Effect of Light on Bacteria-Induced Hypersensitivity in Soybean

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ABSTRACT

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Erwinia amylovora-infiltrated, intact primary soybean leaves, incubated in 24 hr of postinfiltration darkness, expressed more severe hypersensitive response (HR) symptoms than did leaves illuminated for 24 hr at 11.5 and 23 W/m^2. HR symptom severity decreased as the 24-hr postinfiltration light intensity was increased from darkness to 11.5 W/m^2. Variations in postinfiltration photoperiod at 11.5 and 23 W/m^2 resulted in varied HR severity levels in primary leaves. With 11.5 W/m^2 intensity, leaves initially exposed for $\leqslant$4 hr expressed a more intense symptom than did initially dark-incubated counterparts. No significant differences in percent panel necrosis were observed between initial light and dark exposures of 6 hr at that intensity. With an intensity of 23 W/m^2, leaves exposed to $\leqslant$6 hr initial illumination expressed a more intense symptom than initially dark-incubated counterparts. With initial incubations exceeding 6 hr in both light regimes, dark-incubated leaves expressed the more intense symptom. Light increased the in vitro lag phase of *E. amylovora* whereas the lag phase was increased by 4 hr in the dark. The HR, as measured by electrolyte leakage from bacteria-infiltrated leaves, was photosensitive after 6 hr post infiltration and was more severe in dark-incubated tissue. Infiltration of bacterial suspensions in atrazine solutions into leaves reversed the effects of light after 24 hr and induced panel necrosis equal to that of leaves infiltrated with bacteria alone and incubated in the dark for 24 hr. Atrazine (5×10^{-5} M) did not affect the in vitro growth of *E. amylovora*.

The bacteria-inducted hypersensitive response (HR) has been defined as an induced resistance reaction to bacterial invasion in an incompatible host/pathogen system (11,14). More recently the HR has been viewed as a consequence of cellular imcompatibility (27). HR is characterized by increased electrolyte leakage in affected plant tissue (4,23) with tissue necrosis developing within 24 hr of inoculation (4). Three phases of the HR have been differentiated (15,16): an induction phase, after which the host is irreversibly committed to the response; a latent phase, which lasts until the appearance of symptoms; and a collapse phase, during which loss of host cell membrane integrity is noted. The HR is known to be altered by temperature, relative humidity and light (14,16,18). Of these, the effects of light on the HR are ambiguous. In studies on various host-bacterium systems the following conclusions have been drawn: HR is not affected by illumination (7,16,19,28,34); HR is intensified with increased light intensity (18); HR is intensified with dark treatment (12); and darkness can induce a compatible response in leaves that would show an HR if illuminated (18).

Abnormally high electrolyte leakage from affected cells is considered characteristic of the HR. This phenomenon also has been observed as being photo-affected (4). The results of one study showed that leakage was much greater in affected tissues under dark incubation.

Preliminary studies (9) with a soybean/*Erwinia amylovora* system revealed that postinfiltration photoperiod and light intensity influenced the intensity of the HR. The results of research on the effect of light intensity and photoperiod on bacteria-induced hypersensitivity in soybean, and evidence to implicate photosynthesis as the physiological process responsible for altered host response, are presented in this paper.

MATERIALS AND METHODS

In all experiments, light intensities were measured by a Lambda LI-185 Quantum/Radiometer/Photometer (LI-COR Inc., Lincoln, NE 68504). Illumination immediately preceding infiltration was provided by "Gro-Lux" lamps (GTE Sylvania Inc., Danvers, MA 01923); postinfiltration illumination was supplied by four, 100-W Industrial Service bulbs (Westinghouse Electric Company, Bloomfield, NJ 07306). Light treatments were carried out in a Percival environmental chamber (Percival Refrigeration and Mfg. Co., Des Moines, IA 50306), while dark treatments were administered in a Lab-Line incubator (Lab-Line Instrument Inc., Melrose Park, IL 60161. Conductivity was measured on a YSI conductivity bridge (Yellow Springs Instrument Co., Yellow Springs, OH 50306).

