Procedures for Growth and Inoculation of *Xanthomonas fragariae*, Causal Organism of Angular Leaf Spot of Strawberry

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ABSTRACT

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Growth of *Xanthomonas fragariae*, the cause of strawberry leaf spot, was optimal in 0.8% nutrient broth, 0.5% casein hydrolysate, 0.1% yeast extract, 1% glucose, and minerals. Distilled, demineralized water and saline (0.85% NaCl) were satisfactory inoculum diluents. Spray infiltration was the most rapid, sensitive, and reliable method of leaf inoculation. The number of lesions increased linearly with inoculum titer and as leaf age decreased. The number of lesions per 0.8-cm^2 site ranged from 12.04 to 102.44.

Xanthomonas fragariae Kennedy and King 1962 is responsible for sporadic, occasionally serious outbreaks of angular leaf spot of strawberry. The disease has been recorded in Italy, France, New Zealand, Australia, and Greece and in several states, namely, Minnesota, California, Kentucky, Wisconsin, and Florida (2–5,7). Comparative data on optimal conditions for growth of *X. fragariae* in vitro, on inoculum preparation, and on inoculation techniques are not available. This information would be useful in strawberry breeding programs.

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MATERIALS AND METHODS

Bacterial cultures. Cultures of *X. fragariae* NCPPB 1822 (National Collection of Plant Pathogenic Bacteria, Plant Pathology Laboratory, Hatching Green, Harpenden, Hertfordshire, England) were grown for 5 days at 26–28 C on slants of medium B containing 1.5% agar. The cultures were stored at 4 C and transferred monthly.

Media. Several media were used. Basal medium (BM) contained 0.8% nutrient broth (Difco), 0.02% $Ca(NO_3)_2$, 0.001% $FeSO_4 \cdot H_2O$, and 0.001% $MnSO_4$ (8). Medium A (8) was BM containing 0.5% casein hydrolysate and 0.1% yeast extract. Medium B was medium A supplemented with 1% dextrose. Yeast-dextrose-calcium carbonate medium (YDC) contained 1% yeast extract, 2% dextrose, and 2% finely ground calcium carbonate (8). Nutrient broth (NB) contained 0.8% nutrient broth (Difco), 0.05% NaCl, and 0.02% dextrose. Medium D consisted of 16 g of nutrient broth (Difco), 0.05% NaCl, and 1% dextrose. Medium M9 (1) contained 2% casein hydrolysate, 2% dextrose, 0.2% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2$, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, and 0.1% NH_4Cl. All media were adjusted to pH 7.0 before being autoclaved.

In vitro growth studies. Each medium (50 ml in 250-ml flasks) was inoculated with approximately 2×10^5 CFU/ml of 72-hr (solid medium B, 28 C) *X. fragariae* NCPPB 1822 and incubated in shake culture at 27 C. Appropriate dilutions were plated on medium B at 0, 24, and 48 hr. Growth was also monitored by absorbance at 620 nm. A growth curve was constructed; the initial titer was 5.6×10^6 CFU/ml.

Inoculum diluents. Sterile distilled, demineralized water (sdd), phosphate buffer (6.7 g of Na_2HPO_4 and 3.3 g of KH_2PO_4 per liter, pH 7.0), physiological saline (8.5 g of NaCl per liter), and medium B were compared. Cells of *X. fragariae* NCPPB 1822 from 48-hr shake

cultures (medium B) were washed, resuspended, and adjusted to 10^5, 10^6, and 10^7 CFU/ml with each diluent. Fletcher strawberry leaves were inoculated with each inoculum concentration by spray infiltration. Two replicates of one plant each were inoculated. The number of lesions per site (10–18 sites per treatment) was determined 12–20 days after inoculation.

Inoculation techniques. Four methods were compared: midvein injection, wound inoculation, vacuum infiltration, and spray infiltration. Aqueous suspensions of *X. fragariae* NCPPB 1822 at about 10^8 CFU/ml were used for all methods. Inoculum was grown in medium B (shake culture) for 48–72 hr at 27 C and washed with sdd. Syringes with 26-gauge needles were used to inject strawberry leaflet midveins. Wound inoculation was done by rubbing inoculum mixed with Carborundum on the lower leaf surface. For vacuum infiltration, attached strawberry leaves were dipped into an aqueous suspension of *X. fragariae* cells containing Triton X-100 (1:10,000); the wet leaf was then exposed to a partial vacuum of 75 mm Hg for 3 min. An airbrush was used to spray inoculum onto the lower leaf surface at 1 kg/cm^2 and approximately 3 cm from the target for 2–4 sec or until slight water-soaking was evident. Complete water-soaking, indicated by a dark green color, often resulted in localized, nonspecific necrosis and poor development of symptoms. After inoculation, leaves were rinsed with water and incubated in the greenhouse (20–28 C with 10–12 hr of natural light per day) in a mist chamber until lesions were counted (2–3 wk).

