculated with 10^3, 10^2 or 10^1 cfu, but not in any fruit inoculated with 10^0 cfu or saline. *E. amylovora* was isolated from 75% of fruit inoculated with 10^7 cfu, from 10% of fruit inoculated with 10^5 or 10^4 cfu, but not from any other fruit.

After coolstorage and incubation *E. amylovora* was detected in 35% of fruit inoculated with 10^7 cfu, in 3% of fruit inoculated with 10^5 cfu, but not in any other fruit. *E. amylovora* was not isolated from any of the fruit tested.

3.1.2. Coolstorage and incubation in commercial conditions (Table 2).

Forty eight hours after inoculation *E. amylovora* was detected in 75% of fruit with 10^7 or 10^5 cfu, in 25% of fruit inoculated with 10^3 cfu, in 10% of fruit inoculated with 10^1 cfu, but not in fruit inoculated with saline. *E. amylovora* was isolated from 50% of fruit inoculated with 10^7 cfu, from 34% of fruit inoculated with 10^5 cfu, from 10% of fruit inoculated with 10^3 cfu, but not from fruit inoculated with 10^1 cfu or saline.

After coolstorage, *E. amylovora* was detected in 66% of fruit inoculated with 10^7 cfu, in 28% of fruit inoculated with 10^5 cfu, in 10% of fruit inoculated with 10^3 cfu, and in 3% of fruit inoculated with 10^1 cfu, but not in fruit inoculated with saline. *E. amylovora* was isolated from only 7% of fruit inoculated with 10^7 cfu, and not from any other fruit.

After coolstorage and incubation, *E. amylovora* was detected in 36% of fruit inoculated with 10^7 cfu, in only 6% of fruit inoculated with 10^5 cfu, but not in any other fruit. *E. amylovora* was isolated from only 3% of fruit inoculated with 10^7 or 10^5 cfu, but not from any other fruit.

3.2. Orchard-run fruit

3.2.1. Fruit from orchard with fire blight symptoms

E. amylovora was detected in 2% of fruit before coolstorage but not in any fruit after either coolstorage, or coolstorage and incubation. *E. amylovora* was not isolated from any fruit tested.

3.2.2. Fruit from orchard without fire blight symptoms

E. amylovora was neither detected in, nor isolated from any of the fruit tested before or after coolstorage or after coolstorage and incubation.

3.3. *Erwinia amylovora* suspensions (Table 3)

E. amylovora was detected in, and isolated from LB broth immediately after inoculation, and after both coolstorage and coolstorage and incubation. However, *E. amylovora* was only detected in, and isolated from all saline suspensions immediately

after inoculation, and only from coolstored and coolstored and incubated saline suspensions when the inoculation levels were ≥ 10^3 and 10^4 cfu respectively.

4. Discussion

Coolstorage for 25 days, both in the laboratory (0°C ± 0.5°C) and in a commercial packhouse (2°C ± 0.5°C), had a marked effect in reducing the survival of *E. amylovora* in the calyxes of inoculated and naturally-infested apples. Populations of *E. amylovora* did not increase to detectable levels when inoculated and naturally-infested fruit were incubated (c. 20°C) for a further 14 days after the coolstorage treatment. The survival of *E. amylovora* at low concentrations in nutrient broth but not in saline, given the same coolstorage and incubation treatments, suggests that the nutritional and environmental conditions in apple calyxes are not conducive to multiplication of the pathogen. The inability to detect *E. amylovora* in the coolstored and incubated fruit has yet to be adequately explained. However, bacterial inhibitors, such as phenolic compounds, may be released from the dried-up flower parts remaining in the calyx as a result of rehydration during coolstorage.

The commercial coolstorage conditions used in this research are those required for the codling moth disinfestation programme for the export of New Zealand apples to Japan. The results reported, using a sensitive PCR detection method for *E. amylovora*, suggest that these coolstorage conditions will also provide assurance that mature export quality fruit from orchards free of fire blight symptoms, or even with low levels of fire blight, may be exported with a negligible risk of causing the establishment of the disease.

Acknowledgements

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Table 1. Percentage of inoculated apple calyxes with *Erwinia amylovora* after coolstorage, or coolstorage and incubation in laboratory conditions.

	Time after inoculation and fruit treatment		
E. amylovora cfu	Nil	Coolstorage 25 days	Coolstorage 25 days Room Temp. 14 days
10^7	100	90	35
10^5	100	20	3
10^4	100	20	0
10^3	100	< 8	0
10^2	40	< 8	0
10^1	20	< 8	0
10^0	1	0	0
Saline	0	0	0

Table 2. Percentage of apple calyxes inoculated at harvest with *Erwinia amylovora* after coolstorage, or coolstorage and incubation in commercial conditions.

	Time after inoculation and fruit treatments		
E. amylovora cfu	At harvest	Coolstorage 25 days	Coolstorage 25 days Room Temp. 14 days
10^7	77	66	36
10^5	75	28	6
10^3	25	10	0
10^1	10	3	0
Saline	0	0	0

Table 3. Detection of *E. amylovora* in LB broth and saline suspensions after coolstorage, or coolstorage and incubation.

	Time after inoculation and treatment					
	Nil		Coolstorage 25 days		Coolstorage 25 days Room Temp. 14 days	
E. amylovora cfu	LB broth	Saline	LB broth	Saline	LB broth	Saline
10^8	+	+	+	+	+	+
10^6	+	+	+	+	+	+
10^4	+	+	+	+	+	+
10^3	+	+	+	+	+	-
10^2	+	+	+	-	+	-
10^1	+	+	+	-	+	-
10^0	+	+	+	-	+	-
Nil	-	-	-	-	-	-

+ = *E. amylovora* detected.

- = *E. amylovora* not detected.