Soybean seeds, *Glycine max* L., Merrill 'Chippewa' were planted in a steam pasteurized mixture of sand and Hagerstown loam soil (2:1, v/v) in 89-ml plastic cups, one seed per cup. Plants were fertilized with a liquid fertilizer (20-20-20) at planting and 1 wk later. Seeds were germinated and grown in a greenhouse with auxiliary lighting providing a 14-hr photoperiod and light intensity of 5.5 W/m^2. When the primary leaves had unfolded (about 1.5 wk after planting), the plants were covered with vented polyethylene bags and placed under "Gro-Lux" lamps providing a photoperiod of 16 hr and a light intensity of 14.4 W/m^2. Temperature ranged from 22–25 C at leaf height. When the first unfolded trifoliolate leaf was approximately 1 cm long, the bags were removed and the primary leaves were infiltrated under vacuum with glass distilled water or glass distilled water suspensions of log-phase cells of *Erwinia amylovora* (Burrill) Winslow et al. A 3-hr preinfiltration period under "Gro-Lux" lamps was administered throughout experimentation. A single strain of *E. amylovora* obtained from a blighted Bartlett pear tree at Rock Springs, PA in May of 1976 was used in all experiments. Bacteria were stored under sterile mineral oil on nutrient agar slants at 23 C and by lyophilization. Transfers were made to new storage slants each month and virulence periodically was checked on Bartlett pear twigs.

Twenty-two hours prior to infiltration, bacteria were transferred from under oil to 10 ml of sterilized nutrient broth in a 0.1 M solution of phosphate buffer (pH 6.0) and grown for 12 hr at 23 C on a shaker with a 2.5-cm circular orbit at 175 RPM. One-tenth milliliter of this suspension was transferred to 10 ml of buffered broth and incubated as above for 10 hr. Log-phase cells were pelleted by centrifugation of 1,000 *g* for 10 min, washed in glass-distilled water, and repelleted at 1,500 *g* for 10 min. The pellet was resuspended in glass-distilled water to a concentration of 3.1×10^7 cells per milliliter of H_2O, as estimated photometrically at 420 nm and verified by viable cell counts.

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Infiltration of leaves. Infiltration of primary leaves of soybean plants followed the procedures of Pell et al (24) with some modifications. Both leaves of a soybean plant were infiltrated simultaneously while being held in the infiltration solution by a pliable, plastic collar, fitting snuggly against the inner wall of a 100-ml beaker. Infiltration time averaged 1.5 min. Leaf laminae were infiltrated over 90–100% of their total area. Temperatures during infiltration were kept at 18–20 C, which stabilized the leaf response. Following infiltration, leaf surfaces were blotted dry and the plants were transferred to treatment chambers.

Postinfiltration photoperiod studies. Immediately after infiltration, seven groups of six plants each were placed in the dark, or under illumination at 11.5 or 23 W/ m^2. After periods of 2, 4, 6, 8, and 10 hr, groups of six plants were transferred (referred to as a "shift" in the Results section) from light to dark or from dark to light treatments, remaining here for the balance of 24 hr. Bacteria- and glass-distilled water-infiltrated controls remained in darkness, and 11.6 or 23 W/ m^2 for 24 hr. Treatment temperatures and relative humidities ranged from 27–30 C and 60–100% respectively. After treatment, plants were placed in ambient laboratory conditions for 24 hr to allow more contrast to develop between necrotic and living tissue. Forty-eight hours after infiltration, plants were rated for percentage of necrotic area on the third large panel from the leaf base. The rating system used was adapted from that of Horsfall and Barratt (13) and measured 12 levels of necrosis ranging from 1.2 to 98.8% of the panel area.

Effect of light on bacterial growth. Log-phase cells of *E. amylovora,* grown as described above, were used to inoculate 10 ml of buffered nutrient broth contained in 125-ml, side-arm flasks. The flasks either were shielded from light with aluminum foil, or left unwrapped and illuminated (2.9 W/ m^2) while incubated at 23 C on a rotary shaker with a 2.5-cm circular orbit at 175 RPM. This light intensity was calculated as the light intensity affecting bacteria cells infiltrated into leaves exposed to light at 11.5 W/ m^2. The turbidity of the cultures was measured at 0, 4, 5, 7, 8.5, and 10 hr at 420 nm.