Cultivar reactions. Washed cells of *X. fragariae* NCPPB 1822 from 24-hr medium B shake cultures were adjusted to 70% T_{620} ($3–4 \times 10^7$ CFU/ml) in sdd. Tenfold serial dilutions were made in sdd to obtain suspensions containing 10^6 and 10^5 CFU/ml. The 12 cultivars inoculated were Earliglow, Guardian, Sparkle, Stelemaster, Raritan, Midland, Fletcher, Surecrop, Blakemore, Suwannee, Badgerbelle, and Atlas. Inocula were applied by spray infiltration of an 0.8-cm^2 area exposed by a hole in an aluminum foil shield (6); each leaflet had 4–6 inoculation sites. Three ages of leaves were inoculated, the youngest being just fully expanded (2–4 wk old). Each leaflet of a trifoliolate leaf was inoculated with a different inoculum concentration. The experiment was done twice, using one plant per cultivar and two inoculum batches. After inoculation, leaves were rinsed with water and incubated as described previously.

The average number of lesions per site for each leaflet was obtained. The natural logs of these figures (LAVLC) were analyzed, and the antilogs were used. Because the coefficient of variation of the log data (21.6227) was less than that of the original data (42.6039), LAVLC data were more desirable.

RESULTS

Comparison of media. Growth of *X. fragariae* in vitro was slow, taking 24 hr to reach a concentration of 10^7–10^8 CFU/ml from an initial concentration of 2×10^5 CFU/ml (Table 1). This finding was consistent with published reports of *X. fragariae* growth on solid media (2,4,5,7). *X. fragariae* did not grow in medium D, as determined by viable cells or turbidity. In BM and M9 medium, the increase in viable cell number after 24 hr was about 100-fold. Only in BM did the CFU/ml increase significantly between 24 and 48 hr. Medium A and NB supported approximately a four-log increase in cell number after 48 hr. Medium B and YDC supported 3–5 times more viable cells at 24 hr and 5–7 times more cells at 48 hr than did medium A and NB. Calcium carbonate particles precluded the use of YDC medium as a broth when turbidity measurements were required. Medium B containing 1.5% agar was an excellent plating medium, with single colonies appearing after 6–7 days at 28 C; sealing the plates in plastic bags reduced this time to 3–5 days.

In growth of *X. fragariae* NCPPB 1822 in medium B, a long log phase of about 30 hr occurred after a lag of 5–10 hr (Fig. 1). The doubling time was 2 hr. Cell concentration ($1–3 \times 10^{10}$ CFU/ml) was maximum between 48 and 53 hr.

Inoculum diluents. At all three inoculum concentrations, the number of lesions was greatest when *X. fragariae* cells were resuspended in saline and sdd (Table 2). Only about 0.8–9% as many lesions developed with phosphate buffer as with sdd or saline. With medium B, 6–32% as many lesions developed as with sdd or saline.

Inoculation techniques. Midvein injection of inoculum caused variable water-soaking along major veins immediately after inoculation and confluent lesions after 7 days. No interveinal lesions developed. Wound inoculation caused no visible injury or symptoms. Vacuum infiltration produced many dispersed lesions but was time-consuming. Spray infiltration was fast and easy, and many sites on a single leaflet could be inoculated.

About 5 days after plants inoculated by spray infiltration were placed in a mist chamber, small (0.5 mm) water-soaked lesions (dark green with reflected light and translucent green with transmitted light) were evident on the lower leaf surface (Fig. 2). The average diameter of lesions was 1–2 mm on the oldest leaves and 4–5 mm on the youngest leaves.

Antilog LAVLC was directly related to inoculum concentration, especially on the oldest leaves (Table 3). The number of

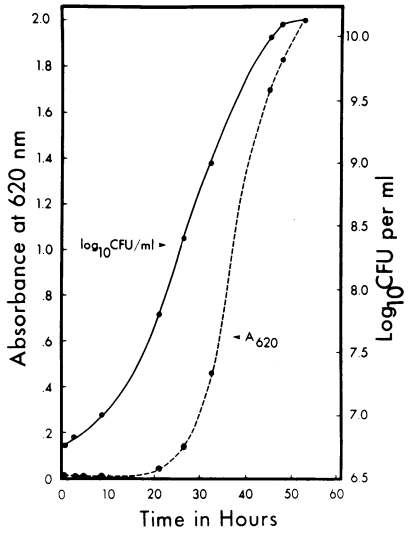

Fig. 1. Growth of *Xanthomonas fragariae* NCPPB 1822 in medium B at 27 C.

