



# Effect of UV - exposure on colony formation of *Xanthomonas fragariae* in vitro

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## Summary

The research described in this report was carried out by Plant Research International by request of Clean Light B.V. The aim of the work was to determine the dose response curve for deactivation of the plant pathogenic bacterium *Xanthomonas fragariae*, the causal organism of bacterial angular leaf spot in strawberry, on R2A agar by exposure to UV-c (254 nm). For this purpose, *X. fragariae* was plated on R2A agar plates and exposed to a range of UV-c dosages. The fraction of bacterial colony forming units (CFU's) surviving treatment was determined and used to calculate the ED<sub>50</sub> (the dose reducing viability of *X. fragariae* on R2A agar by 50%) and ED<sub>90</sub>.

It is concluded that *X. fragariae* is very sensitive to UV-c. Exponential decline functions were fitted to the data.

Estimates for the ED<sub>50</sub>, derived from the fitted exponential decline functions, range between 0.64 and 0.84 mJ/cm<sup>2</sup>.

The ED<sub>90</sub> is estimated in the range between 2.13 and 2.78 mJ/cm<sup>2</sup>.

# 1. Introduction and scope of the research

*Xanthomonas fragariae*, the causal organism of bacterial angular leaf spot of strawberry, is listed as a quarantine organism by the European Plant Protection Organisation (EPPO, A2 status) and by the European Union plant health legislation. It is a potentially serious and insidious disease, first reported in the USA (Kennedy and King, 1962).

*X. fragariae* causes a reduction in fruit yield. In continental Europe, losses of 10–20% in yield have been reported, though in systems where overhead irrigation is used, enabling the bacteria to spread and multiply on wet leaves, higher losses than this are thought to have occurred (Elphinstone, 2005).

Residues of infected leaves and crown infections on runners used for planting are sources of inoculum for primary infections. In the residues of infected leaves and rhizomes, in or on soil, the bacterium survives from one crop to the next. Bacterial cells are transferred from residues to young leaves at the beginning of the growing season. From crown infection pockets, the bacterium causes lesions along the veins at the base of the youngest leaves, which develop in the apical crown region.

The bacterium exudes from primary lesions, and bacterial cells are spread by aerosols, caused by rain and sprinkler irrigation, and by wind to healthy leaves. Penetration occurs through the stomata. Infections of the crowns occur through local wounds or downwards colonization from the affected leaves. During the growing season several cycles of secondary infections may occur. The bacterium attacks all plant parts except fruits and roots. However the calyx of the fruit can be infected too, showing black discoloration (Figure 1). Consequently the marketability of the fruits is reduced.

During epidemics, when environmental conditions favour exudation and spread, the bacterium may cause systemic infections associated with crown pockets. Systemic infections may arise under damp nursery conditions. The conditions favouring infection are moderate to cool daytime temperatures (about 20°C), low night-time temperatures and high humidity's (Maas, 1984; Kennedy & King, 1962; Hildebrand et al., 1967). The bacterium is spread locally by splash (rain and irrigation) and mechanical dispersal (machines, humans and animals moving through the crop). Commercial strawberry runners used for planting may spread the bacterium over short and long distances. They may still bear old, whole or torn, infected leaves or have crown infection pockets. Moreover, almost invisible fragments of infected leaves may be hidden in the apical crown region or between the roots (Kennedy & King, 1962a). Chemical control measures are not available, thus growers have to rely on the use of healthy planting material and avoidance of conditions favouring disease development for disease control. Furthermore a strict hygiene policy has to be followed to prevent the disease from entering the farm and avoid transmission of the bacterium between fields.

*Xanthomonas fragariae* holds a quarantine status in the Netherlands and the rest of Europe. Strawberry runners used for planting thus have to be free of the bacterium. When *X. fragariae* is found in Dutch production fields, harvesting may only continue under strictly controlled conditions. Following the harvest, the plant material has to be destroyed. When *X. fragariae* is found during the production of commercial propagation material, all commercial activities with this material are blocked and the complete crop must be destroyed.



