The Replication of <u>Erwinia carotovora</u> in Natural Surface Water

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Abstract

Four strains of *Erwinia carotovora* were added to 150 ml samples of filtered (0.45um) surface water. Strains tested were: 1) *Eca* SKI blackleg strain; 2) *Eca* non-SKI snow strain; 3) *Ecc* non-XX1X rain strain; and 4) *Ecc* XX1X aerosol strain. Water samples tested were from the Rio Grande and South Platte Rivers (collected January, 1988) and ocean water alone and ocean water plus 1% air-dried sea-weed (collected February, 1987). Filtered deionized water was also included in the study. Each strain was inoculated into separate flasks containing the water sample. Populations were determined by spread plating aliquots at specific time intervals after inoculation. The study was not replicated.

Results showed that average populations (all strains combined) increased to a maximum of ca 20, 60 and 87 times the original population for South Platte, ocean water plus sea-weed and deionized water, respectively. Populations did not appear to increase in Rio Grande and ocean water alone.

When results for strains are compared (all surface water samples combined), strain 1 (*Eca* SKI blackleg strain) did not appear to replicate in any of the water samples. However, the three other strains tested showed maximum increases of ca 90, 20 and 29 times the initial population seeded into the water samples for strains 2, 3, and 4, respectively.

Introduction

Studies have shown that pathogenic *Erwinia carotovora* strains can be recovered from surface water samples. One key questions is whether *E. carotovora* strains are present as contaminants in the water or are actually inhabitants that can replicate under some environmental conditions (currently undefined). Therefore, a preliminary study was done to determine if replication of four *E. carotovora* strains could occur in four surface water samples.

Materials and Methods

Water samples used in the study were collected at Oceanside, California (ocean water) in February, 1987, the Rio Grande River (RG) at Del Norte, Colorado, in January, 1988, and the South Platte River (SP) near Platteville, Colorado, in January, 1988. Brown algae (sea-weed) was also collected in February, 1987, and placed in a separate container. Ocean water and brown algae samples were stored in the cold room at the Potato Virus Lab laboratory at 4°C in 10 l polyethylene jugs (Nalgene). The RG and SP samples were collected in pre-autoclaved polypropylene 1 gal jugs.

All water samples were filtered through Gelman #GN-6 (0.45 um) pre-sterilized membrane filters. The filtrate (150 ml) was placed into each of 4 pre-autoclaved erlenmeyer flasks capped with sterile aluminum foil. Four flasks of filtered deionized water were also included in the study. An additional 4 flasks of ocean

water were included in the study and air-dried sea-weed (dried several days at room temperature in an exhaust hood), previously ground in a surface disinfested mortar and pestle, was added to the water at a rate of 1% (1.5 g per flask). The flasks were placed on a reciprocating shaker table (146 RPM) and incubated at room temperature (ca 24-28C estimated) during the course of the study. Aliquots (0.2 ml per SMP plate, 1 plate per sample per time interval) were spread plated to determine E. carotovora cfu/ml present after inoculation. Assay sensitivity was ca 1.25 cfu/ml. Time intervals tested were immediately prior to and after inoculation and also at post-inoculation times of 1, 2, 4, 8, 16, 32, 72, 126.5 and 336.5 hr. The last two time intervals tested were rounded to 127 and 337 hr, respectively, and are listed as such in the data tables. Inoculum was grown on 4 NA slants per strain at 26C. Slants were streaked on January 12, 1988, and cells were removed by washing on January 14, 1988, with sterile cold deionized water. Absorptance values were determined and concentrations (cfu/ml) calculated using a Bausch and Lomb Spectronic 20, Model 340, as outlined by Aleck (Ph.D. Thesis). Calculations were made to determine cfu/ml and $7.5x10^5$ cfu were removed and placed in 500 ml of sterile cold deionized water to give a final concentration of $1.5x10^3$ cfu/ml. Cells were "rested" at this concentration at 4.5°C for ca 15.75 hr to expend endogenous metabolites. This "rested" suspension was used to inoculate the flasks (1 ml inoculum used/flask) to give a target population of 10 cfu/ml. The four strains used were: 1) IPM649 Eca blackleg (SKI); 2) G424 Eca snow strain (non-SKI); 3) Oregon 057-3 Ecc rain strain (non XX1X) and 4) Oregon OA-2 Ecc aerosol strain (CC501 = XX1X).