Table 1. Growth of *Xanthomonas fragarie* NCPPB 1822 in seven media in shake culture at 27 C

	24 hr[a]		48 hr	
Medium	**$\log_{10}$ CFU/ml**	**A_{620}**	**$\log_{10}$ CFU/ml**	**A_{620}**
B	8.097	0.1337	9.652	1.699
YDC	7.966	...[b]	9.615	...[b]
A	7.720	0.0969	9.101	0.432
NB	7.639	0.0888	8.890	0.409
BM	7.000	0.0110	8.373	0.0731
M9	6.914	0.0223	7.236	0.0434
D	5.000	0.0044	5.000	0.0066

[a] Initial titer of *X. fragariae* NCPPB 1822 = 2×10^5 CFU/ml; initial A_{620} = 0.0088.
[b] Readings not available because of calcium carbonate particles.

Table 2. Effect of inoculum diluent on number of lesions produced on Fletcher strawberry leaves by *Xanthomonas fragariae* NCPPB 1822

	Average number of lesions per site[a]		
Inoculum diluent	**10^7 CFU/ml**	**10^6 CFU/ml**	**10^5 CFU/ml**
Distilled H_2O	48.0	23.8	12.4
0.85% NaCl	64.8	36.3	8.2
0.064 M PO_4 buffer	4.5	0.7	0.1
Medium B	15.4	4.0	0.7

[a] Data are means of two tests, each consisting of 10–18 inoculation sites (0.8 cm^2) per treatment.

lesions per site was inversely related to leaf age, ie, significantly more lesions were produced on the youngest leaves (Table 3).

The reactions of 12 strawberry cultivars in two tests were compared (Table 4). The average number of lesions per site ranged from 12.04 for Atlas to 102.44 for Earliglow. Statistically, Earliglow was the most susceptible and Badgerbelle and Atlas were the least susceptible.

To check the reproducibility of spray infiltration, the number of lesions per site for all leaf ages, inoculum concentrations, and cultivars in each of the two tests were analyzed. The mean number of lesions per site was 83.30 for the first inoculation and 79.12 for the second; the average number of inoculation sites per plant on 13 plants was 42. These data were not significantly different ($P = 0.05$) according to Duncan's multiple range test.

Table 3. Effect of leaf age and inoculum concentration of *Xanthomonas fragariae* NCPPB 1822 on number of lesions produced on 12 strawberry cultivars

Log_{10} CFU/ml[a]	Leaf age[b]	Antilog LAVLC[c]
	1	118.24 a
7	2	104.36 ab
	3	80.91 ab
	1	83.66 ab
6	2	66.17 b
	3	38.48 c
	1	34.92 c
5	2	14.38 d
	3	10.86 d

[a] Washed cells from 24-hr shake cultures.
[b] 1 = youngest (fully expanded) leaf; 2 = second youngest; 3 = third youngest.
[c] Data followed by identical letters are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

Table 4. Reactions of 12 strawberry cultivars to inoculation with *Xanthomonas fragariae* NCPPB 1822

Cultivar	Antilog LAVLC[a]
Earliglow	102.44 a
Guardian A	95.95 ab
Guardian B	84.13 ab
Sparkle	83.47 ab
Stelemaster	83.22 ab
Raritan	58.70 abc
Midland	48.22 abc
Fletcher	45.61 abc
Surecrop	40.28 abc
Blakemore	32.47 abc
Suwannee	26.90 abc
Badgerbelle	18.29 bc
Atlas	12.04 c

[a] Average of two tests with washed cells from 24-hr shake cultures; data followed by identical letters are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