The effect of light on bacterial growth in situ was determined by using intact, primary leaves of soybean plants, infiltrated with bacterial suspensions as described above except that the leaves were washed with distilled water before they were blotted. Following infiltration, plants were incubated in dark or light (11.5 W/ m^2) conditions for 0, 2, 4, 6, 8, and 10 hr. At the end of the exposures, the leaves were excised, their areas were measured, and they were immediately pulverized in 9.9 ml of sterile 0.1 M potassium phosphate-magnesium sulfate buffer (pH 7.2). Leaf bacterial populations were determined using a dilution plating technique.

Postinfiltration electrolyte leakage studies. Intact, primary leaves of 24 groups of two soybean plants each were infiltrated with water or log-phase bacterial suspensions and subjected to light and dark treatment as described previously. At the termination of the treatment exposures, six leaf disks, 8 mm in diameter, were cut from each primary leaf, placed in 4.5 ml of glass-distilled water, and incubated at 23 C for 1.5 hr on a rotary shaker with a 2.5-cm circular orbit at 175 RPM. The bathing solution was measured for conductance.

Effect of atrazine on the cessation of photosynthesis of soybean leaves, bacterial growth, and percent panel necrosis. Intact, primary leaves of soybean plants were infiltrated with water or 5 $\times 10^{-5}$ M atrazine in water (23). Plants were placed under light treatment (11.5 W/ m^2) for 4 hr. Rates of photosynthesis were determined 4 hr after infiltration by using the $^{14}CO_2$-fixation technique of Magyarosy et al (21) in which 10 μCi in 0.1 ml of $NaH^{14}CO_3$ solution in the side arms of modified Warburg flasks.

Atrazine and bacterial growth. Effect of atrazine on bacterial growth in vitro was determined using log-phase cells of *E. amylovora* in 10 ml of nutrient broth made with and without a 5 $\times 10^{-5}$ M solution of atrazine. The media were contained in 125-ml side-arm flasks incubated on a rotary shaker at 23 C. The turbidity of the cultures was measured at 0, 2, 3, 5, 7, 8, 9, and 10 hr after inoculation.

Atrazine and percent panel necrosis. The effect of atrazine treatment on percent panel necrosis was determined using 10 groups of six soybean plants each. Intact, primary leaves were infiltrated with water, 5×10^{-5} M atrazine/H_2O solution, or with bacteria suspended in water or atrazine solution. Following infiltration, the plants were transferred to light (11.5 and 23 W/ m^2) and dark treatments, removed after 24 hr, and rated for HR intensity.

In all experiments, analyses of variance (36) and Duncan's Modified (Bayesian) LSD Tests (8) were used to determine the significance of difference among treatments. The K = 100 level of probability was used in all cases. This value approximates $P = 0.05$ (37).

RESULTS

Hereafter, all references to time will be in hours after infiltration unless otherwise stated. The first visible HR symptoms were observed at 8 hr in both light- and dark-incubated leaves as slight depressions 1–4 mm in diameter on the abaxial leaf surface and as faint, discolored areas (greenish-grey to yellowish-green) on the

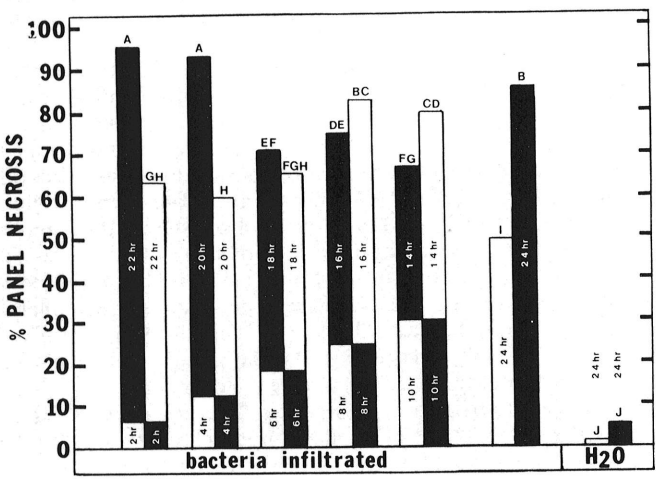

Fig. 1. Percent necrosis in primary leaves of soybean infiltrated with *E. amylovora* and affected by postinfiltration photoperiod at 11.5 watts/ m^2. Percent necrosis was evaluated on a scale from 1.2 to 98.8%. Shaded and unshaded areas of bars represent unilluminated and illuminated regimes, respectively, with portions representing initial exposures touching the baseline. Means represented by the same letter(s) are not significantly different at K = 100 according to Duncan's Modified (Bayesian) LSD Test. Each bar represents an average of 75 observations.