Results

The results are shown in Table 1. When comparisons are made for flasks containing river water (all strains combined), the starting populations (0 hr) showed that an average of 40 cfu/ml (range 20-65 cfu/ml) was seeded into the flasks containing Rio Grande River water for the four strains tested. Average populations started to decline between 1 hr and 2 hr and were undetectable (assay sensitivity ca 1.25 cfu/ml) at 127 hr. Data for the South Platte River water showed that the average populations ranged from 27.5-43.8 cfu/ml during the first 8 hr and increased to 536.2 cfu/ml at 16 hr after inoculation. Populations in the South Platte river water were undetectable at 127 hr after inoculation. However, the population increase that occurred between 8 hr and 16 hr show that replication of Erwinia is possible in South Platte River water to ca 19 times the original population at 16 hr after inoculation.

Data for ocean water samples showed that average populations for the strains rapidly decreased and were undetectable at 16 hr after inoculation. However, when 1% air-dried sea-weed was added to the ocean water sample, average populations were not only present at 16 hr, but had increased to an estimated 2562 cfu/ml by 32 hr after inoculation which was ca 60 times the original average population of 42.5 cfu/ml.

Data for deionized water also showed an average population increase. The increase appeared to start at ca 8 hr and populations were similar to those for the ocean water plus sea-weed at 32 hr with an average of 2501 cfu/ml. However, populations decreased more quickly in the deionized water and were undetectable at 127 hr. In contrast, viable cells (for strain #4) could still be detected in ocean water plus sea-weed at 337 hr after inoculation.

When data (excluding data for deionized water) for strains are compared, the Eca SKI blackleg strain (strain #1) did not appear to replicate in natural water and was undetectable after 72 hr. In contrast, the Eca non-SKI snow strain (strain #2) showed an increase in numbers at 32 hr and 72 hr. However, populations were undetectable at 127 hr.

The Ecc non-XX1X (strain #3) rain strain also showed a large increase in populations at 32 hr but declined sharply by 72 hr and were undetectable at 127 hr. The Ecc XX1X strain (strain #4) showed an increase in cell numbers by 16 hr and a population peak at 32 hr. However, populations persisted and viable cells were still detected at 337 hr after inoculation.

Table 1. Replication of four _Erwinia carotovora_ strains in natural surface water and deionized water. G. D. Franc, January, 1988.

		Time Interval Tested (hr) and Estimated Average CFU/ml									
Water Source	Erwinia Strain[1]	0	1	2	4	8	16	32	72	127	337
Combined[2]	1	26.3	43.8	31.3	11.3	16.3	21.3	30.0	0.0	0.0	0.0
Combined[2]	2	20.0	7.5	13.8	7.5	6.3	85.0	623.7	1791.0	0.0	0.0
Combined[2]	3	45.0	32.5	35.0	28.8	31.3	71.3	862.5	25.0	0.0	0.0
Combined[2]	4	53.8	57.5	43.8	32.5	30.0	423.7	1532.0	207.5	7.5	3.8
Rio Grande	Combined	40.0	42.5	30.0	25.0	18.8	21.3	5.0	1.3	0.0	ND
South Platte	Combined	27.5	35.0	40.0	31.3	43.8	536.2	481.2	103.7	0.0	ND
Ocean	Combined	35.0	30.0	30.0	7.5	3.8	0.0	0.0	0.0	0.0	0.0
Ocean + Sea-Weed	Combined	42.5	33.8	23.8	16.3	17.5	43.8	2562.0	1918.0	7.5	3.8
Deionized	Combined	28.8	31.3	41.3	21.3	23.8	96.3	2501.0	378.7	0.0	ND

[1] 1 = _Eca_ SKI; 2 = _Eca_ non-SKI; 3 = _Ecc_ non-XX1X; 4 = _Ecc_ XX1X.

