Bacterial Pathogens: Reducing Seed and In Vitro Survival by Physical Treatments

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ABSTRACT

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Cotyledon lesions on cucumber seedlings were prevented or greatly reduced in laboratory and field tests when seeds, vacuum-infiltrated with *Pseudomonas lachrymans,* were stored for 3 days at 50 C and 75% relative humidity. The treatment reduced seed germination. *P. lachrymans* did not survive dry in vitro at 50 C and 75% relative humidity for 1 day. This pathogen and eight other plant-pathogenic bacteria survived poorly in vitro at 75% as compared with 0 or 34% relative humidity.

Many plant-pathogenic bacteria are found in protected positions on or in plant tissues, where they survive for years (2). Season-to-season survival in association with seeds or other propagative parts is an important consideration in plant disease control because these materials are so widely disseminated.

Previous work suggested that dry, hypobiotic bacteria (those in a state of reduced metabolism, as would be expected of bacteria surviving with dry seed) do not survive well in vitro when stored at the moderately high relative humidity (RH) of 75% (6). This paper reports on studies of the influence of 75% RH on the survival of dry plant pathogens in vitro and describes tests in which 75% RH was used in attempts to control pathogens associated with seed. A short account of some of the work has been published (3). Most tests were performed with cucumber (*Cucumis sativus* L.) and *Pseudomonas lachrymans.* This pathogen, which incites angular leaf spot, is reported to be seedborne (9).

MATERIALS AND METHODS

Laboratory tests. We vacuum-infiltrated cucumber seed with large numbers of *P. lachrymans* cells to mimic a "worst case" situation in nature, in which the pathogen is within as well as on the seed. Cells were taken from cultures 1 to 2 wk old, when bacteria are resistant to drying in vitro (6). We were unable to obtain naturally infested seed.

Fifteen-gram lots of seed (each about 500 seeds) were immersed in aqueous suspensions containing the surfactant Tween 20 (Baker Chemical Co.) at 1 ml/L and *P. lachrymans* at $1-2 \times 10^7$ colony-forming units per milliliter. The *P. lachrymans* was taken from 1- to 2-wk-old slant cultures on nutrient agar (Difco, 23 g/L). A vacuum of 50 cm of Hg was drawn for 2 min on the vessel containing the seed and then quickly released. A 30-mg seed took up about 13 mg of water. Seed was dried on paper in the laboratory and stored at 16–24 C until used. The procedure without the bacteria did not reduce seed germination.

In most experiments, seed was stored for 3 days at 50 C and 75% RH (50-75 treatment). A 15-g lot of cucumber seed or a 100-g lot of soybean (*Glycine max* L.) seed (about 500 seeds) was distributed on an aluminum screen (mesh 1.5 × 1.5 mm) suspended 6 cm over water with salt (NaCl) added in excess to produce 75% RH in a closed, polyethylene box (23 × 23 × 10 cm high). Seeds gradually became hydrated during the treatment. Control lots were held for 3 days at 30–50% RH and 24 C and remained dry. Hydrated seeds were dried on paper and stored in paper sacks in the laboratory for 1–7 days before assay.

We devised a cucumber seedling assay to estimate the control of *P. lachrymans.* A 15-g lot of inoculated seed was planted in 500 cc of water-saturated vermiculite held by a wire rack (23 × 30 cm) 2 cm above a water reservoir in a pan 31 × 36 × 14 cm high. The vermiculite was kept uniformly moist by placing a muslin cloth under it and into the water. The unit and vermiculite were sterilized before use. Pans were closed with transparent film and placed under fluorescent lights for 16 hr a day at 24 C. After 7–10 days, individual seedlings were inspected for characteristic cotyledon lesions incited by the pathogen.

When a treatment produced a high level of disease control, isolations from suspect lesions were made on selective agar medium M71 (1) to determine if *P. lachrymans* was present. *P. lachrymans* was also detected by plating seed in M71. Good surface contact was ensured between seeds and agar by adding seeds to molten medium (about 45 C). After 3–5 days incubation at 24 C, the pathogen produced a characteristic colony when observed at ×10. Confirming pathogenicity tests (7) were occasionally made with cells from presumed *P. lachrymans* colonies; recognition was better than 95%.

Lots of soybean seed naturally carrying *P. glycinea,* which incites bacterial blight, were also treated. The effectiveness of the treatment was assessed by the seedling assay previously described (5), except that racks and closed pans were used as mentioned above. Characteristic lesions formed on seedling cotyledons.