Figure 1. Symptoms of angular leaf spot, caused by *Xanthomonas fragariae*, on leaves and the calyx of the fruit.

#### Scope of the Research

The research described in this report was carried out by Plant Research International in January 2008 by request of Clean Light B.V. as outlined in contract 08/PRI-0159. Clean Light (P.O.Box 271, 6700 AG Wageningen, the Netherlands, [www.cleanlight.nl](http://www.cleanlight.nl)) is the developer and owner of the UV Crop Protection Technology described in this report. Modification of the experimental procedure with respect to the range of UV dose rates applied was approved by Clean Light prior to both experiments. A 75 Watt UV source (Philips TUV 64T5 4P SE) was received from Clean Light and used for the experiments.

The aim of the work was to determine dose response curves for exposure of *X. fragariae* on R2A agar (a low nutrient medium for enumeration and cultivation of bacteria) to UV-c. *X. fragariae* was exposed to UV-c shortly after plating on R2A agar. Colony formation after incubation was the response variable of choice.

## 2. Materials and methods

The aim of the research was to determine the dose response curve for deactivation of *X. fragariae* on R2A agar by exposure to UV-c (254 nm). For this purpose, *X. fragariae* was plated on R2A agar plates at a density of approximately 100, 1000 or 10000 colony forming units per plate. These plates were exposed to a range of UV-c dosages from 0 to approximately 140 mJ/cm<sup>2</sup>. The fraction of bacterial colony forming units (CFU's) surviving treatment was determined and used to calculate the ED<sub>50</sub> en ED<sub>90</sub>.

A replicated experiment was carried out, independent cultures, suspensions and R2A petri plates were used for the separate experiments. Exposure to UV-c was carried out in the morning and afternoon of 18 January 2008 for experiment 1 and 2 respectively.

### Culturing, preparation of bacterial suspensions and plating

*Xanthomonas fragariae* Kennedy et King (Kennedy and King, 1962) isolate PD 4450, stored as pure culture in the PRI collection of plant pathogenic micro-organisms and originally isolated from infected strawberry, was cultured on Yeast Pepton Chalk (YPC) agar. Forty eight hours prior to the experiment, the bacteria was transferred to Yeast Pepton Glucose (YPG) agar to produce the cultures to be used in the experiment (Figure 2A). At the start of the experiment, bacterial suspensions are produced by suspending *X. fragariae* from YPG agar in ¼ strength Ringers solution (Figure 2B). 1x10<sup>5</sup>, 1x10<sup>6</sup> and 1x10<sup>7</sup> dilutions of this suspension were plated on R2A agar plates using a spiral plater (Figure 3A) to produce R2A agar plates containing approximately 10000, 1000 or 100 CFU/plate respectively on top of the agar. These plates were left alone for 15 minutes to allow the water from the bacterial suspensions to be absorbed by the agar before they were treated with UV-c (Figure 3B).

### UV-c treatment

A 75 Watt UV source (Philips TUV 64T5 4P SE) was received from Clean Light and used for the experiments. The fluorescent tube was enclosed in a quartz glass tube which in turn was surrounded by a Teflon sleeve as protective measures for field applications. The UV-c source was mounted in a Clean Air DLF/BSS 4 down flow at 64 cm from the work bench, switched on at least 30 minutes before the start of the experiment and switched off only after the experiment was finished. A sterile air flow from top to bottom guarantees a sterile work space inside the cabinet. A black cardboard box with a removable lid was placed inside the cabinet (Figure 3B). The front of this black box opened to the outside of the flow cabinet so that petri plates can be placed in the box without exposure to UV-c. In this environment, the UV-c dose rate the petri dishes were receiving was measured to be 0.49 mW/cm<sup>2</sup> using a UV-c sensor (Dr. Groebel RM-21). Prior to exposure, petri dish lids were removed and the petri dishes were placed inside the black box. Exposure started when the lid of the black box was removed and ended when it was placed back in position. *X. fragariae* on the agar surface in the petri plates was thus exposed to a range of twelve UV-c dose rates, 0, 1.0, 2.0, 3.9, 7.4, 14.7, 22.1, 29.4, 39.2, 68.6, 102.9 and 137.2 mJ/cm<sup>2</sup>. Following exposure, the petri plates were incubated for five days in the dark at 25°C. Following incubation, the bacterial colonies formed on the plates were quantified and the fraction of bacteria surviving UV-c exposure was calculated and analyzed statistically to determine the ED<sub>50</sub> and ED<sub>90</sub>.