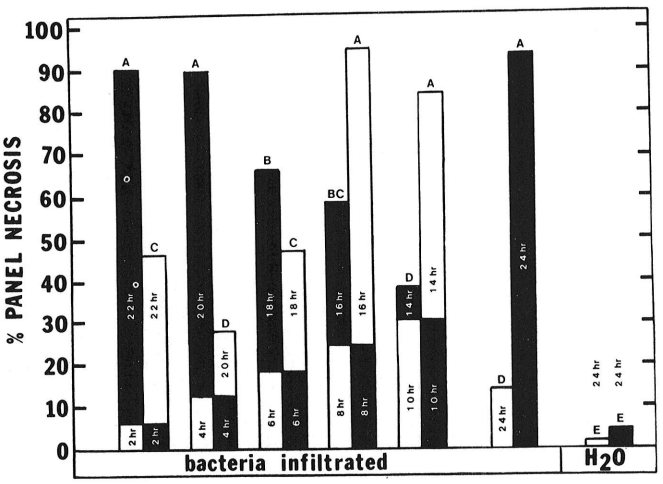

Fig. 2. Percent necrosis in primary leaves of soybean infiltrated with *E. amylovora* and affected by postinfiltration photoperiod at 23 watts/ m^2. Percent necrosis was evaluated on a scale from 1 to 98.8%. Shaded and unshaded areas of bars represent unilluminated and illuminated regimes, respectively, with portions representing initial exposures touching the baseline. Means represented by the same letter(s) are not significantly different at K = 100 according to Duncan's Modified (Bayesian) LSD Test. Each bar represents an average of 36 observations.

adaxial surface. Leaves expressing more intense symptoms (90.63% or greater of the panel area necrosed) were flaccid by 10 hr and often expressed severe curling at 24 hr. Necrotic areas were visible at 24 hr, but had not developed distinct margins. Visible symptoms were identical in appearance in both light- and dark-incubated leaves. Pinpoint necrosis occurred on water-infiltrated leaves incubated in darkness at 48 hr.

Postinfiltration photoperiod and HR severity. Leaves incubated in darkness for 24 hr displayed significantly more necrosis than did leaves incubated for an equivalent time in light at either intensity (Fig. 1). Leaves incubated for 24 hr at 11.5 W/m^2 produced a higher percent necrosis than those incubated at 23 W/m^2.

Leaves initially incubated in light at either intensity for 2 and 4 hr, and for 8 and 10 hr in darkness, expressed a high level of HR severity equal to or greater than the percentage of necrosis with the 24-hr dark treatment. Leaves shifted at 2 and 4 hr and receiving initial light exposures at 11.5 W/m^2 expressed a significantly higher percentage of necrosis than did those leaves initially exposed to darkness for identical intervals (Fig. 1). No significant differences in the percentage of necrosis were observed between initial light and dark treatments of 6 hr at this intensity. A similar relationship was observed at 23 W/m^2 (Fig. 2), except that initial light exposures of 2, 4, and 6 hr resulted in necrosis percentage significantly higher than those of their initially dark-incubated counterparts. With initial light treatments of 8 and 10 hr leaves expressed significantly less necrosis relative to their initially dark-inoculated counterparts at both intensities.