[2] Averages are for surface water only and do not include data for deionized water.

POTATO SEED-CUTTER TEST RESULTS FOR TRANSMISSION OF TUBER-BORNE PATHOGENS

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ABSTRACT

A recently designed seed-cutter, that allows for both chemical and heat disinfestation of the blade between tuber cuts, was tested for transmission of tuber-borne pathogens. Pathogens tested were *Erwinia carotovora* (blackleg and soft-rot), *Corynebacterium sepedonicum* (ringrot) and potato virus X (PVX). Results showed that the heating exposure time during one cycle of the blade had no measurable effect on *C. sepedonicum* populations and only reduced initial *E. carotovora* populations by an estimated 17%. However, Abscind™ reduced populations of both pathogens to undetectable levels during one cycle of the blade. The effectiveness of heat can be increased by prolonging exposure time or by increasing the temperature of the heating unit. Additional tests showed that 10% Clorox (a.i. sodium hypochlorite) may be faster acting than Abscind™ under certain conditions.

INTRODUCTION

A range of plant pathogens are known to be transmitted by the cutting knife during normal potato seed-cutting operations. Some of the pathogens known to be transmitted are *Erwinia carotovora* (the blackleg and soft-rot organism), *Corynebacterium sepedonicum* (the ringrot organism) and Potato Viruses S (PVS) and X (PVX). The recent design of a seed-cutter that allows both chemical and heat disinfestation of the blade between tuber cuts has potential for minimizing the spread of tuber-borne disease. The primary objective of this study was to determine the relative efficacy of the two disinfestation methods for eradication of pathogens from the blade.

Different pathogens may require different treatments to kill or inactivate them and the effectiveness of the various treatments will change when environmental conditions change. In addition, each pathogen has its own special requirements for detection. With these limitations in mind, the work described in this report used several organisms commonly encountered during the seed-cutting operation and tests were made under a limited range of conditions. The organisms studied were *E. carotovora* subsp. *carotovora* (*Ecc*), *E. carotovora* subsp. *atroseptica* (*Eca*), PVX and *C. sepedonicum*. All tests on the Varner cutter were done on September 26, 1987.

MATERIALS AND METHODS

Inoculum preparation and assays. Inoculum for Ecc and Eca was prepared by inoculating individual tubers (provided by John Salazar) with pure cultures of Ecc or Eca several days before the test. On the day of the test, soft-rotted tissue was scraped from tubers and placed in a beaker. Approximately equal volumes of decayed tuber tissue from Ecc- and Eca-inoculated tubers was used. This inoculum was applied to knife surfaces with a paint brush. After treatments were completed, a known surface area (3.629 cm^2 for "normal" studies, 4.839 cm^2 for "death curve" studies) of the blade was swabbed with a sterile cotton swab which was then placed into 5 ml sterile deionized water. Serial dilutions were prepared from this suspension. Assays for Ecc and Eca were done by spread plating 0.1 ml on Stewart's MacConkey pectate medium and the number of colony forming units (CFU) was determined after 48 hr incubation at 26°C.

Ringrot infected tubers were harvested from infected plants after the growing season. Inoculum was squeezed from the vascular tissue of infected tubers and placed in a beaker. Inoculum was applied to knives with the aid of a paint brush. After treatments were applied, a known surface area (4.839 cm^2, 0.750 in^2) was swabbed with a sterile cotton swab and placed into 5 ml sterile deionized water. One ten-fold serial dilution was prepared from this suspension. The suspensions were used to inoculate eggplants, an indicator host (bioassay). Eggplants were observed for up to 40 days after inoculation to determine if and when symptoms developed.