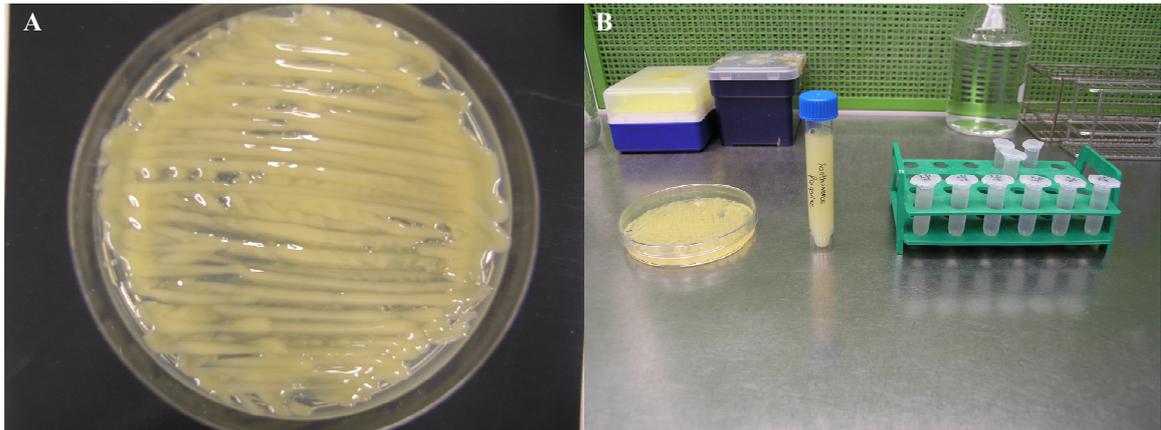


Figure 2. A: *X. fragariae* pure culture on YPG agar.  
B: Pure culture, bacterial suspension in  $\frac{1}{4}$  strength ringers solution and part of the dilution series.

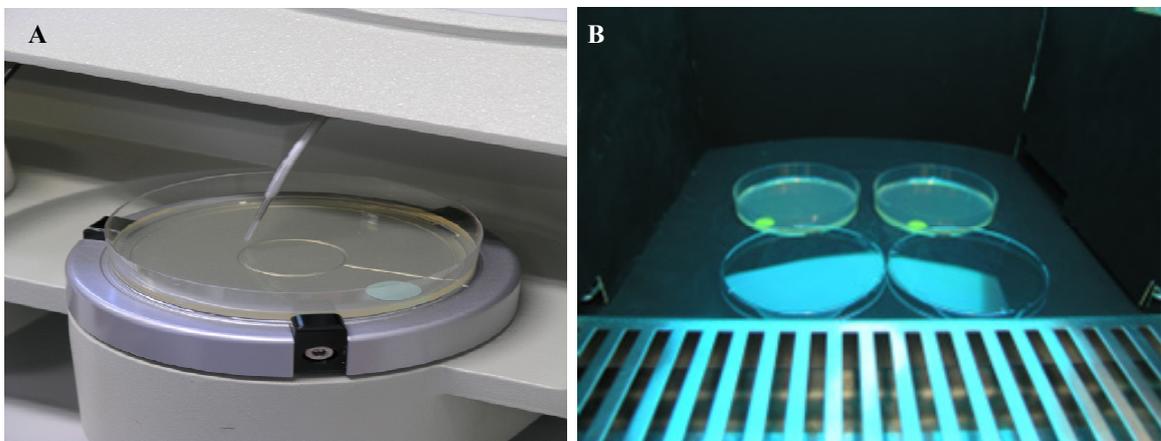


Figure 3. A: Spiral plater, plating the *X. fragariae* suspension on R2A agar.  
B: View of the experimental setup in the clean air downflow cabinet during illumination of *X. fragariae* on R2A agar plates.