Field trials. Trials were made in 1979 to determine the effectiveness of the 50-75 treatment for controlling the seedling phase of angular leaf spot of cucumber. Each treatment consisted of four 12-m-long replicate rows (250 seeds per row) 1 m apart in a Latin square arrangement. Plants bearing lesioned cotyledons were counted soon after cotyledons had opened.

Glass bead tests. In the glass bead test for survival of dry bacteria in vitro, cells suspended in milk are added to small glass beads, which are dried and stored under given temperature and RH conditions (6). Extent of survival is determined by placing beads on an agar medium and noting the proportion giving rise to bacterial colonies. We used cell suspensions from 1- to 2-wk-old cultures of nine bacterial plant pathogens, and beads were dried at 20 C and 0% RH for 3 days before they were stored under differing conditions. RH was maintained with salts (6). At the start and end of tests, colony characteristics were uniform and typical, and cells from representative colonies were pathogenic by methods previously cited (7).

RESULTS

Treatment at 75% RH. In three tests, angular leaf spot was greatly reduced or eliminated completely by the 50-75 treatment of seeds of three cucumber cultivars that had been stored 17 mo since harvest (Table 1). In a test of seeds that

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had been harvested and stored 5 mo, the disease was eliminated from two cultivars and the third had only two lesions on two seedlings.

Data in Table 1 and unpublished information show that with inoculated seeds the 50-75 treatment reduced germination about 10% as compared with the control treatment (24 C, 30–50% RH). Other tests demonstrated that the germination of uninoculated seeds was also reduced about 10% by the 50-75 treatment. Inoculation reduced germination, as can be seen in Table 1 in a comparison of inoculated and uninoculated seeds held at 24 C and 30–50% RH.

Field trials also demonstrated the effectiveness of the 50-75 treatment in controlling the seedling phase of angular leaf spot. Untreated, inoculated seeds of one cultivar produced 21 and 23% diseased seedlings in two trials planted at different dates in different fields; treated seeds of this cultivar produced 0 and 1% diseased seedlings. A second cultivar produced 11 and 25% diseased seedlings when untreated, as compared with 0.7 and 0.3% when treated. However, germination of inoculated seeds was reduced more in field than in laboratory tests for reasons not understood. Germination of the first cultivar was reduced 17 and 65% in the two tests; for the second cultivar, the figures were 18 and 21%.

Isolations from inoculated seeds cultured on M71 agar also showed the effectiveness of the 50-75 treatment in eliminating *P. lachrymans*. Twenty seeds from each of the nine tests represented in Table 1 were plated. The pathogen was not associated with any of the seeds treated at 75% RH and 50 C, whereas 149 of the 180 control seeds stored at 24 C and 30–50% RH carried the pathogen. Moreover, nearly all of the other bacteria that grew from control seeds were eliminated by the treatment, indicating that it inhibited bacteria associated with seed. We did not compare the seedling assay with seeds cultured on M71 agar partly because it was impractical to culture large numbers of seeds. However, some seed lots contained fungi that made detection of *P. lachrymans* uncertain, despite the fungicide in the agar. Unidentified bacteria may also have interfered.

The 50-75 method could not be used to study the control of *P. glycinea* by means of the soybean seedling assay because the treatment entirely prevented seed germination.

Pathogens were not eliminated when seeds were stored at 75% RH and temperatures other than 50 C, according to seedling assays. Three-day storage at 37 C was not effective in controlling *P. lachrymans* in inoculated cucumber seed; at 60 C, seed was killed. In three tests of inoculated seed stored at 24 C for 1, 2, or 3 mo, some seedlings were diseased even after 3 mo, when germination was reduced more than 50%. *P. glycinea* was not eliminated from naturally infected soybean seed when it was stored 3 mo under the same conditions, and germination was also greatly reduced. Therefore, a temperature of about 50 C seems essential.

Other seed treatments. *P. lachrymans* associated with inoculated seed or with seed from inoculated cucumber fruits has been controlled by treating seed with hot water (52 C for 10 min) or by storing dry seed at 70 C for 3 days (8). These treatments did not eliminate the pathogen from inoculated seed in our tests. The hot water treatment of 15-g lots of seed resulted in 29% as compared with 44% diseased seedlings from untreated seed (average of two tests). The dry seed held at 70 C resulted in 20% as compared with 82% diseased seedlings. Thus, these treatments may control the disease under some conditions, but they achieved only partial control with the severe inoculation procedure used here.