PVX inoculum was applied directly to the knives by cutting through infected tubers. Tubers were previously determined to be infected by the enzyme-linked-immunosorbent assay (ELISA). Tubers were also used to inoculate leaves of *Gomphrena globosa* plants as a positive check of the bioassay used. After treatments were applied, a known surface area (3.629 cm^2, 0.563 in^2) was wiped with a sterile cotton swab and placed into 0.25 ml sterile distilled water. This suspension was rubbed onto leaves of *G. globosa* plants previously dusted with carborundum. Plants were observed for the development of local lesions characteristic for PVX.

Treatments tested. Ecc and Eca were tested under "normal" operating conditions of the Varner seedpiece cutter. Under these conditions, the blade required 65 sec to make one cycle (1 revolution). Once a tuber was cut by the knife (the beginning of the cycle; i.e., the time of blade contamination) it required 22 seconds to reach the heating unit; during this time interval, it was exposed to the chemical disinfectant. Once the knife entered the heating unit, it took 6 seconds to pass through; during this time interval, it was exposed to 260°C (500°F) (according to the thermostat setting). After exiting the heating unit, it took 37 seconds for that portion of the blade to complete the cycle and cut the next tuber.

Treatments tested were: 1) Ecc/Eca inoculum (1:1) alone with no chemical or heat; 2) Ecc/Eca inoculum + Abscind™ (1:1:10) alone; 3) Ecc/Eca inoculum + Abscind™ + heat (260°C=500°F) and 4) inoculum + heat alone. All assays were done 65 seconds after inoculating the blade. PVX treatments tested were the same as those described above and were done simultaneously on one of the blades parallel to that used for the *Erwinia* tests. Four

replications of each treatment were done. Scraping pads were in place on the knives for all treatments.

It was not possible to test for the survival of C. sepedonicum (ringrot) directly on the Varner cutter knives because of the risks of contamination. Instead, 18" pre-cut sections of blades were swabbed with inoculum and assayed 65 seconds later. For treatments requiring heat, the blade section was placed in the heater for 6 seconds to simulate the normal cutting operation. The same treatments and number of replications were used as for Erwinia and PVX assays. However, blade sections were not passed through the scraping pads.

"Death curve" studies were done to determine how effective the heating and chemical disinfestation steps were for elimination of Erwinia. Time intervals tested for survival of Erwinia exposed to Abscind™, 10% Clorox and water (positive check) were 0, 1, 5 and 10 minutes. Time intervals tested for exposure to heat (260°C=500°F) were 0, 10, 20 and 40 seconds. Heat treatments tested were: 1) inoculum alone and 2) inoculum + misting the blade to run-off with sterile deionized water. This second treatment was done to determine if disinfestation by heat was less effective if blades were previously wet from chemical disinfectants since continuous wetting of the blade prior to heat would occur during normal operation of the Varner cutter.

Data for Erwinia assays are expressed as the $\log_{10}$ (total CFU present in assay volume)/(surface area assayed). Death curve studies were expressed the same way, except for one additional step; data are plotted as the percentage of the original population (i.e., average of time = 0 data points) remaining. Data for death curve studies were plotted using "Lotus" and predicted values for treatments were plotted using the "/data/regression" function.

Data for ringrot studies are expressed as the percentage of inoculated plants developing symptoms and the average number of days required for symptom expression in the greenhouse.

RESULTS

The effect of disinfestation treatments on the survival of C. sepedonicum, E. carotovora and PVX is shown in Table 1. PVX was not recovered from any of the treatments even though inoculation of G. globosa by rubbing tubers directly on the leaves showed that tubers were infected and assay plants developed the characteristic local lesions. Therefore, it was not possible to make any conclusions regarding this pathogen.

Data for the survival of the ringrot pathogen (Table 1) showed that "heat" (treatment 4) is equivalent to "no heat" (treatment 1). However, exposure to Abscind™ (treatment 2) reduced numbers to undetectable levels during one cycle of the blade (65 seconds). Similar trends are seen for the Erwinia data. Exposure to the chemical disinfectant (treatment 2) reduced numbers to undetectable levels while "heat alone" (treatment 4) still had detectable levels of Erwinia present. However, "heat alone" reduced Erwinia

populations by ca. 56% relative to treatment 1 ("no heat") in contrast to ringrot assays where no reduction, and actually an increase in the percentage of plants infected, was observed.