Light and bacterial growth. Illumination at 2.9 W/m^2 depressed the growth rate of *E. amylovora* between 4 and 5 hr and at 8.5 hr after inoculation in buffered nutrient broth compared to cultures grown in complete darkness (Fig. 3). Bacteria populations in situ remained constant in light- and dark-incubated leaves through 4 hr after infiltration (Fig. 4). After this time, populations in light-incubated leaves were at a significantly higher level than those in dark-incubated leaves. After 6 hr of dark incubation the leaf bacteria populations began to increase at rates similar to populations in light-incubated leaves, but did not reach comparable numbers. The graphed point representing bacterial numbers and 10 hr of light incubation (Fig. 4) was an estimated value. Plate counts in two of the three experimental replications of this treatment exceeded the measurable limits (3).

Light and electrolyte leakage. Electrolyte leakage from bacteria-infiltrated tissue incubated for 0 and 2 hr in light (11.5 W/m^2) or darkness did not vary significantly from that of water-infiltrated tissue undergoing identical exposures and treatments (Fig. 5). At 4 and 6 hr, bacteria-infiltrated tissue in both light regimes exhibited a significant and statistically identical rise in cell leakage over that of water-infiltrated tissue. After 6 hr, leakage from dark-incubated tissue increased significantly above that detected earlier, but leakage from light-treated tissue decreased after this point. Preliminary studies showed electrolyte leakage from noninfiltrated

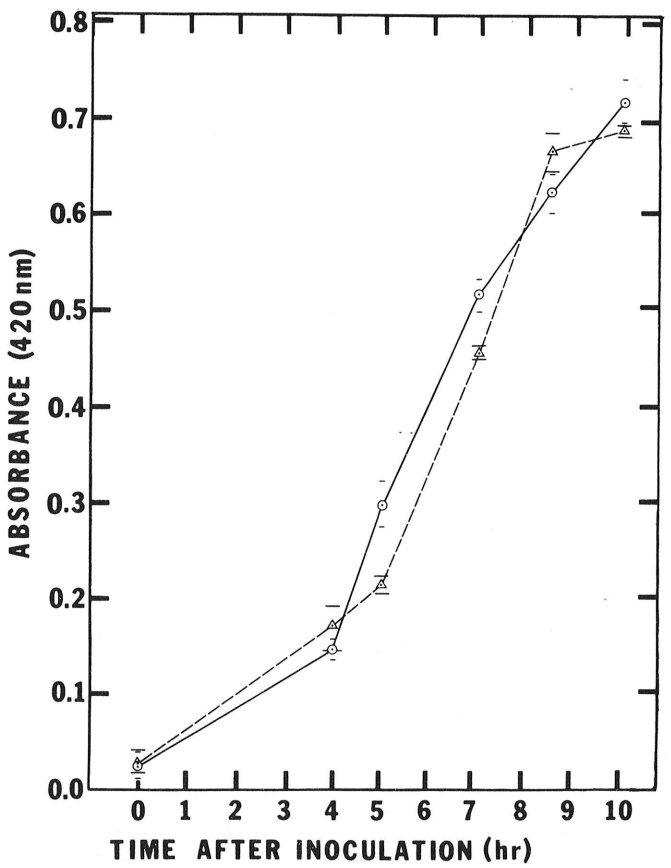

Fig. 3. Growth of *Erwinia amylovora* in buffered nutrient broth incubated in 2.9 watts/m^2 light (dashed line) and darkness (solid line). Error bars denote one standard deviation. Each point represents an average of 12 observations.