Inoculation method. Inoculation by vacuum infiltration placed *P. lachrymans* cells within as well as on seeds. In two tests, inoculated seeds (50 per treatment) were soaked for 10 min in 1% NaClO, rinsed well with deionized water, and cultured in M71 agar. Eleven percent of the treated seeds still carried the pathogen, as compared with 100% of the seeds not treated with NaClO.

Survival in vitro. Glass bead tests were made at 24 and 50 C and at 34 and 75% RH to compare the survival of dried *P. lachrymans* cells in vitro and when associated with seed. Assays were made at 1, 4, and 8 days. *P. lachrymans* survival was compared with that of *Corynebacterium michiganense*, which is resistant to drying (6). Treatment at 75% RH and 50 C was damaging to *P. lachrymans*, killing the organism even during 1 day of storage (Table 2). In general, *C. michiganense* was more resistant to drying than *P. lachrymans*,

Table 1. Storing cucumber seed inoculated with *Pseudomonas lachrymans*: Effects of different temperature and relative humidity (RH) levels on seed germination and incidence of angular leaf spot on seedlings

Cultivar	Stored for 3 days at: Temp (C)	RH (%)	Inoculated	Seeds germinated of ca. 450 planted[a]: No.	%	Diseased seedlings[a]: % of those germinating	No. lesions/ seedling
National Pickling	50	75	Yes	209	46	0[b]	...
	50	30–50	Yes	305	68	84	7
	24	30–50	Yes	229	51	93	9
	24	30–50	No	421	94	0	...
Boston Pickling	50	75	Yes	347	77	0.1[b]	2
	50	30–50	Yes	361	80	87	6
	24	30–50	Yes	376	84	98	7
	24	30–50	No	420	93	0	...
Chicago Pickling	50	75	Yes	345	77	0.3[b]	6
	50	30–50	Yes	402	89	69	6
	24	30–50	Yes	379	84	92	6
	24	30–50	No	439	98	0	0

[a] Mean of three tests at different times for each cultivar.
[b] This treatment resulted in no diseased seedlings in five of the nine tests represented in this table.

Table 2. Survival of *Pseudomonas lachrymans* and *Corynebacterium michiganense* on dry glass beads stored at different temperatures and relative humidity (RH) levels

Days stored	Temp (C)	RH (%)	Beads bearing survivors (%)[a]: *Pseudomonas lachrymans*	*Corynebacterium michiganense*
1	24	34	100	100
	50	34	94	99
	24	75	100	100
	50	75	0	0
4	24	34	100	100
	50	34	16	81
	24	75	100	100
	50	75	0	0
8	24	34	100	100
	50	34	0	55
	24	75	61	99
	50	75	0	0

[a] Mean of three tests for *P. lachrymans* and two for *C. michiganense* at different times. Survival was 100% at start of experiments.

Table 3. Survival of bacterial pathogens dried on glass beads and stored at different relative humidity (RH) levels for 3 mo at 20 C

Bacterium[a]	Beads bearing survivors (%)[b] 0% RH	34% RH	75% RH
Agrobacterium tumefaciens	93	84	0
Corynebacterium nebraskense	99	99	60
Erwinia stewartii	93	81	1
Pseudomonas lachrymans (P. syringae)	52	98	0
P. phaseolicola (P. syringae)	90	82	0
P. solanacearum	6	1	0
P. syringae (from bean)	100	100	17
Xanthomonas campestris	100	100	0
X. nigromaculans f. sp. *zinniae (X. campestris)*	100	100	0

[a] Names in parentheses are preferred in Bergey's Manual of Determinative Bacteriology (8th ed., 1974).
[b] Mean of three experiments at different times. Survival at start of experiments was 100%.

but it was also killed in 1 day by the treatment.

The survival of *P. lachrymans* and eight other plant pathogens was compared at 0, 34, and 75% RH in other glass bead tests similar to those reported previously (6). Survival of all species was markedly inhibited by 75% RH (Table 3). A *Corynebacterium* sp. was again the least affected by the treatment.

DISCUSSION

By storing seed at 75% RH for 3 days at 50 C, we eliminated or greatly reduced a bacterial pathogen that was within as well as on the seed. Because the seed had been vacuum-inoculated, the test was severe. The method entails seed hydration and redrying, which, as we found with soybean, may be excessively harmful to many of the large-seeded species (4). Results presented previously (6) and extended in the present paper indicate that many (possibly all) bacterial pathogens survive poorly when stored dry at 75% RH. Therefore, this treatment may be useful in other pathogen-host combinations when it does not also reduce seed germination unacceptably.

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