Results for "chemical death curve" studies are shown in Figure 1. All counts for 10% Clorox exposure were 0 CFU, even for assays done immediately after applying the treatment. Since the same inoculum was used throughout the study and living cells were easily recovered for treatments in which water or Abscind™ was applied, the data suggest that 10% Clorox was the fastest treatment for reducing Erwinia populations. Although Abscind™ was not as fast as 10% Clorox, it still rapidly reduced populations under the conditions of the test (see "Abs.Pred" line in Figure 1). As expected, the water treatment showed little reduction in populations during the 10 minute test ("Wat.Pred" line in Figure 1). Because of the small sample size and variation in the data, the correlation coefficient for Abscind™ treatment was not significant ($p>0.05$).

Data for death of Erwinia after exposure to heat for 0-40 seconds are shown in Figure 2. The lines for the dry blade ("Dry Pred.") and the wet blade ("Wet Pred.") are nearly identical and have the same approximate slope. This suggests that the effect of the heat is not noticeably affected by the extra wetting of the blade during normal cutting operations. The correlation coefficients (r) for both regression lines are highly significant ($p\leq0.01$) showing that as heating time increases, populations decrease at a linear rate.

DISCUSSION

Assay results for phytopathogens should always be interpreted cautiously. Assays that fail to detect the presence of pathogens do not prove that pathogens are not present. Rather, they may be present in numbers too small to detect, on the average, by the assay method used. For example, the estimated assay sensitivity for E. carotovora detection in Table 1 is ca. 0.302 (scale $\log_{10}$ (CFU)/cm^2) or ca. 3.44 colony forming units per cm^2. It is much more difficult to estimate the assay sensitivity of the ringrot assays used in this study because many variables are involved and most are poorly defined.

The results do show that the chemical disinfectants, Abscind™ or Clorox (10%), sharply reduced both E. carotovora and C. sepedonicum populations under "normal" operating conditions of the Varner cutter. Death curve results show that Clorox was faster in terms of eliminating Erwinia. However, the active ingredient in Clorox (sodium hypochlorite) is readily inactivated by organic matter and debris and would lose effectiveness if blades were allowed to become dirty.

The heat exposure during the normal cutting operation (6 seconds) had no measurable effect on C. sepedonicum (ringrot) and reduced the Erwinia population by only 16.6% during the same time interval (calculated using the equation for the "Wet Pred" regression line in Figure 2 where $y = (-2.473)(x) + 89.28$). Although the reduction of Erwinia counts is good, it may be simultaneously decreasing the contact time with the chemical due to its drying effect. It may be best to delay heating the blade until immediately prior to completing one cycle of the blade to maximize the

wetting and contact time with the chemical disinfectant. Increasing the air temperature, heating time and/or more efficiently increasing the temperature of the blade will probably be necessary to justify the presence of the heating unit in terms of disease control. More work is needed to determine the best combination of heat and chemical disinfestation.

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Table 1. The effect of disinfestation treatments on the survival of C. sepedonicum, E. carotovora and PVX under "normal" cutting operations.
G. D. Franc, CSU, 1987.

Treatment[1]	Ringrot/Eggplant Results[2] % with symptoms	Ringrot/Eggplant Results[2] Average # days for symptom develop.	Erwinia carotovora (blackleg)[3] Average $Log_{10}(CFU)/cm^2$	PVX[4]
1. Inoculum alone	85%	21	1.56	NA
2. Inoculum + Abscind™	0%	>40[5]	0.00	NA
3. Inoculum + Abscind™ + Heat	0%	>40[5]	0.00	NA
4. Inoculum + Heat	100%	18	0.68	NA

[1] Abscind™ was prepared at 1:1:10 (base:activator:water). Exposure to heat was at 260°C (500°F) for 6 seconds.