## Statistical set up and data analysis

A completely randomized experiment with twelve UV-c dosages and four replicate petri plates per UV-c dosage was carried out. Exponential (decline) functions were fitted to data describing the % surviving CFU's, as determined from colony counts, in relation to the UV-c dose received. The general mathematical form of this exponential function is given in Equation 1. For the current dataset, 'y' represents the percentage *X. fragariae* CFU surviving UV-c treatment, 'b' represents the reference level of survival before exposure to UV-c (i.e. the point departure, approximately 100%), 'r' determines the steepness of the curve and 'dose' represents the dose of UV-c received in mJ/cm<sup>2</sup>. GenStat was used to obtain estimates of parameters b and r, including their corresponding standard error. No colonies formed on plates exposed to 7.4 mJ/cm<sup>2</sup> or more. To stabilize the variance with respect to the UV-c dose received and produce more reliable estimates for the standard errors accompanying the parameter estimates, all UV-c dosages greater than 15mJ/cm<sup>2</sup> were left out of the analysis. The experiment was carried out twice and an exponential decline function was fitted for each experiment separately and for the combined dataset from both experiments.

$$Y = b \cdot r^{\text{dose}}$$

Equation 1

### 3. Results

#### UV dose response curves

Colony counts were carried out five days after exposure of the petri plates. No new colonies formed in the week after the colony counts were completed. Results of experiments 1 and 2 are summarized in Figure 4. Figure 4A gives the individual data points from both experiments and the fitted exponential decline function for each separate experiment. Figure 4B gives the average survival per experiment and UV-c dose including a fitted exponential decline function over both experiments. The results of the statistical analysis are summarized in Table 1.