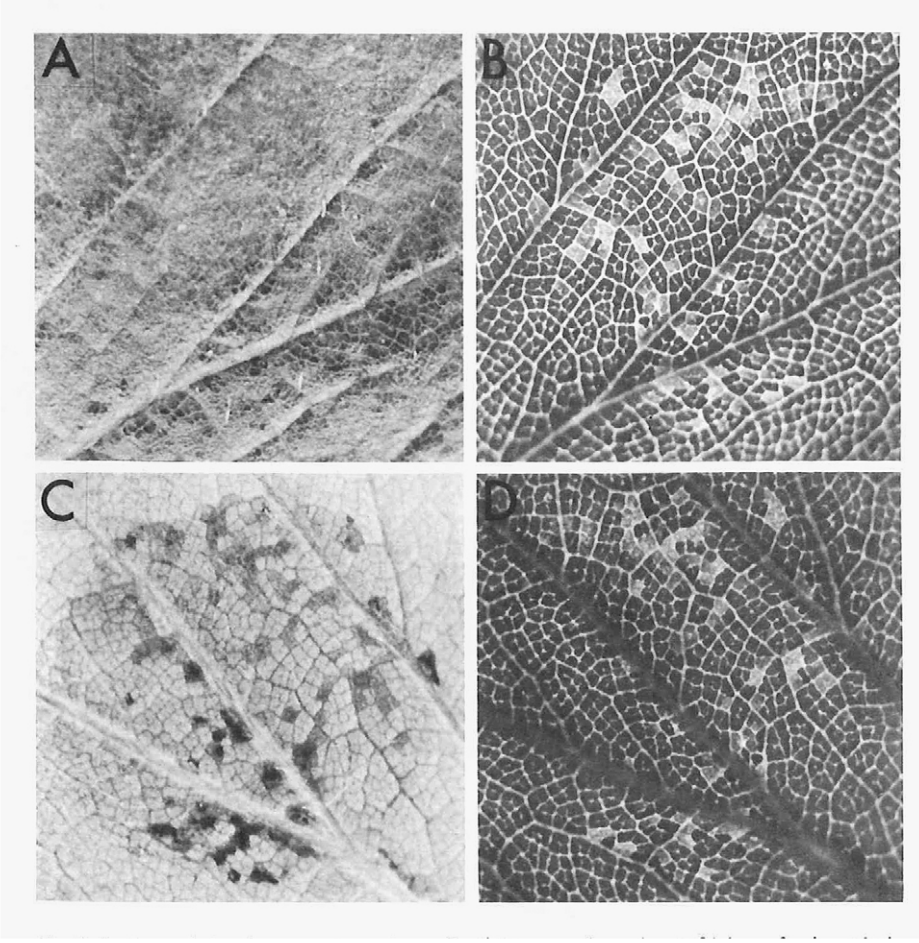

Fig. 2. Lesions of *Xanthomonas fragariae* on Earliglow strawberry leaves 21 days after inoculation by spray infiltration of 10^8 CFU/ml (5.75 ×). **(A,B)** Upper and **(C,D)** lower leaf surfaces viewed with reflected (A,C) and transmitted (B,D) light.

DISCUSSION

X. fragariae grew well in BM and medium A, although neither contained carbohydrate. Growth was not visible on these media, however, when 1.5% agar was added. This finding agrees with published results of *X. fragariae* growth on similar solid media (5). Comparison of viable cell number and turbidity of several media at 48 hr points out the problem encountered when turbidity is used as a measure of growth without establishing a definitive relationship between CFU/ml vs. time and CFU/ml vs. absorbance for each medium.

When medium B is used, *X. fragariae* cells should be harvested at 20–35 hr, washed, and resuspended with either sdd or saline for inoculum preparation, since these diluents allow more lesions to develop.

Spray infiltration is the most rapid and reliable method of inoculating strawberry with *X. fragariae*. When three trifoliolate leaves per plant are spray-infiltrated at six sites per leaflet, 30 or more plants can be inoculated per hour. Because very few new leasions appear 12–14 days after inoculation, the entire test can be completed in 2 wk. The method is reproducible both within the same inoculation test (Guardian A and B, Table 4) and between inoculation tests.

The number of lesions is directly related to *X. fragariae* concentration. We calculate that the minimum inoculum concentration capable of causing visible lesions is $1–7 \times 10^4$ CFU/ml; in fact, about 10^4 CFU/ml is the lowest concentration resulting in lesions.

Lesions were larger and more numerous on the youngest fully expanded leaves (2–4 wk old). Kennedy and King (3), however, reported that leaves 4–8 wk old were more susceptible to *X. fragariae* than younger or older leaves. In our tests, the number of lesions was inversely related to leaf age regardless of inoculum concentration.

Among the 12 strawberry cultivars inoculated, Atlas was the most resistant and Earliglow was the most susceptible. The cultivars may be grouped as follows: 1) Earliglow, Guardian, Sparkle, and Stelemaster, high susceptibility; 2) Raritan, Midland, Fletcher, Surecrop, Blakemore, and Suwannee, intermediate susceptibility; and 3) Badgerbelle and Atlas, low susceptibility. A greater number of replicates would probably establish more definite groupings.

Our methods of inoculum preparation and inoculation should be useful for assessing the virulence of *X. fragariae* isolates and for selecting resistant cultivars in a strawberry breeding program.

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