[2] Each entry represents the average of 4 replications of 12 eggplants each.

[3] Each datum entry is the average of 4 replications. Assay sensitivity is ≤ 0.302.

[4] None of the inoculated Gomphrena globosa developed local lesions except for the positive checks.

[5] Maximum time allowed for symptom development was 40 days. Therefore, symptoms never developed for these treatments. If C. sepedonicum was present, it was undetectable by the methods used.

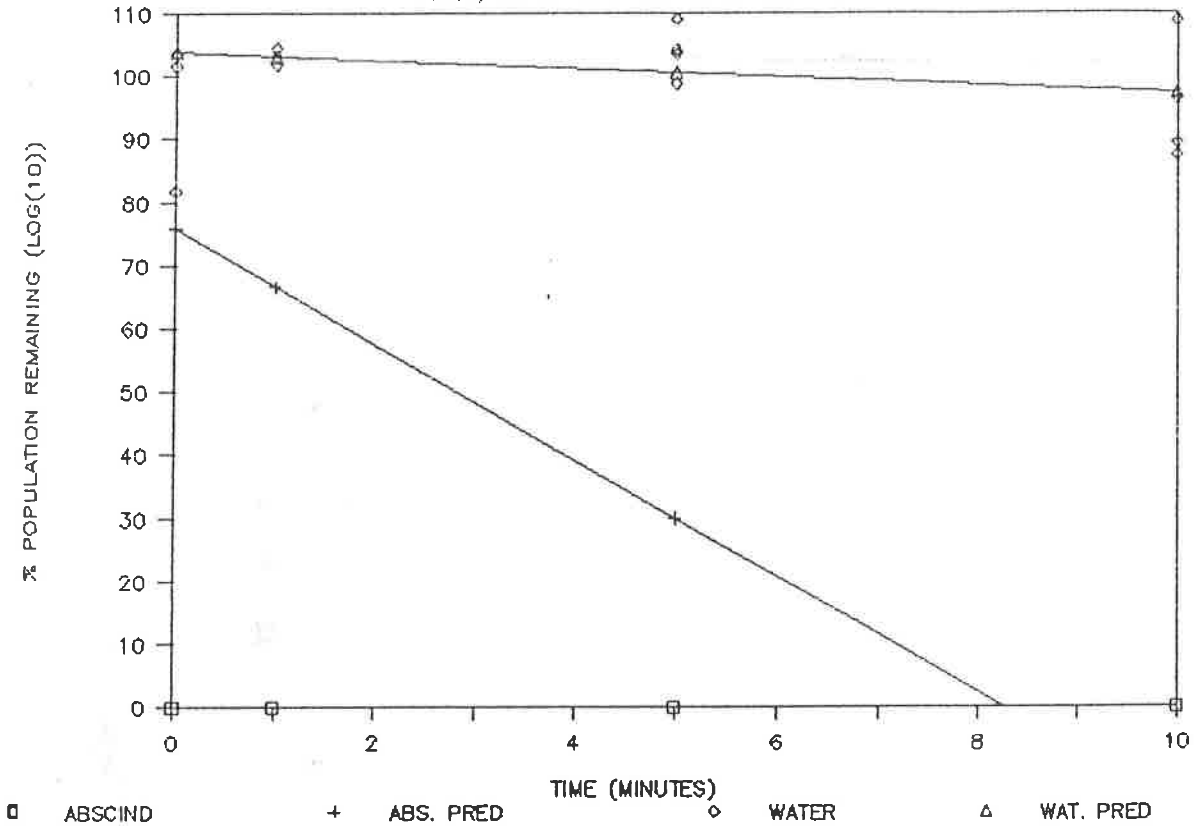

Figure 1. The change in populations of Ecc/Eca ($\log_{10}$(CFU)/cm^2) per unit time after exposure to water, Abscind™ and 10% Clorox. All assays for surfaces exposed to 10% Clorox failed to detect any living cells. The correlation coefficients (r) for the regression line for Abscind™ (Abs.Pred) and Water (Wat.Pred) are not significant ($p \ngtr 0.05$).