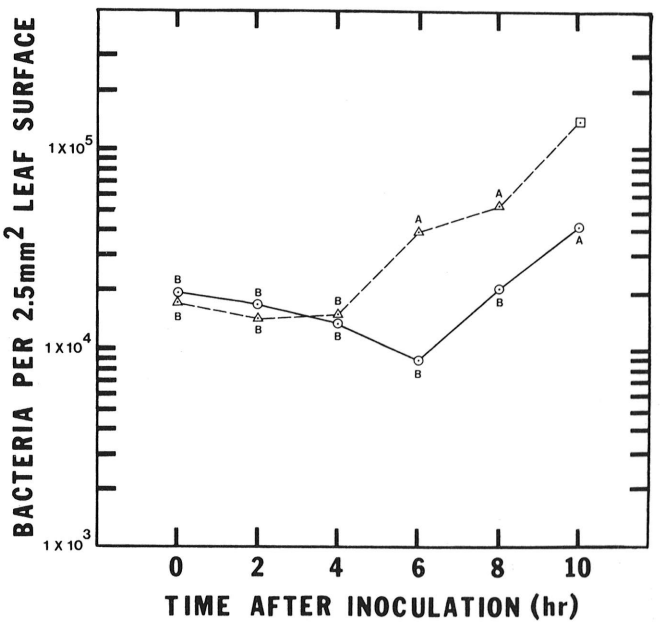

Fig. 4. Postinfiltration growth of *Erwinia amylovora* in primary leaves of soybean incubated in 11.5 watts/m^2 light (dashed line) and darkness (solid line). Means represented by the same letter(s) are not significantly different at K = 100 according to Duncan's Modified (Bayesian) LSD Test. Each point represents an average of nine observations.

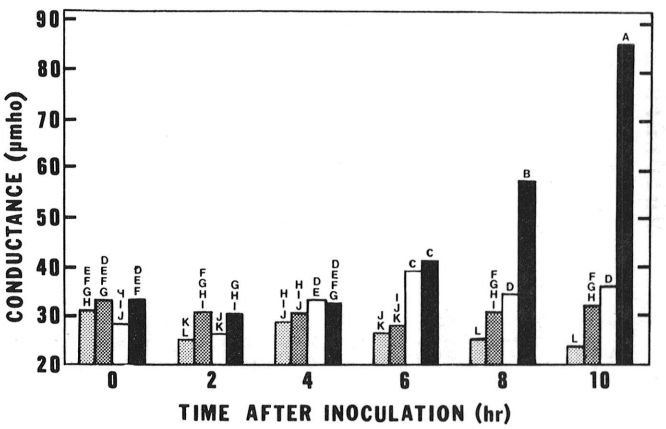

Fig. 5. Conductance of leaf tissue bathing solution with respect to postinfiltration light (11.5 watts/m^2) (*Erwinia amylovora*-infiltrated [open bars], water-infiltrated [20% density bars]) and darkness (*E. amylovora*-infiltrated [solid bars], water-infiltrated [40% density bars]). Means represented by the same letter(s) are not significantly different at K = 100 according to Duncan's Modified (Bayesian) LSD Test. Each bar represents an average of 36 observations.

tissue to be similar to water-infiltrated tissue.

Atrazine and photosynthesis. Photosynthetic activity in primary leaf tissue of soybean was reduced by 97% (2.06×10^5 cps versus 5.00×10^3 cps) following infiltration with 5×10^{-5} M atrazine and a 4 hr light incubation (11.5 W/m^2).

Atrazine and bacterial growth. No statistical difference in the growth rates of *E. amylovora* was measured in buffered nutrient broth with and without 5×10^{-5} M atrazine (Fig. 6).

Atrazine and HR severity. Leaves infiltrated with bacteria suspended in atrazine solution and incubated in darkness, or in light at 11.5 and 23 W/m^2 for 24 hr, exhibited HR severity values not significantly different from leaves infiltrated with bacterial suspensions without atrazine and incubated in darkness for identical exposures (Fig. 7). Leaves infiltrated with bacteria in the absence of atrazine and incubated in light at 11.5 and 23 W/m^2, developed HR severities characteristic of 24-hr treatments at their respective intensities (Figs. 1 and 2). Control leaves, infiltrated with and without atrazine and incubated in light (23 W/m^2) and darkness for 24 hr, expressed minimal symptoms.

DISCUSSION

E. amylovora induces the characteristic HR response in intact, soybean primary leaves (15,16). The ability of light to influence the HR (photo-influence) in this pathogen/host system has been demonstrated with respect to symptom expression and tissue electrolyte leakage.

The 24-hr dark incubation of *E. amylovora*-infiltrated soybean leaves induced a significantly more intense response than identically inoculated leaves in coincident 24-hr illumination at 11.5 and 23 W/m^2 (Figs. 1 and 2). These results agree with those of Hildebrand and Riddle (12) who report that isolates of *Agrobacterium* and *Xanthomonas* in tobacco, cause a more intense HR in dark postinfiltration treatment than in light. Other investigators find no photo-influence on the HR (7,16,19,34), however none of these used identical systems. Our results showed that HR severity decreased as light intensity increased from complete darkness to 23 W/m^2. Lozano and Sequeira (18) evaluated *Pseudomonas solanacearum* in tobacco, and report an opposite relationship. The variability existing in the reported influence of light on the HR supports the observation that the HR induced in different host/pathogen systems is influenced differently by environmental conditions (12). Indeed, it is perhaps unjustified to consider the HR as a physiologically identical response in any incompatible system.