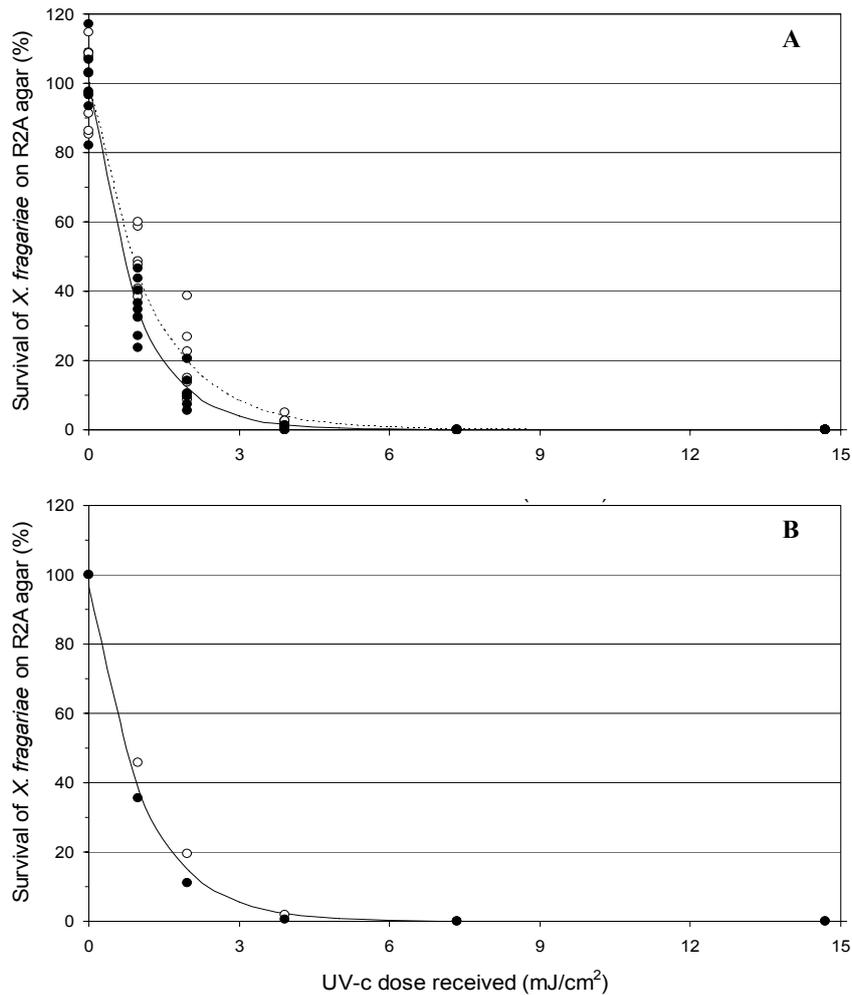


Figure 4. Dose response curves for exposure of *Xanthomonas fragariae* on R2A agar to UV-c (254 nm). A: Individual data points and fitted exponential decline functions per experiment. B: Average survival per UV-c dose and experiment and fitted exponential decline function over both experiments. ○: Experiment 1; ● Experiment 2.