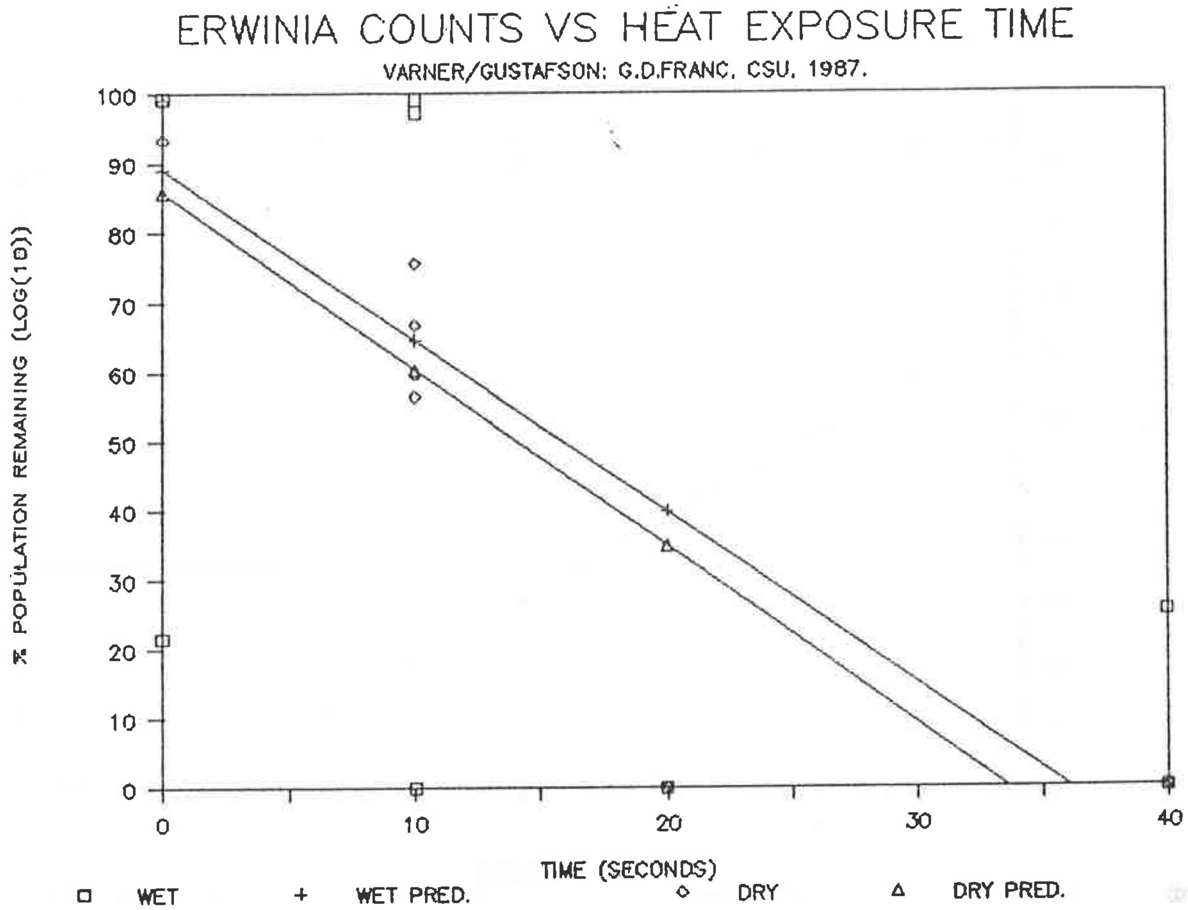

Figure 2. The change in populations of Ecc/Eca ($\log_{10}$(CFU)/cm^2) per unit time after exposure to heat (260°C = 500°F). The correlation coefficients (r) for the regression line for a "wet" blade (Wet.Pred) and a dry blade (Dry.Pred) are highly significant ($p \leq 0.01$).