Variation of the postinfiltration photoperiod of bacteria-infiltrated leaves at the two light intensities varied HR severity. When the HR intensity of leaves exposed for the same initial time periods, but to different light schemes (ie, photo- and nyctoperiodicity), were compared, and inversion in the relative HR severity at 6 to 8 hr after infiltration was observed at both intensities. Those leaves receiving initial light exposures of 4 hr or less at 11.5 W/m^2, and 6 hr or less at 23 W/m^2, showed a more intense level of HR severity than their initially dark-incubated counterparts. At the lower intensity, HR severities on leaves exposed to 6 hr initial light and darkness were not significantly different from one another. When initial light exposures exceeded 6 hr at both intensities, initially dark-treated leaves exhibited the more intense response. The period between 6 and 8 hr after infiltration is an important time in the photo-influence of the HR in soybean. Since inhibition of the HR is even greater at 23 W/m^2 than at 11.5 W/m^2, this inhibition is apparently energy dependent.

The in vitro growth of *E. amylovora* was significantly depressed between 4 and 5 hr at 2.9 W/m^2. However, in situ bacterial growth was enhanced in light-incubated leaves through the photosensitivity of host leaf tissue. Light- and dark-incubated populations remained similar until 4 hr after infiltration after which they increased in light. However, bacterial populations in dark-incubated leaves began increasing only after 6 hr. Since the doubling time of this bacterial isolate was about 2 hr (Fig. 3), the leaf phenomenon responsible for the delayed bacterial growth in dark-incubated leaves would have become effective at or before 2 hr after infiltration and remained effective until 4 hr postinfiltration.

Differences in electrolyte leakage from bacteria-infiltrated, light- and dark-treated leaves were similar to those in pepper (4). Increases in leakage from bacteria-infiltrated leaves at 4 and 6 hr were similar, irrespective of illumination, and we suggest such leakage was due to HR initiation after 2 hr. Only after 6 hr did illumination influence HR-induced leakage, and this length of time appears necessary for expression of photo-influence of the HR

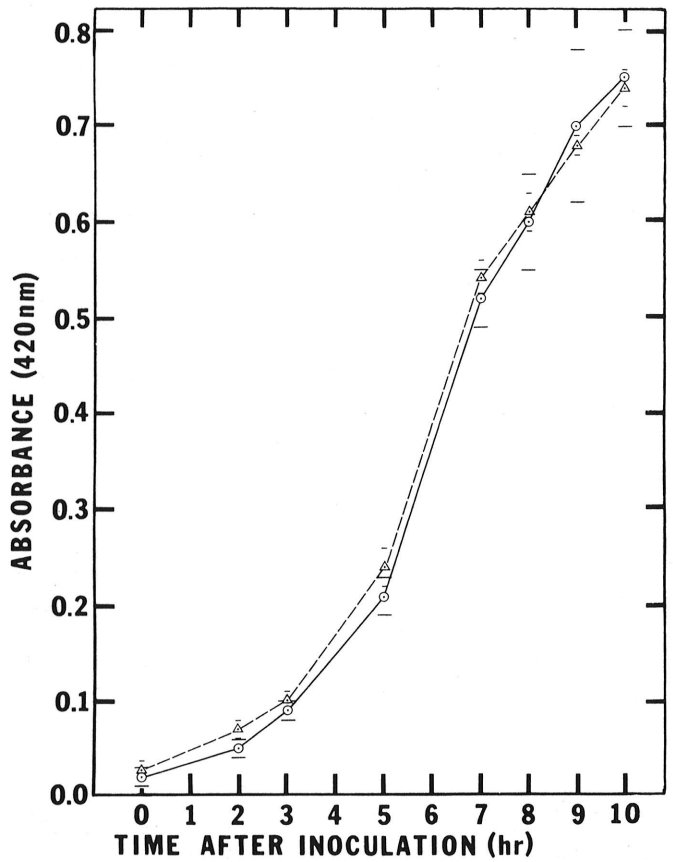

Fig. 6. Growth of *Erwinia amylovora* in buffered nutrient broth (dashed line) and in the presence of 5×10^{-5} M atrazine (solid line). Error bars denote one standard deviation. Each point represents 12 observations.