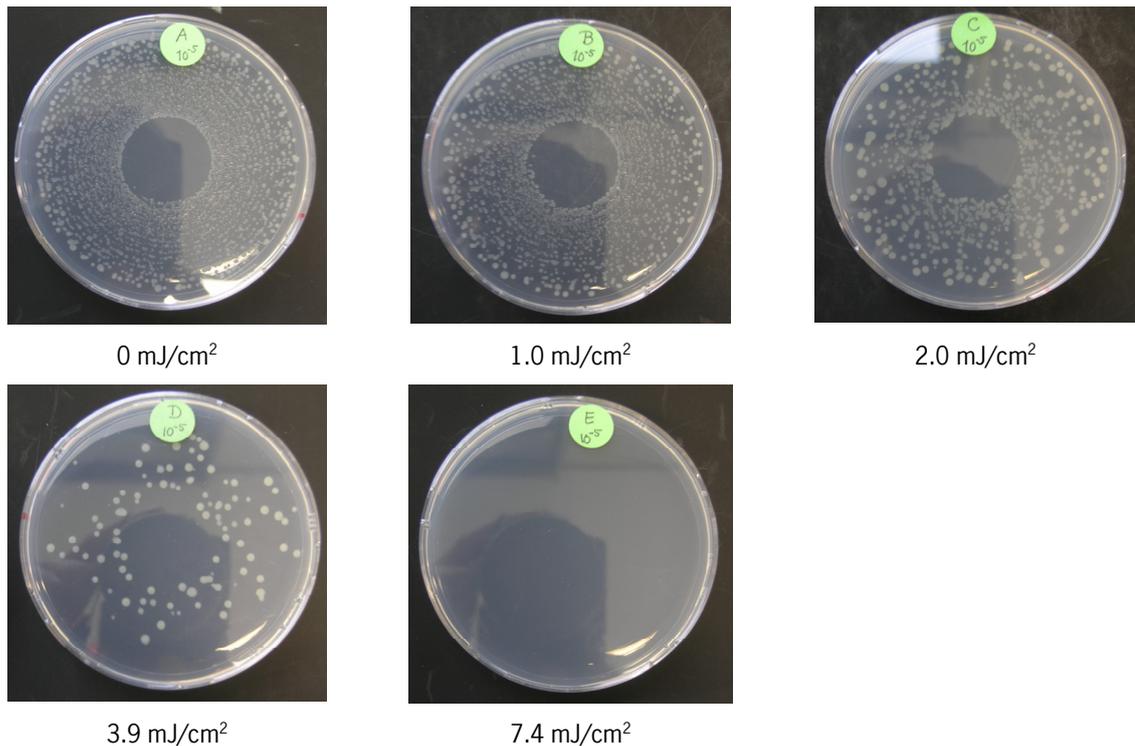


Figure 5. *X. fragariae* colonies on R2A agar following exposure to UV-c. *X. fragariae* was plated on R2A agar using a spiral plater and illuminated with a range of UV-c dosages shortly after plating. The resulting formation of colonies was seriously hampered by increasing dosages of UV-c and completely inhibited at or above 7.4 mJ/cm<sup>2</sup>.

From Table 1 and Figure 4 it can be concluded that *X. fragariae* is very sensitive to UV-c. The dose response curve is well described by an exponential decline function instead of e.g. the sigmoid shaped function that was used for *P. infestans* (Kessel & Forch 2006). Estimates for the ED<sub>50</sub> (the dose reducing viability of *X. fragariae* on R2A agar by 50%) range between 0.64 and 0.84 mJ/cm<sup>2</sup>. The ED<sub>90</sub> is estimated in the range between 2.13 and 2.78 mJ/cm<sup>2</sup>.

Table 1. Summary of the statistical analysis. Parameter estimates and corresponding standard errors (in brackets) following from fitting an exponential decline functions following the general shape of Equation 1.

	Estimate		% variance accounted for	Estimate	
	b	r		ED <sub>50</sub>	ED <sub>90</sub>
Experiment 1	100.38 (2.24)	0.4369 (0.0178)	96.6	0.84 (0.04)	2.78 (0.14)
Experiment 2	100.18 (1.70)	0.3387 (0.0146)	98.0	0.64 (0.03)	2.13 (0.08)
Experiment 1 + 2	96.68 (1.44)	0.3879 (0.0124)	96.9	0.73 (0.02)	2.43 (0.08)

## 4. Discussion

The aim of the research described in this report was to determine dose response curves for exposure of *Xanthomonas fragariae* on agar plates to UV-c (254 nm). For this purpose, *X. fragariae* was plated on R2A agar and illuminated shortly after plating. The effect of UV-c illumination was determined by quantifying the subsequent formation of colonies. The higher the UV-c dose received, the lower the number of colonies (Figure 4, Figure 5) and at or above 7.4 mJ/cm<sup>2</sup> no colonies formed at all. The effect of UV-c exposure on survival of *X. fragariae* on agar was best described by an exponential decline function. Using these fitted functions, the ED<sub>50</sub> was estimated between 0.6 and 0.8 mJ/cm<sup>2</sup>. The ED<sub>90</sub> was estimated between 2.1 and 2.8 mJ/cm<sup>2</sup>.

*X. fragariae* is thus highly sensitive to UV-c. The ED<sub>50</sub> and ED<sub>90</sub> reported fall within the range generally reported for bacteria exposed to UV-c. Comparison to earlier results with sporangia of *Phytophthora infestans*, for which the ED<sub>95</sub> for sporangial germination after UV-c illumination was estimated to be 6 – 8 mJ/cm<sup>2</sup> (Kessel & Forch 2006), learns that *X. fragariae* is much more sensitive than *P. infestans*.

During the current experiments *X. fragariae* was exposed to UV-c on an agar surface. The effect of this matrix, if any, on the experimental results is unknown. With respect to application of UV-c during strawberry cultivation, the current results suggest that low dosages of UV-c are sufficient to eradicate the bacterium from any surface exposed to UV-c. To bridge the gap between the current (laboratory) results and practical application, a logical next step would be to determine the stages of the infection process sensitive to UV-c. These experiments can be done under quarantine greenhouse conditions. The results allow determination of the window of opportunity for eradication of *X. fragariae* in strawberry crops. The final step in the development of an UV-c application against *X. fragariae* in strawberry would be a complete system test under practical conditions.

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