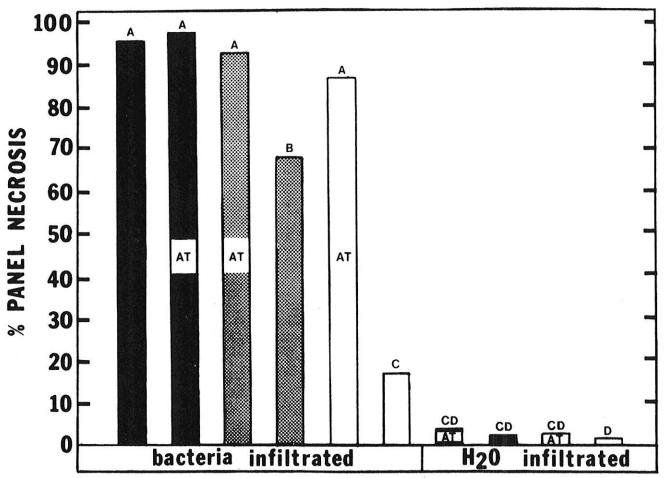

Fig. 7. Percent necrosis in primary leaves of soybean infiltrated with *E. amylovora* and incubated in postinfiltration light (11.5 watts/m^2 [40% density bars], 23 watts/m^2 [open bars]) and darkness (solid bars) for 24 hr with and without 5×10^{-5} M atrazine. Percent necrosis was evaluated on a scale from 1.2 to 98.8%. Means represented by the same letter(s) are not significantly different at K = 100 according to Duncan's Modified (Bayesian) LSD Test. Each bar represents an average of 36 observations.

in soybean (Fig. 1 and 2).

Atrazine, a triazine herbicide which apparently blocks photosynthesis at the point of water photolysis (2,32), cancelled the inhibitory effect of light on HR expression when the herbicide, atrazine, was coinfiltrated with the bacteria (Fig. 7). A similar relationship was reported using *X. phaseoli* and pepper (28). Atrazine treatment did not influence the in vitro growth of *E. amylovora*. No attempt was made to determine the effect of atrazine on in situ bacterial growth. The explanation for light-induced inhibition of HR and apparent reversal of this phenomena when atrazine was added to the bacterial infiltrate may be related to photosynthetic activity of the host. If the photosynthesizing host produces a factor responsible for inhibition of bacterially-induced HR expression, then any mechanism which reduces or prevents photosynthesis should be consistent with more intense HR. Both a prolonged dark period (Figs. 1 and 2) and a chemical inhibitor of photosynthesis (eg, atrazine) (Fig. 7) intensified HR severity in soybean foliage. The report of Le Normand (17) showing the HR to be influenced by light wavelengths associated with photosynthetic activity also is in agreement.

The HR (5,6,11,16,33,35), its photo-influence (4) (Fig. 5), photosynthesis (1,2,20,28) and atrazine (22) all affect ion permeability or membrane integrity in plant cells. While HR and atrazine (36) alter the selective integrity of affected plant cell membranes, photosynthesis (25,26,33) tends to enhance it. Light treatment has been shown to mediate the degree to which membrane integrity is impaired by the HR (Fig. 5). It is possible then to visualize a photo-influenced HR system, acting through light-mediated ion transport conceivably working against the deteriorating effects of membrane integrity induced by the HR in light. Darkness and atrazine treatments, on the other hand, could be portrayed as doing little to hinder HR expression.

The intensity of light and the photoperiod to which bacteria-infiltrated soybean leaves are exposed determines the intensity to HR which results. It appears that when the host photosynthesizes, inhibitors or inhibiting systems to HR development are produced or activated.

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